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Major Article

ERG11 gene polymorphisms and susceptibility to fluconazole in *Candida* isolates from diabetic and kidney transplant patients

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Abstract

Introduction: Candidiasis is the most frequent opportunistic mycosis in humans and can cause mortality, particularly in immunodeficient patients. One major concern is the increasing number of infections caused by drug-resistant *Candida*s trains, as these cannot be efficiently treated with standard therapeutics. The most common mechanism of fluconazole resistance in *Candida* is mutation of *ERG11,* a gene involved in the biosynthesis of ergosterol, a compound essential for cell integrity and membrane function. **Methods:** Based on this knowledge, we investigated polymorphisms in the *ERG11* gene of 3 *Candida* species isolated from immunocompromised and immunocompetent patients. In addition, we correlated the genetic data with the fluconazole susceptibility profile of the *Candida* isolates. **Results:** A total of 80 *Candida albicans*, 8 *Candida tropicalis* and 6 *Candida glabrata* isolates were obtained from the saliva of diabetic, kidney transplant and immunocompetent patients. Isolates were considered susceptible to fluconazole if the minimum inhibitory concentration was lower than 8 μg/mL. The amino acid mutations F105L, D116E, K119N, S137L, and K128T were observed in *C. albicans* isolates, and T224C and G263A were found in *C. tropicalis* isolates. **Conclusions:** Despite the high number of polymorphisms observed, the mutations occurred in regions that are not predicted to interfere with ergosterol synthesis, and therefore are not related to fluconazole resistance.

Keywords: *Candida albicans*. Drug Resistance. Fluconazole. Sterol. 14α-demethylase.

INTRODUCTION

Candidiasis is the most frequent opportunistic mycosis in humans and is one of the major causes of mortality in immunocompromised patients[1,2]. The incidence of systemic fungal infections has increased steadily over recent years, mainly due to the growing number of organ and bone marrow transplants, cancer treatments, and AIDS cases, all of which are detrimental to the body's natural defense system[3]. In view of the compromised immune system in such cases, the availability of effective antifungal agents is particularly critical to treatment. However, treatment can be hindered by the presence of *Candida* species displaying multiple drug resistance (MDR), which is becoming more frequently reported[4,5].

Treatment for *Candida* infections normally involves triazole antifungals, of which fluconazole is the most commonly prescribed[6,7]. The azolic antifungals act by inactivating lanosterol 14α-demethylase, thereby inhibiting biosynthesis of ergosterol, a compound essential for cell integrity and membrane function[8,9]. This results in a decrease in the level of ergosterol available for membrane function and a concomitant increase in the number of intermediate metabolites[10]. Resistance to azole antifungals is commonly associated with continuous drug use, but can also because by intrinsic factors[11,12], such as mutations or alterations in the expression of the *CDR1*, *CDR2*, *PDR5*[13-14], *ERG3*[15], *MDR1*, *FLU1*[16], and *ERG11*[17] genes. The *ERG11* and *ERG3* genes encode proteins involved in the biosynthesis of ergosterol, and mutations at specific points in these two genes can critically alter the effectiveness of azolic antifungal drugs[18].

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Thus, this study aims to investigate polymorphisms in the *ERG11* gene, and correlate this genetic data to the fluconazole susceptibility profile of different *Candida* species isolated from diabetic and kidney transplant patients.

METHODS

Biological material

Samples of saliva were collected from 90 outpatients, including 40 diabetic patients (12 male and 28 female), 19 kidney transplant recipients (9 male and 10 female), and 31 immunocompetent patients (3 male and 28 female; control group). The 40 diabetic patients were between 40 and 70 years old and had been diagnosed with type II diabetes over five years prior. All had hypertension but were not using insulin, and 40 were diagnosed with hyperglycemia. All transplant patients were between 30 and 60 years old, and had received a kidney transplant over one year before the study. In addition, 19 of the transplant patients were under treatment with prednisone, azathioprine, and cyclosporine. The control group was composed of individuals between the ages of 18 and 30, who had not been diagnosed with any disease, and were not using drugs with antimicrobial or anti-inflammatory activities. The patients within the kidney transplant, diabetic and control groups were not paired by gender or age. A total of 94 *Candida* isolates were obtained, and the species were identified by Benedetti *et al*.[19]. Among the 70 isolates used in this study, 58 were classified as *Candida albicans*, 6 as *Candida glabrata* and 8 as *Candida tropicalis*.

Ethical standards

This study was performed in accordance with the ethical standards of the 1964 Helsinki declaration, and was approved by the Ethics Committee (*Conselho Nacional de Ética em Pesquisa - CONEP*) under registration no. 19885/2011, Universidade Paranaense (Parana-Brazil). Informed consent was obtained from all individual participants involved in the study.

Fluconazole minimum inhibitory concentration

The fluconazole minimum inhibitory concentration (MIC) of *Candida* isolates was determined using an E-Test Kit (Biomerieux, France) in Mueller-Hinton media (Difco, USA), supplemented with 2% glucose and 0.5 µg/mL methylene blue, according to the protocol of the Clinical and Laboratory Standards Institute[20]. The isolates were classified as susceptible to fluconazole if their MIC was ≤8 µg/mL; dose-dependent MIC values were from 8 to 32 µg/mL; and resistant isolates had MIC ≤ 32 µg/mL[21]. As controls, three reference strains were used: *C. albicans* ATCC – 44858 (MIC - 1 µg/mL), *C. glabrata* ATCC – 2001(MIC - 4 µg/mL), and *C. tropicalis* ATCC – 28707 (MIC - 2 µg/mL). Analysis of variance (ANOVA), was used to compare MIC values of individual isolates, as well as isolate groups (diabetic, kidney transplant, and immunocompetent control) using GraphPad Prism software, version 5 (GraphPad, USA) with a significance level of 0.05. When the MIC value was higher than that reported for each species, the differences between reference strains and the isolates were confirmed with a t-test.

DNA isolation, amplification, and partial sequencing of the *ERG11* gene

Total genomic DNA was purified from 2 day-old single colony cultures growing on Mueller-Hinton media (Difco, USA) using the Ultraclean Microbial DNA Isolation Kit(MoBio, USA) according to the manufacturer's instructions. Partial sequences of the *ERG11* gene were amplified using the following primer pairs: *C. albicans*[9], Sec1A (forward); 5'-TTAGTGTTTTATTGGATTCCTTGGTT-3', Sec1B (reverse); 5'-TCTCATTTCATCACCAAATAAAGATC-3',*C. tropicalis*[22], CtERGr1 - F (forward); 5'-TCTTTTGTCAACACAGTAATGGC-3', CtERGr1 - R (reverse); 5'–AACACCTTTACCAAAAACAGGAG-3', *C. glabrata*[23], CgERGr1 - F (forward); 5'-ACTACAATAACATGTCCACTGA-3', CgERGr1 – R (reverse); 5'-GGGTGGTCAAGTGGGAGTAA-3'. The amplification was performed as described by Xu *et al.*[9] in a final volume of 12.5 µL, containing 1 X Tris Base buffer solution, dNTPs (0.2 µM, Invitrogen, USA), $MgCl_2$ (1.6 mM), primers (15 pmol each), Taq DNA polymerase (0.5 U) (Invitrogen, USA), and template DNA (20 ng). The PCR products were purified using 7.5 M ammonium acetate (15 µL) and absolute ethanol (74 µL). Samples were incubated on ice for one hour, followed by centrifugation for 45 min at 23,100 *g*. The pellet was re-suspended in 12 µL of Milli-Q water. Sequencing of the PCR products was performed using a Big Dye Kit (Applied Biosystems, USA), followed by purification using Sephadex G-50 fine DNA grade resin (GE Healthcare, UK) in a MultiScreen Column Loader (Merck Millipore, USA), and analysis by electrophoresis in an ABI3500 Automatic Sequencer (Applied Biosystems, USA). The obtained sequences were visually inspected using the BioEdit program version 7.2.5[24], aligned using ClustalW[25], and manually adjusted using MEGA software, version 6[26]. Additional sequences were obtained from GenBank (**Table 1**) and alignments were generated in ClustalW. Amino acid prediction was performed using MEGA software, version 6[26].

RESULTS

Fluconazole minimum inhibitory concentration

All isolates with MIC values ≤ 8 µg/mL[21] were classified as susceptible to fluconazole (**Table 1**). The mean MIC values was higher for the species *C. glabrata* (p=0.02), the mean MIC were 1.43 µg/mL for *C. albicans,* 1.71 µg/mL for *C. tropicalis*, and 3.0 µg/mL for *C. glabrata*. Interestingly, although all *C. albicans* isolates were characterized as susceptible to fluconazole (MIC ≤ 8 µg/mL)[21], strains isolated from transplanted (MIC = 1.86 µg/mL) and diabetic (MIC =1.63 µg/mL) patients had higher fluconazole MIC values than the control group (MIC =0.94 µg/mL) (p=0.001) (**Table 1**).

Analysis of polymorphic sites in the *ERG11* gene of *Candida* isolates

The GenBank codes for the partial sequences of the *ERG11* gene from *Candida* isolates are listed in **Table 1**. The alignment of the *ERG11* sequence of the *C. albicans* isolates was 482 bp-

TABLE 1: Polymorphic sites in sequences of the *ERG11* gene from isolated species of *C. albicans*, *C. tropicalis* and *C. glabrata*, and associated amino acid prediction, fluconazole MIC values and GenBank codes.

Species	Isolate	Source	Mutation type*	Amino acid prediction**	MIC (μg/mL)	GenBank codes
C. albicans	ATCC- 44858	ATCC	Synonymous	F105L / D116E / S137L	1.0	MF411488
	CA_112_PC	Control	Missense	K128T	0.2	MF411470
	CA_2_PC	Control	Synonymous / Missense	F105L / K119N / K128T	0.5	MF411410
	CA_13_PC	Control	Synonymous	F105L / K119N	0.5	MF411414
	CA_136_PC	Control	Synonymous / Missense	F105L / K119N / K128T	0.5	MF411482
	CA_141_PC	Control	Synonymous	F105L	0.5	MF411487
	CA_115_PC	Control	Synonymous / Missense	F105L / S137L/ K128T	0.75	MF411471
	CA_124_PC	Control	Synonymous	F105L / D116E	0.75	MF411478
	CA_12_PC	Control	Synonymous / Missense	F105L / K128T	1.0	MF411413
	CA_128_PC	Control	Synonymous	F105L / D116E / K119N /S137L	1.0	MF411481
	CA_137_PC	Control	Synonymous	F105L / S137L	1.0	MF411484
	CA_9_PC	Control	Synonymous / Missense	F105L / K119N / K128T	1.5	MF411412
	CA_118_PC	Control	Synonymous / Missense	F105L / K119N / K128T	1.5	MF411473
	CA_122_PC	Control	Synonymous	F105L	1.5	MF411477
	CA_121_PC	Control	Synonymous / Missense	F105L / K128T	2.0	MF411476
Arithmetic mean					**0.94**	
Geometric mean					**0.69**	
	CA_30_PD	Diabetic	Synonymous / Missense	F105L / K128T	0.5	MF411422
	CA_35_PD	Diabetic	Synonymous / Missense	F105L / K119N / S137L / K128T	0.75	MF411426
	CA_79_PD	Diabetic	Synonymous / Missense	F105L / S137L/ K128T	0.75	MF411450
	CA_21_PD	Diabetic	Synonymous / Missense	F105L / K128T	1.0	MF411416
	CA_25_PD	Diabetic	Synonymous	F105L	1.0	MF411418
	CA_29_PD	Diabetic	Synonymous	F105L/ D116E/ K119N / S137L	1.0	MF411421
	CA_38_PD	Diabetic	Synonymous / Missense	F105L / K119N / S137L /K128T	1.0	MF411427
	CA_91_PD	Diabetic	Synonymous	F105L	1.0	MF411457
	CA_100_PD	Diabetic	Synonymous / Missense	F105L / K119N /K128T	1.0	MF411464
	CA_135_PD	Diabetic	Synonymous	F105L	1.0	MF411483
	CA_22_PD	Diabetic	Synonymous / Missense	F105L/ K119N / K128T	1.5	MF411417
	CA_28_PD	Diabetic	Synonymous	F105L	1.5	MF411420
	CA_31_PD	Diabetic	Synonymous	F105L	1.5	MF411423
	CA_33_PD	Diabetic	Synonymous / Missense	F105L / D116E / S137L / K128T	1.5	MF411425
	CA_39_PD	Diabetic	Synonymous / Missense	F105L / K128T	1.5	MF411428
	CA_76_PD	Diabetic	Synonymous	F105L / D116E / K119N / S137L	1.5	MF411449
	CA_81_PD	Diabetic	Synonymous / Missense	F105L / S137L/ K128T	1.5	MF411451
	CA_86_PD	Diabetic	Synonymous	F105L / D116E / K119N	1.5	MF411454
	CA_90_PD	Diabetic	Synonymous	F105L	1.5	MF411456
	CA_104_PD	Diabetic	Synonymous / Missense	F105L / K119N / K128T	1.5	MF411466
	CA_19_PD	Diabetic	Synonymous	F105L	2.0	MF411415
	CA_32_PD	Diabetic	Synonymous	F105L / D116E	2.0	MF411424
	CA_43_PD	Diabetic	Synonymous	F105L / D116E	2.0	MF411429
	CA_69_PD	Diabetic	Synonymous	F105L / K119N / S137L	2.0	MF411444
	CA_70_PD	Diabetic	Synonymous	F105L / D116E / K119N	2.0	MF411445
	CA_72_PD	Diabetic	Synonymous	F105L	2.0	MF411447
	CA_73_PD	Diabetic	Synonymous / Missense	F105L / K128T	2.0	MF411448
	CA_84_PD	Diabetic	Synonymous	F105L /D116E /K119N /S137L	2.0	MF411452
	CA_92_PD	Diabetic	Synonymous / Missense	F105L /D116E /K119N/S137L/ K128T	2.0	MF411458
	CA_93_PD	Diabetic	Synonymous / Missense	F105L / K128T	2.0	MF411459
	CA_98_PD	Diabetic	Synonymous / Missense	K119N / K128T	2.0	MF411462
	CA_99_PD	Diabetic	Synonymous	F105L	2.0	MF411463
	CA_85_PD	Diabetic	Synonymous	F105L /D116E / K119N /S137L	3.0	MF411453
	CA_96_PD	Diabetic	Synonymous	F105L	3.0	MF411461
	CA_102_PD	Diabetic	Synonymous	F105L	3.0	MF411465
Arithmetic mean					**1.63**	
Geometric mean					**1.49**	
	CA_57_PT	Transplanted	Synonymous	F105L	0.75	MF411436
	CA_46_PT	Transplanted	Synonymous / Missense	F105L / K119N / K128T	1.0	MF411430
	CA_51_PT	Transplanted	Synonymous	F105L / K119N	1.5	MF411433
	CA_52_PT	Transplanted	Synonymous / Missense	F105L / K128T	1.5	MF411434
	CA_49_PT	Transplanted	Synonymous	F105L	2.0	MF411432
	CA_58_PT	Transplanted	Synonymous	F105L / K119N	2.0	MF411437
	CA_63_PT	Transplanted	Synonymous	F105L / K119N / S137L	2.0	MF411441
	CA_62_PT	Transplanted	Synonymous	F105L	3.0	MF411440
	CA_67_PT	Transplanted	Synonymous / Missense	F105L / K119N / K128T	3.0	MF411443
Arithmetic mean					**1.86**	
Geometric mean					**1.70**	
C. tropicalis	ATCC- 28707	ATCC	Synonymous	T224C / G263A	2.0	MF414164
	CT_40_PD	Diabetic	Synonymous	G263A	1.5	MF414156
	CT_42_PD	Diabetic	Synonymous	G263A	1.5	MF414157
	CT_55_PT	Transplanted	Synonymous	T224C / G263A	1.5	MF414158
	CT_129_PC	Control	Synonymous	T224C / G263A	1.5	MF414163
	CT_77_PD	Diabetic	Synonymous	T224C / G263A	2.0	MF414160
	CT_105_PD	Diabetic	Synonymous	T224C / G263A	2.0	MF414161
	CT_106_PD	Diabetic	Synonymous	T224C / G263A	2.0	MF414162
Arithmetic mean					**1.71**	
Geometric mean					**1.73**	
C. glabrata	ATCC -2001	ATCC	Not found	—	4.0	MF414155
	CG_113_PC	Control	Not found	—	1.5	MF414152
	CG_123_PC	Control	Not found	—	1.5	MF414153
	CG_45_PT	Transplanted	Not found	—	4.0	MF414149
	CG_48_PT	Transplanted	Not found	—	4.0	MF414150
	CG_78_PT	Diabetic	Not found	—	4.0	MF414151
Arithmetic mean					**3.0**	
Geometric mean					**2.88**	

*Mutations F105L, D116E, K119N, S137L, T224C and G263A were classified as synonymous and K128T as missense. **Mutations previously described.

long, with 134 polymorphic sites. The 407 bp-long alignment of the *C. tropicalis* isolates to the *ERG11* genus contained 12 polymorphic sites. No polymorphisms were found in the partial sequence alignment of the *ERG11* gene of the six *C. glabrata* isolates.

On comparing the obtained *ERG11* sequences to the published *ERG11* sequences of fluconazole-sensitive *Candida* species, we observed that 32.9% (n=31) of isolates showed no nucleotide alteration in their partial *ERG11* sequences. The remaining 67.1% (n=63) of isolates showed at least one mutation of these, 19.1% (n=18) had a unique polymorphic site, 21.3% (n=20) showed two substitutions, 17.1% (n=16) had three polymorphic sites, and 9.6% (n=9) had more than three variable sites in the partial sequence of the *ERG11* gene (**Table 1**).

Analysis of the predicted amino acid sequence of *ERG11* revealed that 82.9% (121 bp) of variable sites resulted in synonymous mutations, with no change in the predicted amino acid sequence in *C. albicans* or *C. tropicalis* isolates, while 17.1% (25 bp) resulted in missense mutations, which were observed only in *C. albicans* isolates. In addition, 41.8% (56 bp) of mutations in *C. albicans* isolates occurred in the amino acid lysine (Lys) position F105L, 18.7% (25) in arginine (Arg) position K119N, 11.1% (15) in phenylalanine (Phe) position S137L, and 9.7% (13) in leucine (Leu) position D116E. In *C. tropicalis*, the most frequent mutation (58.3%) was in the amino acid asparagine (Asn) position G263A, followed by threonine (Thr) position T224C (41.7%). The missense nucleotide mutation observed in the *ERG11* gene of *C. albicans* isolates was a substitution of adenine with cytosine at codon 530, which resulted in the amino acid cysteine instead of phenylalanine (**Table 1**). In addition, of the 25 missense mutations observed, 92% (n=23) were heterozygous, and 8% were homozygous.

DISCUSSION

Candida infections are the most frequent cause of opportunistic diseases in immunocompromised patients[27], and fluconazole is the first treatment option for such cases. However, several studies have documented the ability of *Candida* to develop high-level resistance to azole compound[28], either through efflux pumps or alterations in the sterol 14-α-demethylase caused by mutations in the *ERG11* gene[29,30]. For effective treatment of *Candida* infections, and selection of the most efficient prophylactic measures, it is necessary to identify the *Candida* species, evaluate its susceptibility profile to different antifungals, and determine the mechanisms involved in any observed resistance[31].

The *Candida* isolates used in this study had been classified in a previous study[21] as the three different species; *C. albicans*, *C. glabrata,* and *C. tropicalis*. All isolates (70) were classified as susceptible to fluconazole, with MIC ≤ 8 μg/mL[20]. However, there were significant differences in the susceptibility ranges, with isolates from the control group displaying higher susceptibility to fluconazole than isolates from diabetic and kidney transplant patients (**Table 1**). This higher susceptibility of isolates from the control group maybe related to the absence of continuous fluconazole use, which has been suggested as one of the principal causes of fluconazole resistance in *Candida*[12,32,33]. In contrast, the higher fluconazole MIC values of strains isolated from transplant patients may be related to frequent contact with hospital patients, which facilitates oral colonization by less susceptible strains. Another contributor to this lower susceptibility may be the high cellular stress level of this group, caused by exposure to other drugs such as chemotherapeutics, corticosteroids, and antibiotics, which have been shown to activate resistance mechanisms in yeast[34,35].

In addition, we observed a difference in the mean fluconazole MIC values between different *Candida* species (**Table 1**). The lowest fluconazole MIC values were observed in *C. albicans*, followed by *C. tropicalis,* then *C. glabrata*. These differences may be related to intrinsic factors specific to each species[36,37] (**Table 1**). In the case of *C. glabrata* isolates, the relatively high tolerance to fluconazole is supported by the absence of any mutations in the *ERG11* gene (**Table 1**), suggesting that the higher tolerance may be a common characteristic of this species. Consistent with this notion, previous studies have demonstrated that after exposure to fluconazole, *C. glabrata* isolates showed lower susceptibility profiles compared to other *Candida* species[36,37].

Analysis of the *ERG11* gene sequence showed that, despite the high number of polymorphic sites observed in *C. albicans* and *C. tropicalis*, none occurred in the region coding for the binding site of the antifungal, the enzyme sterol 14-α-demethylase[38,39]. Although specific *ERG11* amino acid substitutions are known to be responsible for resistance to azolic compounds[40,41], the mutations characterized as F105L, D116E, K119N, K128T and S137L in *C. albicans* isolates, and T224C and G263A in *C. tropicalis* (**Table 1**) were not associated with antifungal resistance[9,23,42,43,44], in agreement with the MIC profiles. Although these mutations are synonymous, it is possible that they could influence the efficiency of translation, resulting in alterations in protein production, as has been suggested previously[45]. For example, the F105L mutation was previously correlated with fluconazole resistance[40], where as another study suggested that this mutation alone does not result in an alteration in the active site of 14-α-demethylase[46].

Several studies have recently investigated the fluconazole susceptibility of *Candida* isolates from HIV, cancer, and immunocompromised patients in Brazil[47,48]. However, no other studies have investigated the fluconazole susceptibility of *Candida* species associated with diabetic and kidney transplant patients. Although Chaves *et al.*[49] evaluated the diversity of *Candida* isolates from kidney transplant recipients in Brazil, the authors did not investigate the fluconazole susceptibility profiles

We therefore provide the first comparison of fluconazole susceptibility profiles of *Candida* species isolated from diabetic and kidney transplant patients in Brazil. Although the therapeutic and prophylactic use of fluconazole is widespread, fluconazole resistant *Candida* isolates were not observed in this study. However, fluconazole MIC values were higher for isolates from diabetic and kidney transplant patients compared with the control group. Further studies should therefore be performed to verify whether continued exposure to fluconazole may result in the identification of resistant *Candida* strains in these patients.

Conflict of interest: The authors declare that there is no conflict of interest.

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