Screening for amino acid substitutions in the *Candida albicans Erg11* protein of azole-susceptible and -resistant clinical isolates: new substitutions and a review of the literature

Running title: *ERG11* mutations and azole susceptibility in *C. albicans*

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Abstract

For several years, azole antifungal drugs have been a treatment option for potentially life-threatening *Candida* infections. However, azole resistance can occur through various mechanisms such as alterations in *ERG11*, encoding lanosterol 14α-demethylase (CYP51). In this study, we investigated the antifungal susceptibility to fluconazole, itraconazole and voriconazole of 73 clinical isolates of *C. albicans*. Screening for amino acid substitutions in Erg11 was performed on each of the 73 isolates. Twenty isolates displayed a marked decrease in azole susceptibility. Amino acid substitutions were detected in more than two-thirds of the strains. In all, 23 distinct substitutions were identified. Four have not been described previously among which N136Y and Y447H are suspected to be involved in azole resistance. We suggest that the high genetic polymorphism of *ERG11* must be considered in the rationale design of new azole compounds targeting lanosterol 14α-demethylase. A review of all Erg11 amino acid polymorphisms described to date is given.

Introduction

Candida albicans is responsible for a wide spectrum of clinical infections in humans, ranging from mucosal infections such as vaginitis or oropharyngeal candidiasis to potentially life-threatening systemic infections such as candidemia. Because of their safety profile and high therapeutic index, azole antifungal drugs have been used to treat *Candida* infections for many years, either as first-line therapy, antifungal prophylaxis, empirical or pre-emptive treatment. However, as a consequence of long-term exposure to azole drugs, resistance can arise. To date, at least four distinct mechanisms have been shown to confer azole resistance in *C. albicans*: (i) reduced intracellular accumulation of azoles due to the overexpression of genes encoding efflux transporters belonging to the ATP-binding cassette superfamily (*CaCDR1* and *CaCDR2*) or major facilitator superfamily (*CaMDR1*); (ii) genetic alterations in the *ERG11* gene encoding lanosterol 14α-demethylase (CA-CYP51), the primary target of azoles; (iii) overexpression of the *ERG11* gene; and (iv) alterations in the ergosterol biosynthetic pathway (Sanglard and Odds, 2002). Importantly, these mechanisms are often combined in clinical isolates (Cernicka *et al.*, 2006; Chau *et al.*, 2004; Coste *et al.*, 2007; Franz *et al.*, 1998; Goldman *et al.*, 2004). Recently, Selmecki et al. demonstrated that azole resistance could also result from the formation of an isochromosome harboring *ERG11* and *TAC1* genes (encoding a transcription factor involved in *CDR1* and *CDR2* upregulation) through segmental aneuploidy (Selmecki *et al.*, 2006).

To our knowledge, more than 140 different amino acid substitutions have been reported to date in Erg11 of clinical isolates of *C. albicans* (reviewed in Table 1). This high genetic polymorphism suggests that lanosterol demethylase, is highly permissive to structural changes. Interestingly, most of these substitutions, instead of being randomly dispersed, are clustered into three hot spot regions ranging from amino acids 105 to 165, 266 to 287 and 405 to 488 (Marichal *et al.*, 1999). Several lines of evidence indicate that these amino acid changes do not contribute equally to azole resistance. Whereas some substitutions such as K143R, S405F, G464S, R467K or I471T have been recovered

exclusively from azole-resistant strains and their involvement in azole resistance has been confirmed using *in vitro* experiments (heterologous gene expression, affinity between azoles and CA-CYP51), others, such as E266D or V488I, probably do not contribute to azole resistance because they are found in both azole-resistant and azole-susceptible strains (Chau *et al.*, 2004; Kakeya *et al.*, 2000; Lamb *et al.*, 2000; Loffler *et al.*, 1997; Sanglard *et al.*, 1998). Finally, T315A, Y118A, Y118F, and Y118T have been clearly associated with resistance but have not yet been detected in clinical isolates (Chen *et al.*, 2007; Lamb *et al.*, 1997). Such findings clearly show that further study of azole-susceptible and azole-resistant strains is required for a better understanding of azole resistance mechanisms. Additionally, there is now increasing evidence that a mapping of all Erg11 amino acid changes involved in azole resistance, onto the active site and channels of the 3D modeled structure of CA-CYP51 could help in the design of new azole antifungals with potent activity against resistant strains (Chen *et al.*, 2007; Fukuoka *et al.*, 2003; Rupp *et al.*, 2005; Sheng *et al.*, 2004; Xiao *et al.*, 2004, Sheng *et al.*, 2009).

During the course of an ongoing project in our laboratory, aimed at the design and synthesis of new azole antifungal drugs through a modeling approach, Erg11 amino acid substitutions were screened in a large number of azole-susceptible and -resistant clinical isolates of *C. albicans* (Giraud *et al.*, 2008; Lebouvier *et al.*, 2007; Pagniez *et al.*, 2002). All of the amino acid changes occurring in Erg11 are reviewed with reference to the literature.

Materials and Methods

C. albicans strains

A collection of 73 clinical isolates of *C. albicans* from the Mycobank of the Laboratory of Parasitology and Medical Mycology, Nantes University Hospital, France, was investigated. Most of the strains (n=62) were isolated during routine laboratory procedures from different hospitals. The remaining isolates were provided by the French National Reference Center for Mycoses and

Antifungals (Institut Pasteur, Paris, France; n=11). All strains were identified using either the VITEK2 system® or chromogenic medium Candida ID2® (bioMérieux, Marcy l'Etoile, France). These strains, selected for their antifungal susceptibility profile (azole-susceptible as well as azole-resistant strains), represented a large variety of clinical samples. Eighteen isolates were recovered from blood cultures (Table 2).

Antifungal drug susceptibility testing

Antifungal susceptibility to fluconazole, itraconazole and voriconazole was determined for each isolate using the broth microdilution reference method as recommended by the Clinical Laboratory Standards Institute (CLSI) document M27-A2 (NCCLS, 2002). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls. Fluconazole was purchased from Sigma (Saint Quentin Fallavier, France), itraconazole from Janssen-Cilag (Neuss, Germany), and voriconazole was obtained from Mycobiotics (Université de Nantes, France). Minimum inhibitory concentration (MIC) that is the lowest drug concentration that resulted in 50% growth inhibition relative to the growth in the control well, was determined visually after 48h of incubation at 35°C. MIC values for fluconazole, itraconazole and voriconazole were compared to the CLSI interpretative guidelines on antifungal susceptibility testing. Briefly, MICs ≤8 μg/mL were considered as susceptible (S), 16–32 μg/mL as susceptible dose-dependent (SDD) and ≥64 μg/mL as resistant (R) for fluconazole, MICs ≤0.125 μg/mL as susceptible, 0.25–0.5 μg/mL as SDD and ≥1 μg/mL as resistant for itraconazole, and MICs ≤1 μg/mL as susceptible, 2 μg/mL as SDD and ≥4 μg/mL as resistant for voriconazole.

***ERG11* gene amplification and sequencing**

Amplification was performed directly from colonies grown on Sabouraud's agar plates without prior DNA extraction. Briefly, a single colony was gently removed with a micropipette tip and transferred

directly to a PCR tube. The complete *ERG11* open reading frame (1451 bp) was amplified with a PTC-100 thermocycler (MJ Research Inc., Walthal, MA, USA) using the primers described previously: ERG11ORF-F - GAAAGGGAATTCAATCG and ERG11ORF-R - TGTTAATCCAACTAAGTAAC (Lee *et al.*, 2004). Reaction mixtures contained 1 µM of each primer, 10 µL of 5X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.1 U of GoTaq® Flexi DNA polymerase (Promega, Madison, Wisc., USA) and sterile water up to a final volume of 50 µL. Amplification parameters were as follows: initial denaturation at 96°C for 3 min followed by 35 cycles of annealing at 56°C for 1 min, elongation at 72°C for 2 min, and denaturation at 95°C for 1 min. PCR products were purified and sequencing was performed using a BigDye terminator sequencing kit on a ABI PrismR 3130 genetic analyzer (Applied Biosystems, Foster City, CA) using additional primers (Lee *et al.*, 2004). Nucleotide sequences were assembled using Seqscape Navigator software (Applied Biosystems). For each strain, the entire *ERG11* open reading frame sequence was compared to a previously described *ERG11* sequence (accession number X13296) obtained from a fluconazole-susceptible strain (Lai *et al.*, 1989). Silent mutations were not considered. Each substitution leading to an amino acid change was inspected visually for allelic dosage (homozygosity or heterozygosity). Each of the new amino acid change was checked by a second round of amplification and sequencing.

Nucleotide sequence accession number

The *ERG11* sequences of the strains displaying new amino acid substitutions have been submitted to GenBank database under accession numbers EU885932 to EU885938.

Results

Antifungal susceptibility testing

The results of *in vitro* susceptibility testing for the 73 clinical isolates of *C. albicans* are shown in Table 2. According to the CLSI breakpoints, most of the isolates were susceptible to fluconazole, itraconazole and voriconazole (n=53, 73%). Twenty isolates (27%) exhibited a marked decrease in azole susceptibility, including nine which were resistant to at least one of the three antifungal drugs. More precisely, 15 isolates had reduced susceptibility to fluconazole (7 SDD and 8 R), 18 to itraconazole (12 SDD and 6 R) and six to voriconazole (1 SDD and 5 R). Cross-reduced azole susceptibility was observed for six isolates. Most of these isolates were recovered from the mouth or respiratory tract. All but one of the 18 bloodstream isolates were azole-susceptible.

Analysis of Erg11 amino acid substitutions

Amplification of the complete *ERG11* gene and screening for amino acid substitutions was performed for each of the 73 clinical isolates (Table 2). As expected from previous studies, a large number of silent polymorphisms were identified (data not shown). Only 13 isolates had no amino acid substitution. All were azole-susceptible and six were bloodstream isolates. Interestingly, among the remaining 60 isolates carrying amino acid substitutions in Erg11 (40 azole-susceptible and 20 isolates with reduced azole susceptibility), the number of amino acid substitutions varied between the isolates and ranged from one (n=13) to four substitutions per strain (n=4). Most of the isolates had two amino acid changes (n=35). In all, 23 distinct amino acid substitutions were identified including some which have previously been associated with resistance. Interestingly, the following four are new: N136Y, Y221H, L276S and Y447H. Most of the substitutions (88/123, 72%) occurred in both *ERG11* alleles (i.e. homozygous). The 23 distinct amino acid changes identified in our study could be divided into two subsets. The first subset consisted of substitutions that appeared to be restricted to isolates displaying a reduced azole susceptibility phenotype. The following 12 substitutions fall into this category: Y132F, Y132H, K143R, F145L, G307S, S405F, G448E, G448V, G450E, V456I and the new amino acid changes N136Y and Y447H. Of note, these two new

substitutions were recovered from two isolates displaying reduced susceptibility to more than one antifungal (CAAL-61 and CAAL-70). Strikingly, CAAL-61, which contained Y447H, was resistant to fluconazole (MIC >64 µg/mL) and itraconazole (MIC >16 µg/mL) but remained highly susceptible to voriconazole (MIC =0.125 µg/mL). The second subset concerned substitutions recovered from either azole-susceptible or reduced susceptibility isolates and included 11 amino acid changes: D116E, K128T, G129A, D153E, V159I, E266D, V437I, G464S, V488I, and the new substitutions Y221H and L276S. Y221H and L276S occurred in a single allele in two susceptible strains (CAAL-62 and CAAL-100 respectively). D116E, K128T, E266D, and V488I appeared in a large number of isolates (n=29, 19, 18 and 17 isolates, respectively). D116E was frequently recovered in combination with K128T (n=19 isolates) whereas E266D was recovered with V488I (n=16). G129A occurred in a single allele in combination with D116E and E266D in a single azole-susceptible strain. Obviously, other mechanisms are required to explain azole resistance in some of our strains such as CAAL-90 harboring only this kind of amino acid substitutions.

Alignment of 15 different CYP51 amino acid sequences from various organisms, focusing on residues surrounding the four new amino acid changes (residues 136, 221, 276 and 447) as shown in Table 3, revealed that the two new amino acid changes with a potential involvement in azole resistance (N136Y and Y447H) affect highly conserved residues.

Discussion

Mutations in the *ERG11* gene sequence leading to amino acid substitutions represents one of the main mechanisms contributing to azole resistance in clinical isolates of *C. albicans*. To date, more than 140 distinct amino acid substitutions have been reported in the literature but only a few of these have been clearly associated with azole resistance. In this study, our aim was to screen for Erg11 amino acid substitutions in a large collection of *C. albicans* clinical isolates displaying various levels of azole susceptibility. In order to provide as complete a picture as possible of the genetic alterations

occurring in this gene, we chose to study the complete *ERG11* coding sequence instead of focusing on the three hot spot regions described previously (Marichal *et al.*, 1999). For each isolate, the complete *ERG11* gene was amplified using a colony-PCR approach, a quick and easy method based on amplification of a target gene directly from a single colony without any prior DNA extraction step (Luo *et al.*, 2002).

Our data clearly show that point mutations leading to amino acid changes are a frequent event in *ERG11* observed not only in azole-resistant strains but also in azole-susceptible ones. Indeed, only 13 of the 73 isolates (18%) displayed the wild-type sequence (i.e. no amino acid change), including six bloodstream isolates. In all, of the 23 distinct amino acid substitutions identified, 19 have been reported previously. Thus, four substitutions described in this study are new (N136Y, Y221H, L276S and Y447H). Except for Y221H, all substitutions were located in the three hotspot regions described previously (Marichal *et al.*, 1999). In agreement with a previous study, we report a high frequency of D116E, K128T, and E266D (White *et al.*, 2002). Moreover, a large number of clinical strains exhibited the same pattern of amino acid substitutions (D116E and K128T; 19/73, 26%).

Although all isolates displaying a phenotype of reduced azole susceptibility (20/20, 100%) had genetic alterations in Erg11, amino acid polymorphisms were also identified in susceptible isolates (40/53, 75%). This means that the presence or absence of amino acid polymorphism is usually not sufficient to predict azole susceptibility. Finally, allelic dosage (homozygosity *vs*. heterozygosity) must also be taken into consideration in this diploid species (Jones *et al.*, 2004). In this way, the finding that 11 of the 23 amino acid changes (D116E, K128T, G129A, D153E, V159I, E266D, V437I, G464S, V488I, and the two new Y221H and L276S) recorded here were found in both azole-susceptible and -resistant strains strongly suggests that these substitutions are probably not associated with resistance. Whereas, this is largely supported by previous studies for D116E, K128T, D153E, E266D, V437I, and V488I, the case of G129A and G464S needs to be discussed in light of our results (Chau *et al.*, 2004; Favre *et al.*, 1999; Franz *et al.*, 1998; Li *et al.*, 2004; Marichal *et al.*,

1999; Park *et al.*, 2005; Perea *et al.*, 2001; White *et al.*, 2002; Xu *et al.*, 2008). According to a previous report, G129A alone is not sufficient to confer azole resistance but has been shown to be responsible for a 16-fold increase in fluconazole MIC when combined with G464S (Sanglard *et al.*, 1998). Here, the recovery of G129A in an azole-susceptible strain support the hypothesis that G129A must be associated with specific substitutions to contribute to azole resistance in *C. albicans*. Regarding G464S, previously associated with resistance by various methods (Chau *et al.*, 2004; Sanglard *et al.*, 1998), no robust hypothesis can be proposed to explain why this amino acid change occurred here in three highly azole-susceptible isolates. Similar discrepancies have been also reported in the literature for Y132H (Bellamine *et al.*, 2004; Sanglard *et al.*, 1998). Because the new substitutions Y221H and L276S were recovered from azole-susceptible isolates they are unlikely to be associated with resistance. V159I, a substitution that was rarely described in the literature occurred here in azole-susceptible isolates (CAAL-15 and CAAL-101).

As suggested previously, amino acid substitutions that appear to be restricted to reduced susceptibility isolates could be useful as predictive markers of azole resistance as well as for the rationale design of new azole antifungal drugs using homology models of CA-CYP51 constructed based on the X-ray crystal structure of *Mycobacterium tuberculosis* CYP51 (MT-CYP51) (Fukuoka *et al.*, 2003; Macchiarulo *et al.*, 2002; Park *et al.*, 2005; Podust *et al.*, 2001; Xiao *et al.*, 2004). Twelve substitutions recovered in our study fit this criterion because they are restricted to reduced susceptibility isolates: Y132F, Y132H, K143R, F145L, G307S, S405F, G448E, G448V, G450E, V456I and the two new substitutions N136Y and Y447H. Except for F145L, previously reported in susceptible as well as resistant strains, and V456I reported by Sanglard without information relative to the susceptibility of the corresponding strains (Sanglard and Bille, 2002), these findings are in agreement with previous reports. The contribution of some of the amino acid changes (Y132F, K143R, G307F, and S405F) to azole resistance has been confirmed using *in vitro* experiments (Chau *et al.*, 2004; Kakeya *et al.*, 2000; Lee *et al.*, 2004; Loffler *et al.*, 1997; Perea *et al.*, 2001; Sanglard *et*

al., 1998; White *et al.*, 2002). Hence, our data support the involvement of Y132F, Y132H, K143R, G307S, S405F, G448E, G448V, and G450E in azole resistance and their potential use as predictive markers of azole resistance.

The two new amino acid substitutions N136Y and Y447H were only recovered from isolates with a reduced azole susceptibility phenotype and are therefore likely to be involved in azole resistance. N136Y occurred in both alleles in a single isolate with reduced susceptibility to fluconazole, itraconazole and voriconazole. Complementary investigations revealed that this isolate also had a moderately high MIC to posaconazole (E-test, MIC =0.5 µg/mL). According to the secondary structure of CA-CYP51, residue 136 is close to the C helix, a region that is in close proximity to the heme and ligand binding site (Xiao *et al.*, 2004). In the MT-CYP51/FLZ crystal structure, the open BC loop and C helix exhibited high thermal motion suggesting significant changes in protein conformation. Being localized in the region of the mouth of channel 1, this residue could interfere with azole entry or its binding to the active site if the BC loop adopts a closed conformation (Podust *et al.*, 2001). Moreover, the finding that this residue is conserved between fungal CYP51s suggests that it may play an important role in CYP51s tri-dimensional conformation. Because N136Y occurred simultaneously with Y132H, more studies are now warranted to determine whether the resistant phenotype is the result of Y132H alone or is due to an additive effect with Y132H. Y447H was recovered from a single isolate displaying resistance to both fluconazole and itraconazole but that remained susceptible to voriconazole simultaneously with G307S a substitution that could be associated with azole-resistance according to Perea (Perea *et al.*, 2001). Y447H is close to the C terminus part of CA-CYP51 near the L helix and cysteine residue 470, and would therefore be too far away to interact directly with azole binding (Xiao *et al.*, 2004). However, this residue that appear to be conserved among *Candida* species is part of a large insertion located from residues 439 to 457 that was not modeled in detail in this report (Xiao *et al.*, 2004).

Obviously, the reduced susceptibility phenotype observed in some of our strains could not be explained by only studying the genetic alterations in *ERG11*. This again shows that several azole resistance mechanisms are frequently combined in clinical strains of *C. albicans* (Cernicka *et al.*, 2006; Chau *et al.*, 2004; Coste *et al.*, 2007; Franz *et al.*, 1998; Goldman *et al.*, 2004).

To conclude, our study highlights the high diversity and frequency of amino acid substitutions in Erg11, the primary target of azole antifungal drugs. This genetic polymorphism should be taken into consideration in the rationale design of new antifungal compounds targeting lanosterol 14α-demethylase with potent activity against resistant strains. Further experiments will provide answers regarding the contribution of the new amino acid substitutions to azole resistance.

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Figure legends

Table 1. Review of all Erg11 amino acid substitutions described to date in clinical isolates of *C. albicans*.

For each amino acid change, notes regarding the putative involvement in azole resistance and references are given. The new mutations identified in this study are underlined. Amino acid changes depicted in bold face have been clearly associated with azole resistance using the following *in vitro* experiments: increased MIC after heterologous gene expression in *Saccharomyces cerevisiae* by either site directed mutagenesis (a) or functional expression of *C. albicans* PCR-amplified *ERG11* (b), or decreased affinity of lanosterol 14α-demethylase for azole (c). The location of the three hot spot regions (I, II and III) is indicated. FLZ: fluconazole; ITZ: itraconazole; VOR: voriconazole; POS: posaconazole.

Table 2. Results of *in vitro* antifungal susceptibility testing and amino acid substitutions in Erg11 for the 73 clinical isolates of *C. albicans*.

MIC: minimum inhibitory concentration; FLZ: fluconazole; ITZ: itraconazole; VOR: voriconazole; BAL: bronchoalveolar lavage; h: heterozygous (i.e. mutation in a single allele). The new substitutions are shown in bold.

Table 3. Alignment of 15 CYP51 amino acid sequences from various organisms.

Only residues surrounding the four new amino acid substitutions N136Y, Y221H, L276S and Y447H are shown. The alignment was generated using the ClustalW program. Amino acid numbering is taken from the *C. albicans* gene sequence. Arrow heads indicate new amino acid changes. '-' is indicative of a gap in the protein sequence. Amino acids conserved across fungi are underlined in gray and amino acids conserved across all 15 CYP51 sequences are underlined in black. The following accession numbers have been used: *Candida albicans,* X13296; *Candida dubliniensis,* AY034876; *Candida tropicalis,* M23673; *Candida glabrata,* S75389; *Candida krusei,* S75391; *Saccharomyces cerevisiae,* M18109; *Aspergillus fumigatus,* AAF338659; *Schizosaccharomyces pombe,* Q09736; *Ustilago maydis,* Z48164; *Penicillium italicum,* Z49750; *Homo sapiens,* D55653; *Rattus rattus,* D55681, *Mycobacterium tuberculosis,* 1EA1_A; *Arabidopsis thaliana,* AB014459; *Dictyostelium discoideum,* XM 001134568.