Biofilm Alterations on the Stepwise Acquisition of Fluconazole-resistant Candida Albicans Isolates

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Abstract

Objectives: By assessing and comparing the phenotypic changes on the stepwise acquisition of fluconazole resistant Candida albicans isolates, we could find and describe the relationship between drug resistance and biofilm formation ability in a series of clonal strains.

Methods: We performed antifungal susceptibility of five drugs (fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B) to further verify the antifungal activity of the six isolates in vitro. Then we combined hyphal formation assay, cell surface hydrophobicity test positively related to adherence ability, and biofilm assays in vitro to observe and compare the phenotypic characteristics of our six clonal strains.

Results: Biofilm capability is enhanced for four drug-intermediate strains, whereas the initial susceptible strain and the final resistant strain are both poor in adherence, hyphal growth and biofilm formation.

Conclusions: It was suggested that the biofilm formation ability were not absolutely related to the degree of fluconazole resistance.

Keywords: Candida albicans, stepwise acquired azole resistance in vitro, adherence, hyphal formation, biofilm

Introduction

Microbial biofilms are considered to be the most common growth state for many microbial species, and 80% of human microbial infections are related to biofilms.1,2 Candida albicans is the most commonly-diagnosed fungal pathogen of the human microbiota and can cause pervasive infections ranging from superficial mucosal and dermal infections to life threatening systemic infections.3,4 The ability to grow biofilms complicates the eradication of C. albicans infections, particularly regarding its tendency to colonize the surfaces of medical devices and its persistence in different host tissues.1,2 Cells in C. albicans biofilm are also often resistant to a variety of conventional antifungal agents, whose minimal inhibitory concentrations (MICs) can then be up to 1000 times higher than those found for planktonic cells.5 Current studies related to C. albicans biofilms mainly focus on describing differences in the biofilm formation process for Candida species, or on comparing the biofilm-formation ability of C. albicans strains in different niches in the host, or in exploring interactions between C. albicans and bacteria species in the context of dual-species biofilms.6,7 However, the relationship between biofilm formation ability and the degree of
drug resistance in vivo in sequential clonal C. albicans isolates has never been investigated. Then how did the biofilm change during the evolution of acquired drug resistance in vivo? Here, we select a series of six C. albicans considered as having acquired azole resistance in vivo. These isolates were taken from an HIV-infected patient receiving progressively higher doses of fluconazole for recurrent oropharyngeal candidiasis over two years. The biofilm-related parameters we employed in this study include adherence represented by cell surface hydrophobicity here, hyphal growth and biofilm mass.

Materials and methods

Ethical statement

No ethical approval was required as the research in this article related to micro-organisms.

Strains and growth conditions

Candida albicans strains (Ca1, Ca2, Ca5, Ca8, Ca14 and Ca17) used in this study were kindly provided by professor T. C. White (Seattle Biomedical Research Institute, Seattle). A colony was obtained from SDA medium (1% peptone, 2% dextrose, 1.5% agar, PH 7.0) after a 72 hours culture at 30 °C and transferred to YPD medium (1% yeast extract, 2% peptone and 2% dextrose). Then the strains were incubated overnight in 5 mL of YPD broth at 30 °C with rotation (150 rpm).

Antifungal susceptibility testing against C. albicans in vitro

Antifungal susceptibility testing of five drugs (fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B) was performed using a broth micro-dilution method according to the CLSI (Clinical and Laboratory Standards Institute) in document M27–A3. The MIC was defined visually as the lowest concentration of drug that caused a prominent decrease when compared to the growth control. All tests were performed in triplicate and were repeated at least three times.

Hyphal formation assay

A 10 µL of cell suspension (5 × 10⁵ CFU/mL) was spread onto Spider agar (10 g/liter nutrient broth, 10 g/liter mannitol, 2 g/liter K2HPO4, 20 g/liter agar, pH 7.2) and photographed after 3 days growth at 37 °C. After culturing, we transferred a colony with 1mm diameter into 5ml physiological saline. After shaking, 20 µL of the mixture was dropped onto a glass slide and observed under inverted microscope. In concert with this, filamentation was tested in liquid Spider medium. Yeast suspensions (3 × 10⁶ CFU/mL) were incubated in liquid Spider medium at 37 °C and 200 r/minute for 24 hours. 10 µL portion of the suspension was dropped on a blood cell counting plate to count the number of hyphae and yeasts of the various strains. The proportion of hyphal cells was calculated as [number of hyphae / (number of yeasts + number of hyphae)] × 100. Each leg of this portion of the experiment was repeated at least 3 times.

Cell surface hydrophobicity assay

The Cell surface hydrophobicity (CSH) assay of C. albicans was performed as previously described. Briefly, C. albicans cultures were adjusted to a cell suspension with an OD600 (optical density at 600 nm) of 1.0 in YPD medium. A total of 2.5 mL of the suspension from each group was drawn into a clean glass tube, mixed with 0.5 mL of xylene at 37 °C for 10 minutes, and shaken for 30 seconds. Then these tubes were left for 30 minutes at 37 °C in order to facilitate separation of the phases. The turbidity of the aqueous phase was read at 600 nm. The OD600 for the group before xylene treatment was used as the negative control. The HI (hydrophobicity index) was calculated as [(OD600 of the control − OD600 after xylene overlay)/OD600 of the control] × 100. Three repeats were performed for each group.

Biofilm Formation

500ul standardized C. albicans cell suspensions (OD600 of 0.5 in liquid Spider medium) were introduced into the wells of 24-well tissue culture plates (Corning Inc., Corning, NY) and incubated at 37 °C.

Inverted microscopy

The medium was aspirated at 2, 4, 8, 12 and 24 hours of the incubation period. Biofilms were harvested and washed three times in sterile phosphate-buffered saline (PBS) [10 mmol/L phosphate buffer, 2.7 mmol/L potassium chloride, 137 mmol/L sodium chloride (pH 7.4)] and observed by inverted microscopy.

Confocal laser scanning microscopy (CLSM)

After 24 hours of biofilm growth in 24-well plate, biofilms were washed three times with PBS. Two hundreds micrometers of 25 µmol/L FUN-1(1 g dextrose, 1.1915 g 10 mmol/L Na-Hepes, 50 mL H2O, pH 7.2) was added to each well and biofilms were then cultured at room temperature in the dark. After 30 minutes, biofilms were washed gently with PBS and observed under confocal laser scanning microscope. The emission wavelength was 488 nm.

XTT Reduction Assay

Cells were washed three times with PBS before adding 500 µL XTT/VitK3 mixture prepared by mixing 1 µL 10 mmol/L VitK3 with 10 mL 0.5 mg/mL XTT ([2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]) solution, and the resulting mixture was then incubated in the dark at 37 °C for 3 hours. The metabolically active cells reduced the substrate to orange-colored formazan which was measured spectrophotometrically at 490 nm. Results are given as mean ± standard error in the mean for three independent experiments.

Statistical analysis

Data analysis was performed using SPSS (IBM Corp., Armonk, NY, USA). For hyphal growth rate, CSH value and XTT assays, multiple comparison was performed by one-way ANOVA and Turkey HSD was used for post-hoc
Results

Antifungal effects against planktonic cells of C. albicans in vitro

Antifungal susceptibility profiles of the tested isolates are shown in Table 1. The MIC of fluconazole against six oral isolates range from 0.5 μg/mL to 128 μg/mL. The series clearly shows progressive increases in MICs against itraconazole and voriconazole as well, perhaps due to cross-resistance mechanism of azoles. In addition, all the C. albicans strains tested in this study are susceptible to amphotericin B and caspofungin.

Hyphal growth and CSH values

Hyphal growth on Spider agar revealed that Ca1 and -17 formed creamy colonies with smooth surfaces, whereas Ca2, -5, -8 and -14 exhibited a more wrinkled morphology (Fig. 1A). Moreover, under light microscopy, Ca1 and -17 formed colonies predominantly composed of yeast cells, but appeared to be morphologically distinct from Ca2, -5, -8 and -14 in that considerably more hyphae were observed (Fig. 1B). From Figure 1C, similar results were obtained from the liquid Spider medium test as well (F = 85.641, P < 0.01). In addition, CSH values of the other four strains were significantly higher than that of Ca1 or -17 (F = 256.322, P < 0.01) (Fig. 1D), which was consistent with the results attained form hyphal formation assays.

Visualization of biofilm formation under inverted microscopy

We then further characterized the architecture of the biofilms by inverted microscopy. When cultured for 2 hours, the strains form a biofilm with a monolayer of round budding yeast cells (Fig. 2A). After 2 hours, pseudohyphal and hyphal cells begin to form from these dividing yeast cells and microcolonies appear interspersed to form a membrane architecture at 4 hours (Fig. 2B). When incubated for 12 hours, a multi-layer membrane structure is formed with yeast cells and hyphal cells (Fig. 2C). Biofilms become thicker, with more extracellular matrix encompassing all cells after 24 hours, presenting with a dense multilayer membrane structure (Fig. 2D). In general, Ca2, -5, -8 and -14 formed thicker biofilms, consisting of denser hyphal networks and smaller pore spaces than Ca1 or -17.

CLSM image and XTT reduction assay

C. albicans biofilms were considered mature at 24 hours and assessed by confocal laser scanning microscopy (CLSM). In contrast to the less developed biofilms of Ca1 and Ca17, the CLSM images show strains Ca2, -5, -8 and -14 with multiple layers of hyphal cells and a compact architecture of biofilm matrices (Fig. 3A). In addition, from Figure 3b, XTT reduction assay used to quantify the biofilm activity of experimental strains showed the following ranking (from most complex to least complex): Ca2 > Ca8 > Ca5 > Ca14 > Ca1 > Ca17 (F = 149.122, P < 0.01).

Discussion

Polyenes, azoles and echinocandins are three major classes of antifungal drugs used in invasive fungal infections. Among them, azoles, especially fluconazole, are the most commonly therapeutic drugs for C. albicans infections due to their high efficiency and low toxicity. However, they lack fungicidal activity and prolonged usage of fluconazole results in the emergence of resistant strains of C. albicans during treatment. Ca1 was a fluconazole-susceptible strain (0.5 μg/mL) initially isolated in the series through treatment with low doses of fluconazole (100 mg/d). Ca17 was a final isolate of this series with the highest resistance to fluconazole (greater than 128 μg/mL), which was collected when the patient was receiving 800 mg of fluconazole per day. The remaining four strains gathered in this study (Ca2, -5, -8 and -14) and the concomitant increases in MIC values (from 2 μg/mL to 32 μg/mL) occurred in a step-wise pattern, which matched the step-wise increases in the dosage of fluconazole (from 100 mg/d to 400 mg/d) prescribed for the patient.

As a resident of the commensal microbial flora, C. albicans can form biofilms on host mucosal tissue primarily in the oral cavity causing oropharyngeal candidiasis. Additionally, cells in C. albicans biofilms can evade the host immune responses, combat conventional drugs, and seed new infections, making them a troublesome clinical problem. Overall, C. albicans biofilm formation is a systematic, highly controlled process partitioned into four basic stages: adherence, initiation, maturation and dispersion. Microbial adhesion is considered the crucial first step for biofilm formation process and cell surface hydrophobicity (CSH) plays an important role in the adhesion of pathogenic microorganisms to abiotic and biotic surfaces. In the case of C. albicans, a positive association between CSH and adhesion has been reported. One consensus is that C. albicans filamentous forms are naturally invasive, able to penetrate tissues and cause damage in the host. Indeed, hyphal formation in C. albicans is also closely related to biofilm maturation, and mutants defective in hyphal formation demonstrated defects in biofilm formation as well. The ability of C. albicans to switch from yeast to filaments plays a crucial role in enhancing virulence. Many factors can induce hyphal formation, namely, serum, neutral PH, 37 °C, Spider medium,

| MICs of five antifungal drugs against C. albicans planktonic cells. |
|-----------------------------------------------|
| **C. albicans strains** | **FLC** | **ITR** | **VOC** | **AMB** | **CAS** |
| Ca1 | 0.5 | 0.0313 | 0.0313 | 0.25 | 0.0313 |
| Ca2 | 2.0 | 0.0313 | 0.0313 | 0.25 | 0.0625 |
| Ca5 | 8.0 | 0.0313 | 0.0313 | 0.50 | 0.0625 |
| Ca8 | 8.0 | 0.0313 | 0.0313 | 0.50 | 0.1250 |
| Ca14 | 32.0 | 0.0625 | 0.1250 | 0.25 | 0.1250 |
| Ca17 | 128.0 | 0.5000 | 1.0000 | 0.50 | 0.1250 |

FLC, fluconazole; ITR, itraconazole; VOC, voriconazole; AMB, amphotericin B; CAS, caspofungin.
and Roswell Park Memorial Institute-1640 medium. Because biofilm formation in C. albicans has been associated with hyphal formation and adherence ability, we assessed the ability of the six strains to form hyphae on Spider medium and recorded CSH values (referred to adherence) during the biofilm development. Overall, with the exception of Ca17, strains Ca2, -5, -8 and -14 presented a stronger capacity for hyphal growth and CSH values than Ca1. Since C. albicans biofilms begin with the adhesion of yeast cells to a solid surface, growth to form an anchoring layer, and yeast-to-hypha transition. We preliminarily inferred that the biofilm formation ability of C. albicans strains Ca2, -5, -8 and -14 could be stronger than that of Ca1 and Ca17, which was absolutely verified in the biofilm assays.

A correlation between biofilm formation and antibiotic resistance has been noted recently in some bacteria. For example, majority of methicillin-resistant Staphylococcus aureus (MRSA) isolates showed stronger biofilm formation than methicillin-sensitive S. aureus (MSSA) isolates in two different studies. On the other hand, the weak biofilm-forming Enterococcus faecalis isolates were uniformly resistant to ampicillin and vancomycin, compared to their strong biofilm-forming counterparts. Interestingly, in the eukaryotic microorganism C. albicans, our study was the first to observe an unexpected phenomenon between biofilm formation and drug resistance of C. albicans. As described in more detail below, all tested strains were divided into two groups based on their biofilm-producing ability: one group with weak biofilm formation ability (the initially sensitive strain Ca1 and the final resistant strain Ca17) and the other group with strong biofilm formation ability (the four drug-intermediate strains Ca2, -5, -8 and -14). It seems that intermediate fluconazole resistance in Ca2, -5, -8 and -14 promote biofilm formation in vitro, while the high fluconazole resistance (200 times that of the original isolate Ca1) of Ca17 inhibits the biofilm formation. However, an alternative explanation for this last data point could be that length of time and the high dosage of fluconazole treatment in vivo somehow restricted some critical biofilm-related cellular process or processes in Ca17 during co-evolution with that strain’s drug resistance. As is mentioned in one study, mutations in genes related to cell adhesion (agglutinin-like protein 3, 5, and HYR3), filamentous growth (filamentous growth regulator 14, filamentous growth regulator 28, and transcriptional regulator EFH1) and biofilm formation (biofilm and cell wall regulator 1 and serine/threonine protein kinase 1)
occurred in several sequential isolates, suggesting a co-evolution between virulence and drug resistance. Since the fungistatic (growth inhibiting but not necessarily lethal) nature of azoles imposes strong directional selection for the evolution of resistance, only the cells with acquired resistance-related alterations could survive in the presence of the drug. Over the past 20 years, many studies have addressed the same series of isolates we used in this study.

Figure 2. Biofilms under an inverted microscope. *C. albicans* biofilms were cultured and photographed under an inverted microscope at 2 hours (A), 4 hours (B), 12 hours (C) and 24 hours (D), respectively.
to understand the evolutionary emergence of drug resistance in \textit{C. albicans in vivo}. They found gain of function (GOF) mutations in the transcriptional regulator sterol uptake control protein 2 (UPC2) caused the overexpression of lanosterol 14-alpha demethylase, the gene encoding the fluconazole target.\textsuperscript{23-25} Moreover, GOF mutations in transcriptional activator (TAC1) mediated overexpression of the multidrug efflux pumps pleiotropic ABC efflux transporter of multiple drugs\textsuperscript{1} and CDR2.\textsuperscript{26} Similarly, the observed overexpression of multidrug resistance protein 1 efflux pumps was also detected, which was associated with GOF mutations in \textit{MRR1}.\textsuperscript{24,27} Noteworthy, it was proposed that the development of \textit{C. albicans} resistance to azoles occur in a stepwise manner in which loss-of-heterozygosity (LOH) events commonly followed the acquisition of mutations in drug resistance genes.\textsuperscript{26,28} Not surprisingly, as reported in previous studies, \textit{Ca17} contains hyperactive forms of all three zinc cluster transcription factors (ZnTFs) Mr1, Tac1, and Upc2 that are well associated with high resistance to antifungal drugs.\textsuperscript{27}

As biofilms are able to dynamically respond to various stresses and act as a “protective net” where fungus are afforded a stable environment and tolerate high antifungal concentrations, they usually result in the induction of resistance genes when exposed to antimicrobial agents.\textsuperscript{3,29} However, Mukherjee \textit{et al.} found the expression of drug efflux pump genes (CDR and MDR) decreased as the biofilms aged, suggesting that drug efflux pumps do not play a significant role in biofilm resistance to azoles.\textsuperscript{30}

Indeed, acquired resistance \textit{in vivo} is a long-term gradual development process involving a multi-level network regulatory system.\textsuperscript{31} If the intermediate process is ignored, it is possible to draw a one-sided conclusion that biofilm-related virulence is irrelevant to drug resistance and events that play an important role would be omitted, which is not helpful in the search for the basis of this adaptive evolution. From our perspective, \textit{C. albicans} combats drug pressures by several efficient means, such as the upregulation of genes encoding efflux pumps and the enhanced expression of genes encoding the drug target enzyme or mutations.\textsuperscript{32} In order to reduce the “cost” of gene mutations, the organism exhibits a preference for suppressing other cellular processes including the biofilm formation we studied here.\textsuperscript{29,33} During long-term fluconazole treatment or under an escalating dosage schedule, the capacity to form biofilm as a “protective net” gradually increase until it reaches a plateau and only declines later once the MIC passes a second threshold.

In summary, our research broadens knowledge on the relationship between biofilm formation and the evolution of fluconazole resistance in \textit{C. albicans}, which appears to be more complicated than we had first thought. However, our study has some limitations: (1) We only focused on changes of \textit{C. albicans in vitro}. As the cause of most infections in humans, biofilm formation \textit{in vivo} embraces conditions considerably different from those in standard \textit{in vitro} assays, such as liquid flow, host factors, and different components of the host’s immune response.\textsuperscript{34} (2) The durations of the early phase and maturation stage of biofilm formation \textit{in vivo} seem to be shorter.\textsuperscript{35} Therefore, the potential of these findings needs to be confirmed by results with models \textit{in vivo}. (3) due to the small number of samples examined, additional studies will be needed to examine the relationship between biofilm formation and resistance in larger cohorts of patients.

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Figure 2. Continued.
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References

[1] Lohse MB, Gulati M, Johnson AD, et al. Development and regulation of single- and multi-species Candida albicans biofilms. Nat Rev Microbiol 2018;16(1):19–31. doi:10.1038/nrmicro.2017.107.

[2] Cavalheiro M, Teixeira MC. Candida Biofilms: Threats, Challenges, and Promising Strategies. Front Med (Lausanne) 2018;5:28. doi:10.3389/fmed.2018.00028.

[3] Mathé L, Van Dijck P. Recent insights into Candida albicans biofilm resistance mechanisms. Curr Genet 2013;59(4):251–264. doi:10.1007/s00294-013-0400-3.

[4] Williams DW, Jordan RP, Wei XQ, et al. Interactions of Candida albicans with host epithelial surfaces. J Oral Microbiol 2013;5. doi:10.3402/jom.v5i0.22434.

[5] Winter MB, Salcedo EC, Lohse MB, et al. Global Identification of Biofilm-Specific Proteolysis in Candida albicans. mBio 2016;7(5):e01514-16. doi:10.1128/mBio.01514-16.

[6] Li X, Yan Z, Xu J. Quantitative variation of biofilms among strains in natural populations of Candida albicans. Microbiology (Reading) 2003;149(Pr 2):353–362. doi:10.1099/mic.0.25932-0.

[7] Förster TM, Mogavero S, Dräger A, et al. Enemies and brothers in arms: Candida albicans and gram-positive bacteria. Cell Microbiol 2016;18(12):1709–1715. doi:10.1111/cmi.12637.
[8] White TC, Pfaffer MA, Rinaldi MG, et al. Stable azole drug resistance associated with a strain of Candida albicans from an HIV-infected patient. Oral Dis 1997;3(Suppl 1):S102–S109. doi:10.1111/j.1601-0825.1997.tb00336.x.

[9] Klotz SA, Drutz DJ, Zajic JE. Factors governing adherence of Candida species to plastic surfaces. Infect Immun 1985;50(1):97–101. doi:10.1128/iai.50.1.97-101.1985.

[10] Pais P, Galocha M, Viana R, et al. Microevolution of the pathogenic yeasts Candida albicans and Candida glabrata during antifungal therapy and host infection. Microb Cell 2019;6(3):142–159. doi:10.15698/mic.2019.03.670.

[11] Ford CB, Funt JM, Abbey D, et al. The evolution of drug resistance in clinical isolates of Candida albicans. Ellef 2015;4:e00662. doi:10.7554/eLife.00662.

[12] Chandra J, Mukherjee PK. Candida Biofilms: Development, Architecture, and Resistance. Microbiol Spectr 2015;3(4). doi:10.1128/microbiolspec.MB-0020-2015.

[13] Krasowska A, Sigler K. How microorganisms use hydrophobicity and what does this mean for human needs. Front Cell Infect Microbiol 2014;4:112. doi:10.3389/fcimb.2014.00112.

[14] Samaranayake YH, Wu PC, Samaranayake LP, et al. Relationship between the cell surface hydrophobicity and adherence of Candida krusei and Candida albicans to epithelial and denture acrylic surfaces. APMIS 1995;103(10):707–713.

[15] Romo JA, Zhang H, Cai H, et al. Global Transcriptomic Analysis of the Candida albicans Response to Treatment with a Novel Inhibitor of Filamentation. mSphere 2019;4(5):e00620-19. doi:10.1128/mSphere.00620-19.

[16] Konstantinidou N, Morrissey JP. Co-occurrence of filamentation defects and impaired biofilms in Candida albicans protein kinase mutants. FEMS Yeast Res 2015;15(8):fov092. doi:10.1093/femsyr/fov092.

[17] Carlisle PL, Banerjee M, Lazzell A, et al. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. Proc Natl Acad Sci USA 2009;106(23):959–64. doi:10.1073/pnas.0804061106.

[18] Sudbery PF. Growth of Candida albicans hyphae. Nat Rev Microbiol 2011;9(10):737–748. doi:10.1038/nrmicro2636.

[19] Park YN, Srikantha T, Daniels KJ, et al. Protocol for identifying natural associates that selectively affect adhesion, thickness, architecture, cellular phenotypes, extracellular matrix, and human white blood cell penetrability of Candida albicans biofilms. Antimicrob Agents Chemother 2017;61(7):e00584-17. doi:10.1128/AAC.00584-17.

[20] Coste A, Selmecki A, Forche A, et al. Genotypic evolution of azole resistance mechanisms in sequential Candida albicans isolates. Eur J Cell Biol 2007;86(10):1889–1904. doi:10.1016/j.ejcb.2007.06.012.

[21] Coste A, Turner V, Ilicher F, et al. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics 2006;172(4):2139–2156. doi:10.1534/genetics.105.054767.

[22] Popp C, Hampe I, Hertlein T, et al. Competitive Fitness of Fluconazole-Resistant Clinical Candida albicans Strains. Antimicrob Agents Chemother 2017;61(7):e00584-17. doi:10.1128/AAC.00584-17.

[23] Nobile CJ, Fox EP, Nett JE, et al. A recently evolved transcriptional control model of the Candida albicans Response to Treatment with a Novel Inhibitor of Filamentation. mSphere 2019;4(5):e00620-19. doi:10.1128/mSphere.00620-19.

[24] Prasad R, Shah AH, Rawal MK. Antifungals: Mechanism of Action and Drug Resistance. Adv Exp Med Biol 2016;892:327–349. doi:10.1007/978-3-319-25304-6_14.

[25] White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother 2011;55(2):940–942. doi:10.1128/AAC.00995-10.

[26] Hoot SJ, Smith AR, Brown RP, et al. An A643V amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in Candida albicans. Antimicrob Agents Chemother 1997;41(7):1488–1494. doi:10.1128/AAC.41.7.1488.

[27] Coste A, Turner V, Ilicher F, et al. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics 2006;172(4):2139–2156. doi:10.1534/genetics.105.054767.

[28] Seneviratne CJ, Suriyanarayanan T, Swarup S, et al. Transcriptomics Analysis Reveals Putative Genes Involved in Biofilm Formation and Antifungal Resistance of Enterococcus faecalis. J Endod 2017;43(6):949–955. doi:10.1016/j.joen.2017.01.020.

[29] Hoot SJ, Smith AR, Brown RP, et al. An A643V amino acid substitution in Upc2p contributes to azole resistance in well-characterized clinical isolates of Candida albicans. Antimicrob Agents Chemother 2011;55(2):940–942. doi:10.1128/AAC.00995-10.

[30] White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother 1997;41(7):1488–1494. doi:10.1128/AAC.41.7.1488.

[31] Ford CB, Funt JM, Abbey D, et al. Microevolution of the pathogenic yeasts Candida albicans and Candida glabrata during antifungal therapy and host infection. Microb Cell 2019;6(3):142–159. doi:10.15698/mic.2019.03.670.

[32] Carlisle PL, Banerjee M, Lazzell A, et al. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. Proc Natl Acad Sci USA 2009;106(23):959–64. doi:10.1073/pnas.0804061106.

[33] Sudbery PF. Growth of Candida albicans hyphae. Nat Rev Microbiol 2011;9(10):737–748. doi:10.1038/nrmicro2636.

[34] Park YN, Srikantha T, Daniels KJ, et al. Protocol for identifying natural associates that selectively affect adhesion, thickness, architecture, cellular phenotypes, extracellular matrix, and human white blood cell penetrability of Candida albicans biofilms. Antimicrob Agents Chemother 2017;61(11):e01319-17. doi:10.1128/AAC.01319-17.

[35] Hosseini M, Shapouri Moghaddam A, Derakhshan S, et al. Correlation between biofilm formation and antibiotic resistance in MRSA and MSSA isolated from Clinical Samples in Iran: A Systematic Review and Meta-Analysis. Microb Drug Resist 2020;26(9):1071–1080. doi:10.1089/mdr.2020.00031.

[36] Yang X, Dong F, Qian S, et al. Accessory gene regulator (agr) dysfunction was unusual in Staphylococcus aureus isolated from Chinese children. BMC Microbiol 2019;19(1):95. doi:10.1186/s12866-019-1465-z.