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*Review*

# Genetic determinants of antifungal resistance in *Candida* species

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**In the previous decades, it has been an increase in cases of resistance to antifungal agents used in the prophylaxis and treatment of infections caused by *Candida* species. The emergence of resistance to drug classes, it is usually explained by genome alterations ranging from point mutations to gain or loss of whole chromosomes. Therefore, identify and understand into the molecular mechanisms that underlie resistance of the *Candida* spp. is important because this information can provide appropriate treatments in emergency candidemia cases. In this sense, this review have the aim of discuss about the genetic variations associated the antifungal resistance in *Candida* spp.**

**Key words:** Antifungal agents, candidiasis, molecular characterization, mutations, overexpression.

## INTRODUCTION

*Candida* spp. can cause several types of infections with a wide spectrum of clinical presentations, from benign skin-mucosal forms to invasive ones that compromise various human organs (Pfaller and Diekema, 2010). Prophylaxis or prolonged treatment with antifungal agents has increased the incidence of clinical isolates of *Candida* spp. that are resistant to these drugs (Sanglard et al., 2003). Although 50% of worldwide candidemia cases are caused by *C. albicans*, it has been an increase in cases with non-*albicans* species over the past 20 years (Diekema et al., 2012; Healey et al., 2016). Therefore, the identification and characterization of such isolates at the molecular level is important for understanding the spread of *Candida* spp. and the mechanisms of their resistance to antifungal agents (Silva et al., 2016).

The resistance is typically assessed by measuring drug MICs and comparing results to reference breakpoints. Reference broth microdilution testing methods and clinical breakpoint MICs for drug against *Candida* spp. have been developed by the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (CLSI, 2012; Leclercq et al., 2013). Also there are the methods instead employ commercial assays such as Sensititre YeastOne (SYO; Trek Diagnostics) and Etest (bioMérieux) or automated systems like the Vitek 2 (bioMérieux) antifungal testing instrument (Shields et al., 2015).

The antifungal drugs are limited to treat candidiasis. The classes of medications most commonly are polyenes

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(amphotericin B and nistadine), but this class use is more restricted due to nephrotoxicity; pyrimidine analogs (flucytosine); azoles (fluconazole, itraconazole, and voriconazole) and echinocandins (caspofungin, anidufungina, and micafungin). Resistance to antifungal agents may be intrinsic to an organism or may be the result of extensive drug use in the prophylaxis or treatment of fungal infections (Da Matta et al., 2007). Typically, resistance can be attributed to mutations in or increased expression of genes that encode ATP-binding cassette proteins (ABC-type proteins), enzymes that are responsible for ergosterol and glucan biosynthesis, or transcription factors (Barker and Rogers, 2006).

Antifungal drug resistance is nearly always due to fungal genome alterations ranging from point mutations to gain or loss of whole chromosomes. The challenge is to identify these mutations to provide insights into the molecular mechanisms that underlie resistance of the *Candida* spp. This review is about the genetic variations associated the antifungal resistance in *Candida* spp.

## POLYENES

Amphotericin B is one of the earliest antifungals from the polyene class and is considered the reference drug for treatment of most of systemic fungal infections. Amphotericin B acts by binding to sterols in cell membranes, resulting in a leakage of cellular components and cell death. Its action spectrum includes all *Candida* spp., some species of *Aspergillus*, *Blastomyces dermatitidis*, and other fungi. This drug is not absorbed by the gastrointestinal tract and must be administered intravenously with hospital supervision (Batista et al., 1999).

Resistance mechanisms for this class of drugs have not yet been fully elucidated. However, it is believed that alterations in the steroids of the cell membrane and their phospholipid profile, protection against oxidative damage, and mutations in genes involved in ergosterol biosynthesis, especially in *ERG6*, may be related to resistance in *Candida* spp. (Perea and Patterson, 2002).

Vandeputte et al. (2008) identified a specific non-sense mutation (encoding a stop codon) in *ERG6* that resulted in a decrease in the ergosterol content of a clinical isolate of *C. glabrata* resistant to amphotericin B (Table 1). Hull et al. (2012) identified two mutations (T121V and T121I) in *ERG2* in two isolates of *C. glabrata* with decreased sensitivity to amphotericin B. Replacement of the threonine in the 121$^{st}$ position by valine or isoleucine affected *ERG2* function, promoting a reduction in the sensitivity of these isolates to amphotericin B (Table 1).

## PYRIMIDINE ANALOGS

Flucytosine (5-fluorocytosine or 5-FC) is a pyrimidine that transforms itself within the fungal cell into 5-fluorouracil and then to 5-fluordesoxiuridine. These latter molecules behave as antimetabolites and interfere with the normal biosynthesis of nucleic acids and nucleotides vital for fungal growth. This drug is indicated for infections caused by *Cryptococcus neoformans*, *Candida* spp., *Torulopsis* spp., and *Aspergillus* (Vermes et al., 2000).

Mutations that lead to a decrease or halt in the drug's import or its intracellular conversion are often responsible for resistance to pyrimidine analogs. The most frequent mechanism of resistance is a mutation in *FUR1* (5-fluorouridine resistant 1), which encodes the enzyme responsible for the intracellular conversion of 5-fluorouracil into metabolites capable of being integrated into cytosine metabolism. A point mutation that resulted in the replacement of arginine with cysteine at the 101$^{st}$ position of *FUR1* was found to be associated with resistance to 5-FC in *C. albicans* by Hope et al. (2004) (Table 1).

Florent et al. (2009) demonstrated that the mutation T26C, which results in the amino acid change M19T of the FCY1 (fluorocytosine resistance 1) gene that encodes cytosine deaminase, which is responsible for the conversion of 5-FC to 5-fluorouridine monophosphate, was associated with a *C. lusitane* isolate's resistance to 5-FC.

## AZOLES

The azoles have a wide spectrum of action, and so are the most commonly used drug class in the treatment of diseases caused by fungi, particularly by *Candida* spp. Ergosterol is an essential component in maintaining both the integrity and the function of the fungal plasma membrane and is the target of many antifungal agents, including the azoles. The ergosterol biosynthetic pathway converts acetic acid into ergosterol using a number of enzymes, similar to what occurs in the biosynthesis of cholesterol in mammals.

Fluconazole, voriconazole, itraconazole, and posaconazole act on the ergosterol biosynthetic pathway of the fungal membrane through inhibition of the enzyme 14α-demethylase (*Erg11p* or *14DM*), which is dependent on cytochrome P450. Thus, the conversion oflanosterol into ergosterolis prevented, increasing both the permeability and the progressive instability of the fungal cell (Barker and Rogers, 2006; Vandeputte et al., 2005).

Several studies have been conducted for the elucidation of the molecular mechanisms responsible for development of resistance to azoles in clinical isolates of *Candida* spp. Defect in DNA repair may account for accelerated emergence of various genetic changes responsible for drug resistance. The role of DNA repair in fungal pathogens, especially in the emergence of antifungal resistance, has not been explored in depth (Healey et al., 2016).

**Table 1.** Genes associated with antifungal resistance in clinically relevant *Candida* species.

| Class of molecule | Gene name | Location of genes | Mechanism | Fungal species | Class of antifungal | References |
|---|---|---|---|---|---|---|
| Transport proteins of the type ABC | CDR1 | Chr 3 | Overexpression | *C. albicans* | Azoles | Holmes et al. (2006) |
| | CDR2 | Chr 3 | Overexpression | *C. albicans* | Azoles | |
| Enzyme complex in the biosynthesis of ergosterol | ERG2 | Chr 1 | Point mutation | *C. glabrata* | Polyenes | Hull et al. (2012) |
| | ERG3 | Chr 1 | Point mutation | *C. albicans* | Azoles | Sanglard et al. (2003) |
| | ERG6 | Chr 3 | Point mutation | *C. glabrata* | Polyenes | Vandeputte et al. (2008) |
| | ERG11 | Chr 5 | Overexpression<br>Point mutation | *C. albicans*<br>*C. dubliniensis*<br>*C. glabrata*<br>*C. krusei*<br>*C. tropicalis* | Azoles | Carvalho et al. (2013), Eddouzi et al. (2013), Jiang et al. (2012) and Silva et al. (2016) |
| Enzyme complex of the glucan biosynthesis | FKS1 | Chr 1 | Point mutation | *C. glabrata* | Polyenes | Zimbeck et al. (2010); Naicker et al. (2016) |
| | FKS2 | Chr R | Point mutation | | | |
| Transcription factors | UPC2 | Chr 1 | Unknown | *C. albicans* | Azoles | Heilmann et al. (2010) |
| | TAC1 | Chr 5 | Point mutation | *C. albicans* | Azoles | Coste et al. (2004) |
| | PDR1 | Chr A | Point mutation | *C. glabrata* | Azoles | Tsai et al. (2010) |
| | MRR1 | Chr 3 | Point mutation | *C albicans* | Azoles | Dunkel et al. (2008) |
| Major facilitator superfamily | MDR1 | Chr 6 | Overexpression | *C. albicans,*<br>*C. dubliniensis* | Azoles | Chau et al. (2004) and Akins (2005) |
| | PDH1 | Chr F | Overexpression | *C. glabrata* | Azoles | Izumikawa et al. (2003) |
| | PDR16 | Chr 1 | Overexpression | *C. albicans* | Azoles | Saidane et al. (2006) |
| Other | FCY1 | Chr 6 | Point mutation | *C. lusitaniae* | Pyrimidine analogs and Azoles | Florent et al. (2009) |
| | FCY2 | Chr 3 | Point mutation | *C. lusitaniae* | Pyrimidine analogs and Azoles | Florent et al. (2009) |
| | FCY22 | Chr 2 | Point mutation | *C. albicans* | Pyrimidine analogs | Chapeland-Leclerc et al. (2005) |
| | FCA1 | Chr 6 | Point mutation | *C. albicans* | Pyrimidine analogs | Hope et al. (2004) |
| | FUR1 | Chr5 | Point mutation | *C. albicans* | Pyrimidine analogs | Hope et al. (2004) |

ABC: ATP-binding cassette. MFS: Major Facilitator Superfamily; Chr R, 1, 2, 3. 5 and 6: Chromosome location based on the complete genome of *C. albicans* SC5314.Chr A and F: Location of the chromosome based on complete genome of *C. glabrata* CBS138.

Recently, Healey et al. (2016) demonstrated that a mutator phenotype caused by a mismatch repair defect is prevalent in *C. glabrata* clinical isolates. Strains carrying alterations in mismatch repair gene MSH2 exhibit a higher propensity to breakthrough antifungal treatment in vitro and are recovered at a high rate from patients. This genetic mechanism promotes the acquisition of resistance to multiple antifungals, at least partially explaining the elevated rates of triazole and multi-drug resistance associated with *C. glabrata*. The identification the MSH2 defects in infecting strains

may influence the management of patients on antifungal drug therapy.

One such mechanism involves efflux pumps that export the antifungal agent from the intracellular environment to the extracellular environment, thus reducing its intracellular concentration (Holmes et al., 2006). Studies conducted with fluconazole have demonstrated that this drug is actively transported into the extracellular environment by fungal cells in an energy-dependent manner and that most antifungal efflux is caused by the overexpression of genes that encode membrane transport proteins (Holmes et al., 2006).

Two families of efflux membrane transporters can be distinguished in yeasts according to the energy source used for extrusion of substrates (Marie and White, 2009). The genes *CDR1* and *CDR2* (*Candida* drug resistance 1 and 2) encode the ABC-type transport proteins that act as transmembrane efflux pumps, using ATP hydrolysis to transport substrates across the membrane. The expression of *CDR1* and *CDR2* is regulated by *TAC1* (Transcriptional activator of CDR genes 1). Hyper-activation of the *TAC1* transcription factor is conferred by gain-of-function mutations that consequently promote the overexpression of *CDR1* and *CDR2* (Coste et al., 2004).

In addition to *CDR1* and *CDR2*, the *MDR1* gene (Multi-drug resistance 1) is directly involved in fluconazole resistance. This gene encodes a permease protein of the *MFS* (Major facilitator superfamily) type, acting as a membrane carrier and using a proton electrochemical gradient for the transport of substrates. The expression of *MDR1* is regulated by at least three transcription factors, the most commonly described among them being the *MRR1* transcription factor (multi-drug resistance regulator 1). The hyperactivation of *MRR1* is conferred by gain-of-function mutations in *C. albicans* and *C. dubliniensis* (Table 1), leading to overexpression of *MDR1* (Schubert et al., 2008).

Changes in ergosterol biosynthesis represent another mechanism of azole resistance. This mechanism bypasses the inactivation of the sterol enzyme Δ5,6-desaturase (encoded by the gene *ERG3*) that acts just upstream of 14α-demethylase in the ergosterol biosynthesis pathway, converting 14α-metilfecoesterol into 14α-methyl-3,6-diol. As the sterol 14α-metilfecoesterolis capable of supporting fungal cell growth and 14α-methyl-3,6-diol is toxic, inactivation of the Δ5,6-desaturase sterol promotes resistance to azoles (Chau et al., 2005). Thus, isolates with changes in this enzymatic step show a selective advantage when subjected to the action of azoles (Akins, 2005).

Mutations in the *ERG11* gene of *Candida* spp. are also involved in resistance to azoles. *ERG11* is located on chromosome 5 and demonstrates significant variation in size according to the species, from 1,569 bp in *C. parapsilosis*to 2,669 bp in *C. glabrata*. Mutations in *ERG11* confer resistance to azoles by reducing the binding affinity of the drug (Barker and Rogers, 2006).

Several studies have compared *ERG11*gene sequences from isolates of different *Candida* spp. that are susceptible and resistant to azoles, *C. albicans* being the most studied species in this regard (Carvalho et al., 2013; Chau et al., 2004).

Vandeputte et al. (2005) studied isolates of *C. tropicalis* that were resistant to fluconazole, they discovered a missense mutation (Y132F) responsible for resistance that had been previously reported in *C. albicans* by Chau et al. (2004). Carvalho et al. (2013) investigated *ERG11* mutations in clinical isolates of *C. albicans*, *C. glabrata* and *C. tropicalis* that had been previously evaluated by fluconazole-susceptibility tests, identified fourteen different missense mutations, five of which had not been previously described, including a new L321F mutation identified in an isolate of *C. albicans* resistant to fluconazole.

Silva et al. (2016) identified three new synonymous mutations in the ERG11 gene in the isolates of *C. glabrata* (C108G, C423T and A1581G) and two new nonsynonymous mutations in the isolates of *C. krusei*:-A497C (Y166S) and G1570A (G524R), with dose dependent sensitivity to voriconazole. The functional consequence of these nonsynonymous mutations was predicted using evolutionary conservation scores. The Y166S mutation can affect the 14α-demethylase. This observation suggests a possible link between the mutation and dose-dependent sensitivity to voriconazole in the clinical isolate of *C. krusei*.

An increase in *ERG11* gene expression also results in resistance to antifungal agents because such an increase results in an elevated concentration of 14α-demethylase in the intracellular environment, requiring larger amounts of antifungals to inhibit enzyme activity. This mechanism has been revealed in various isolates of *C. albicans* resistant to fluconazole (Xu et al., 2015). *ERG11* gene expression is regulated by the *UPC2* (uptake control 2) transcription factor. In response to azole agents, gain-of-function mutations were identified in the *UPC2* gene, which led to hyperactivity. Such hyperactivity leads to the over-activation of*ERG11* gene expression. Over-expression of *ERG11*, in turn, significantly reduces the effect of the antifungal agent in cells, decreasing the cell's sensitivity (Heilmann et al., 2010).

## ECHINOCANDINS

Echinocandins are the most recent class of antifungal agent to be introduced in clinical practice for the treatment of infections caused by fungal species, especially those of the *Candida* genus (Cappelletty and Eiselstein-Mckitrick, 2007). The three antifungal drugs in this class caspofungin, micafungin, and anidulafungin have proven effective in treating candidiasis (Chandrasekar and Sobel, 2006). These drugs inhibit β-(1,3)-D-glucansynthetase, which is composed of a

complex of proteins and polycarbohydrates and is responsible for fungal cell wall synthesis. Blocking of this enzyme causes osmotic instability, compromising the integrity of the fungal membrane and causing mortality (Morris and Villmann, 2006).

Although the use of echinocandins in treatment is recent, strains resistant to this type of drug have already been described. The drop in sensitivity to echinocandins has been found to be associated to mutations in the Fks1p and Fks2p (FK506 sensitivity protein 1 and 2) subunits of the β-1,3-glucansynthetase complex, which is necessary for the production of β-1,3 glucan, an essential component of the *Candida* cell wall (Desnos-Ollivier et al., 2008 ).

Specifically, mutations have been found in two regions, hot spot 1 and hot spot 2 (composed of nine and eight amino acids, respectively), which show up in both genes. These mutations in *FKS1* and *FKS2* result in an inability of the echinocandins to inhibit the production of 1, 3-β- glucan (Perlin, 2007). Mutations in hot spot 1 of *FKS1* and *FKS2* are the most prevalent among a variety of fungal species resistant to this class of drugs. Zimbeck et al. (2010) found associations between mutations in hot spot1 of both *FKS1* and *FKS2* and *C. glabrata* resistant to echinocandins (Table 1).

Mutations in the FKS2 gene were found in two *C. glabrata* isolates with echinocandin resistance mediated (Naicker et al., 2016). Both isolates were cultured from urine specimens from private-sector patients. A mutation was detected in the hotspot 1 region of the FKS2 gene where serine was replaced by phenylalanine at position 663 (S663F). Other mutation, had change from arginine to lysine at amino acid position 1377 (R1377K) was identified in the FKS2 hotspot 2 region.

Shields et al. (2015) determined the FKS mutation rates in *Candida* spp. by systematic sequencing of at-risk isolates and to determine if discrepant echinocandin susceptibility results were associated with agent-specific FKS mutations. FKS mutations were detected in 5% of sequenced isolates and 2% of isolates overall. Corresponding rates among *C. glabrata* isolates were 8 and 4%, respectively. Among *C albicans* isolates, rates were 5 and <1%, respectively. Mutations occurred exclusively with prior echinocandin exposure and were not detected in other species. Isolates with discrepant susceptibility results did not harbor FKS mutations. Mutation rates among isolates resistant to ≥2, 1, and 0 agents were 75, 13 and 0%, respectively. The authors concluded that FKS mutations were uncommon among non-*C. glabrata* species, even with prior echinocandin exposure.

## CONCLUSION

Infections caused by *Candida* spp. are frequent complications of other conditions, such as pregnancy, use the medications (birth control pills, antibiotics, and steroids), Immune-suppressing diseases (including HIV), diabetes, obesity and others. Although the susceptibility of *Candida* to the currently available antifungal agents can be predicted, candidiasis infections can lead to serious health problems or cause death if left untreated. Lot of molecular mechanisms related to resistencia intrinsic or acquired *Candida* spp. to antifungals has been elucidated, however there are still many species that the mechanisms are even mysterious. In this sense, the genome sequencing of *Candida* species provides the opportunity to elucidate some of the mechanisms involved in intrinsic or acquired resistance by these yeasts. The scientific community has sought to develop strategies to understand and solve problems related to resistance, and one of these alternatives is the search for new bioactive molecules with antifungal activity based on the genetic and molecular characterization of resistant isolates, providing appropriate treatments in emergency cases that are not only socioeconomically, technologically, and industrially viable but are also based on the best specificity of new molecule activity.

## Conflict of interests

The authors have not declared any conflict of interest.

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