Title: ERG11 polymorphism in voriconazole-resistant *Candida tropicalis*. Weak role of ERG11 expression, ergosterol content and membrane permeability.

Running title: ***ERG11* in voriconazole resistant *Candida tropicalis***

Patricia Navarro-Rodríguez[a], Loida López-Fernández[a], Adela Martin-Vicente[a†], Josep Guarro [a] and Javier Capilla [a #]

[a] Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Tarragona, Spain;

† Current affiliation: Department of Clinical Pharmacy and Translational Science, University of Tennessee Health Science Center, Memphis, TN, USA

#Corresponding author:

Javier Capilla Ph.D.

Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut,

Universitat Rovira i Virgili, Reus, Spain.

Phone 0034-977-759381.

Fax: 0034-977-759322.

E-mail: javier.capilla@urv.cat

Abstract

Mutations on ERG11 were detected by gene sequencing and amino acid alignment in 18 *Candida tropicalis* strains with different degrees of sensibility to VRC. ERG11 expression, sterols content and membrane permeability were also evaluated. We report three missense mutations in ERG11 that resulted in resistance to VRC. The transcriptional levels of ERG11 as well as the ergosterol content and membrane permeability do no demonstrated plenty correlation with the obtained MIC values but a tendency.

Despite the advances in the management of the invasive candidiasis (IC), the growing acquisition of azole resistance in *Candida* spp. aggravates the already poor therapeutic options. *Candida tropicalis* is the third commonest *Candida* spp. causing IC in Europe and the most frequent in the Asia-Pacific region (1). Point mutations on the lanosterol 14α-demethylase gene (*ERG11*), and up-regulation of multidrug efflux pumps (ABC ATP-binding cassette) are involved in the acquisition of azole resistance in *C. albicans* and *C. glabrata* (2). Nevertheless, few studies have explore their role in *C. tropicalis*.

We determined the MICs of voriconazole (VRC), posaconazole (PSC) and itraconazole (ITC) against 18 strains of *C. tropicalis* by microdilution (3) resulting in four susceptible strains, twelve resistant and two showing intermedium susceptibility to VRC according to the proposed clinical break points (4). MICs of ITC or PSC above the epidemiological cut off values (ECVs) were obtained in 90% of strains (Table 1).

Twelve strains representative of the obtained MIC of VRC were selected for *in vivo* efficacy. Cyclophosphamide-treated OF-1 male mice (Charles River Laboratories; Criffa SA, Barcelona, Spain) challenged with 1×10^5 CFUs of each isolate received oral VRC (VFEND®; Pfizer S.A., Madrid, Spain) at 40 mg/kg/day for 10 days post-infection (5). VRC only prolonged survival and reduced fungal load in kidneys inthose mice challenged with any of the susceptible (MIC ≤ 0.12µg/mL) or intermediate (MIC = 0.25-0.5 µg/mL) strains (*P* value = 0.02), correlating with the proposed CBPs for VRC. No increase on survival was observed in animals infected with resistant strains and burden reduction was detected only in two cases (Table 1).

ERG11 from all 18 strains was amplified using the oligonucleotides shown on table 2 and then sequenced at Macrogen Europe (Macrogen Inc. Amsterdam, The Netherlands). Multiple sequence alignments were done by using ClustalW algorithm on MEGA Software v. 7 and MegAlign DNASTAR Lasergene Software (6) with the Erg11 of *C. tropicalis* ATCC 750 (GenBank accession number M23673) as reference sequence. Comparative alignment revealed amino acid changes in 3 (25%) of the resistant strains, consisting on two missense mutations in strains FMR 14602 and FMR14603 and one frameshift resulting in 126 amino acids lost at the carboxyl end in strain FMR 8898. Their complete nucleotide sequences and deduced amino acid sequences have been deposited in the ENA (European Nucleotide Archive) under accession numbers LR030273, LR030275 and LR030274 respectively. Comparative analysis of the deduced *ERG11* protein in *C. tropicalis* (M23673) and its predicted 3D- structure with their orthologs in *C. albicans* (C5_006600cp_a) and *Saccharomyces cerevisiae* (YHR007C), showed that the absence of the 126 amino might affects the attachment of the heme moiety and its binding affinity to azole compounds resulting in resistance (data not shown).

Expression of ERG11 was performed in all *C. tropicalis* strains after exposure to 10 µg/mL of VRC during 18 h at 28ºC to. Single-strand cDNA from total RNA as well as amplification of *ERG11* and the housekeeping genes 5.8S ribosomal RNA (*RDN 5.8*) (7) and Actine 1 (*ACT1*), was performed using specific primers (Table 2). Quantitative real-time PCR (RT-qPCR) was done by triplicate using FastStart Universal SYBR Green Master (Roche Diagnostics SL, Sant Cugat del Valles, Spain) in 15 µl PCR reaction on StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Waltham, USA). Cycle threshold (Ct) values of *ERG11*

transcripts were normalized to the Ct corresponding to the housekeeping *RDN 5.8.* The relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (8). In absence of VRC, there was no overexpression of ERG11 in resistant strains compared to susceptible however, after drug exposure, *ERG11* was overexpressed in 16 (88.8%) of the studied strains demonstrating no correlation with resistance (Figure 1) and other mechanisms and genes must be involved. In this sense, recently has been reported that overexpression of ERG6 and interestingly, its maintained expression, can also be involved in resistance to azoles in *C. tropicalis* (9).

In order to determine sterols content in sensible and resistant strains, ergosterol and dehydroergosterol (DHE) was determined by spectrophotometry as percentage of wet weight (10). At baseline all strains showed similar ergosterol content (2,33 - 0,87 %) independently of their VRC susceptibility (Figure 2). However, after exposure to VRC all strains except two showing resistant phenotype, displayed 100% depletion of ergosterol. Interestingly. VRC caused from marked decrease to undetectable levels of DHE in sensible strains while in resistant strain were similar or even increased (Figure 2). Sterols content has been reported to affect azole sensibility in *Candida* spp. due to their role in biofilm formation and membrane permeability (11). In order to evaluate membrane permeability, strains were cultured in Synthetic Medium with 0.0125% or 0.025% SDS. In general, VRC-resistant strains showed higher radial growth than susceptible ones (Figure 2) indicating that membrane permeability can also contribute to susceptibility.

Overall, we report for first time the missense mutations Δ126aa, D448G and D445V in VRC-resistant strains of *C. tropicalis* however, other factors and

mechanisms must be involved. Expression of ERG11, sterols content and membrane permeability have shown only lightly correlation with resistance. More studies are needed in order to decipher the mechanisms driving azole resistance in *C. tropicalis.*

References

1. Quindos G. 2014. Epidemiology of candidaemia and invasive candidiasis. A changing face. Rev Iberoam Micol 31:42-8.
2. Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G. 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. Antimicrob Agents Chemother 49:668-79. https://doi: 10.1128/AAC.49.2.668-679.2005. https://doi: 10.1128/AAC.49.2.668-679.2005.
3. Clinical and Laboratory Standards Institute (CLSI). 2008. Reference method for broth dilution antifungal susceptibility testing of yeast; approved standard—3rd ed. CLSI document M27–3A. Clinical and Laboratory Standards Institute, Wayne, PA.
4. Orasch C, Marchetti O, Garbino J, Schrenzel J, Zimmerli S, Muhlethaler K, Pfyffer G, Ruef C, Fehr J, Zbinden R, Calandra T, Bille J, Funginos. 2014. *Candida* species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs. old Clinical and Laboratory Standards Institute clinical breakpoints: a 6-year prospective candidaemia survey from the fungal infection network of Switzerland. Clin Microbiol Infect 20:698-705. https://doi:10.1111/1469-0691.12440

5. Andes D, Marchillo K, Stamstad T, Conklin R. 2003. *In vivo* pharmacokinetics and pharmacodynamics of a new triazole, voriconazole, in a murine candidiasis model. Antimicrob Agents Chemother 47:3165-9. https://doi:10.1128/AAC.47.10.3165-3169.2003.

6. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725-9. https://doi:10.1093/molbev/mst197

7. Müller FM, Werner KE, Kasai M, Francesconi A, Chanock SJ, Walsh TJ. 1998. Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. J Clin Microbiol 36:1625-9. https://doi.org/10.1128/JCM.36.6.1625-1629.1998

8. Livak K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 408:402-8. https://doi:10.1006/meth.2001.1262

9. Fernandes T, Silva S, Henriques M. (2016) Effect of voriconazole on Candida tropicalis biofilms: Relation with ERG Genes expression. Mycopathologia 81:643–651. https://doi:10.1007/s11046-016-0023-6.

10. Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. 1999. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of Candida albicans. J Clin Microbiol 37:3332-7. https://doi: 10.1128/JCM.37.10.3332-3337.1999

11. Pais P, California R, Galocha M, Viana R, Ola M, Cavalheiro M, Takahashi-Nakaguchi A, Chibana H, Butler G, Teixeira MC. 2020. Candida glabrata transcription factor Rpn4 mediates fluconazole resistance through regulation of ergosterol biosynthesis and plasma membrane permeability.

158 Antimicrob Agents Chemother 64:e00554-20.
159 https//doi:10.1128/AAC.00554-20.
160

161 **Table 1.** Results of *in vitro* antifungal susceptibility and *in vivo* efficacy of

162 voriconazole (VRC). Mutations and overexpression of ERG11 are shown.

Strains	MIC (µg/mL)			*In vivo* efficacy of VRC 40 mg/kg		ERG11 gene	
	VRC	PSC	ITC	Burden reduction (log10 CFU/g)	Survival prolongation	Missense mutation	Overexpression after VRC exposure
VRC sensible							
FMR 10231	0.06	0.25	1	2.42 *	Yes*	No	Yes
FMR 10240	0.06	0.12	0.25	2.28 *	Yes*	No	Yes
FMR 13407	0.06	0.06	0.25	2.29 *	Yes*	No	Yes
FMR 8895	0.12	0.12	>16	-	-	No	Yes
VRC intermediate							
FMR 10239	0.25	0.06	>16	2.46 *	Yes*	No	Yes
FMR 10241	0.25	0.25	0.25	2.91 *	Yes*	No	Yes
VRC resistant							
FMR 12743	1	0.25	2	1.15	No	No	Yes
FMR 14049	1	0.12	0.5	-	-	No	Yes
FMR 14771	1	0.25	1	-	-	No	Yes
FMR 14600	1	0.25	2	2.37 *	No	No	Yes
FMR 14603	2	0.5	2	-	-	D445V	Yes
FMR 14769	2	0.5	1	-	-	No	Yes
FMR 14773	2	0.12	0.25	0	No	No	Yes
FMR 14772	2	0.25	0.5	-	-	No	Yes
FMR 14770	4	0.5	1	1.96	No	No	Yes
FMR 14768	4	0.5	0.5	0.86	No	No	Yes
FMR 14602	16	0.25	8	2.91 *	No	D448G	Yes
FMR 8898	16	>16	16	0.45	No	Δ126aa	Yes

163 * P value < 0.05

Table 2. Primers used for *ERG11* amplification and sequencing and for the evaluation of gene expression. Positions are referred to the start codon. (+) downstream or (-) upstream of ATG.

Gene	Primer	Sequence (5' – 3')	Use	Position ATG
ERG11	ERG11F	TACACTACCGCCATTTCTCA	Sequencing	- 318
	ERG11R	AGTCACCACCCTTTTCTTTC	Sequencing	+1665
	ERG11S1	TCCTGTTTTTGGTAAAGGTGT	Sequencing	+1011
	ERG11S2	ATGGTCTCTTTCCTTGGTTTT	Sequencing	+1171
	ERG11R2	GTGATCTGTGTCGGTTGGT	qRT-PCR/ sequencing	+2436
	ERG11R3	CTGGAACCTTATCACCGTTT	qRT-PCR/ sequencing	+2127
RDN5.8	RDN5.8-F	AACTTTCAACAACGGATCTCT	qRT-PCR	0
	RDN5.8-R	GAGAAATGACGCTCAAACAG	qRT-PCR	+113

FIGURE LEGENDS

Figure 1. *ERG11* expression in eighteen strains of *C. tropicalis* grown in control condition without VRC and with 10 µg/mL of VRC during 18 h, determined by RT-qPCR. **A.** Expression of *ERG11* relative to the housekeeping gene *RDN5.8* using the $2^{-\Delta Ct}$ method. **B**. Expression fold change of *ERG11* under induction with VRC using the comparative $2^{-\Delta\Delta Ct}$ method. Error bars indicate standard deviations calculated from three independent experiments.

Figure 2. A. Fungal colony growth of eighteen strains of *C. tropicalis* grown during 2-5 days at 30 ºC on YPD, SM and SM containing 0.0125% and 0.025% of SDS. **B.** Percentage of ergosterol levels in VRC susceptible and resistant strains after 18h of exposure to VRC. Data represent the mean of two independent experiments with standard error. **C.** UV spectrophotometric sterol profiles of representative VRC susceptible and resistant *C. tropicalis* strains after 18h of culture in YPG with (green and blue curves) or without (red curve) VRC.