G484S Amino Acid Substitution in Lanosterol 14-α Demethylase (ERG11) Is Related to Fluconazole Resistance in a Recurrent Cryptococcus neoformans Clinical Isolate

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Antimicrob Agents Chemother. 2003 Nov;47(11):3653-6.

Running Title: *ERG11* mutation in a fluconazole-resistant *C. neoformans*.

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Abstract

Five sequential isolates of *Cryptococcus neoformans* were recovered from an AIDS patient with recurrent episodes of meningitis and long fluconazole exposure. Isolates 1st to 4th were fluconazole susceptible and the fifth isolate had a MIC of 16 μg/ml. PCR amplification and sequencing of the gene encoding 14α-lanosterol demethylase showed a point mutation responsible for the amino acid substitution G484S in the resistant strain only.

Cryptococcal meningitis or meningoencephalitis is the most frequent encountered manifestation of infections due to *C. neoformans*. Treatment, in patients with AIDS, can be complicate owing to the severe immunosuppression. There is a very high relapse rate after receiving standard regimens of amphotericin B, and this finding have led to the use of lifelong chronic suppressive therapy. The current recommendations for maintenance treatment is oral fluconazole (15). Long term usage of fluconazole for fungal infections has been documented in several studies as a cause of emergence of azole resistance. Paugam *et* al. and Friese *et* al. have reported cases of recurrent cryptococcosis during the period of chronic suppressive therapy. Sequential isolates from these patients were found to have increased fluconazole MICs (4;11).

Mechanisms of azole resistance in yeasts already described include altered affinity of the 14-α lanosterol demethylase (ERG11) to azole drugs due to target site mutation or its overexpression, and decreased accumulation of drugs due to enhanced energy-dependent drug efflux (8;18). The biochemical basis of fluconazole resistance in *C. neoformans* have been explored focusing specially in the reduction of azole cellular content and the altered activity of ERG11 (7). Changes in the azole affinity for the ERG11 have been already related to low-level fluconazole resistance in *C. neoformans* isolates (20). In addition, the decrease affinity of ERG11 for azole derivatives due to mutations that contributes to increase the MICs of fluconazole, has been described in sequential clinical isolates of *Candida albicans* (8;9). To elucidate if this mechanism could also be implicated in *C. neoformans* azole resistance, we compared the *ERG11* genomic sequence in five sequential isolates recovered from recurrent episodes of cryptococcal meningitis. The first four isolates were fluconazole susceptible, and the fifth strain was in vitro resistant.

Clinical case: The five strains of *C. neoformans* were isolated from a 33 year-old male patient, HIV positive since 1990, presenting advanced AIDS (CD4+ count <100 cells/mm3) and recurrent cryptococcosis. The first episode of cryptococcosis was diagnosed in August of 1997, at Hospital Fernandez, Buenos Aires, Argentina. During the following 15 months, four more episodes of cryptococcal meningitis (CM) were detected and documented by cultures recovered from cerebrospinal fluid (CSF). In all episodes the titer of *C. neoformans* CSF-antigen was determined (Latex Cryptococcus Antigen Detection System, Immuno-Mycologics, Inc. OK, USA). The first episode (strain CN-1) showed a CSF-antigen titer (AT) of 1:4096 and the patient was treated with 0.7 mg/kg/day of parenteral amphotericin B for two weeks. After that, a treatment of 0.7 mg/kg/day, three times a week was prescribed, but the patient failed to comply

with it after three weeks of therapy. Fifteen days later (October 1997), he was hospitalized with a second episode (strain CN-2), with a CSF-AT of 1:1024. Therapy established at this moment was amphotericin B (0.7 mg/kg/day) combined with oral fluconazole (800 mg/day) during a month. In spite of it, the patient showed clinical signs of meningitis, and the third isolate was recovered from de CSF (strain CN-3). No changes were observed in the CSF-AT, and therapy was intensified with parenteral fluconazole, with doses of 1600 mg/day during 15 days. After that, a fluconazole maintenance therapy of 800 mg/day was established. Six months after (June 1998), the four episode occurred (strain CN-4) with no variations at the CSF-AT, and parenteral fluconazole was prescribed (1200 mg/day for a month). The patient recovered again and fluconazole suppressive therapy was re-established. However, another relapse occurred in November of 1998 (strain CN-5) without changes at the CSF-AT (1:1024). A new treatment with parenteral amphotericin B was prescribed, but the patient failed to comply with the therapy and left the hospital. He did not attend Hospital Fernandez for other medical examinations. The five isolates from each episode were identified as *C. neoformans* var. *grubii* by the following parameters: morphology, assimilation and fermentation of carbon and nitrogen compounds and molecular taxonomy**.**

Susceptibility testing of the five *C. neoformans* isolates were performed by microdilution and E-test methods. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 were used as quality control strains throughout the experiments (10). **Microdilution method**: The susceptibility testing followed the NCCLS recommendations (10) for the microdilution procedure but included some modifications previously described (14). Briefly, the susceptibility testing included RPMI supplemented with 2% glucose as assay medium (RPMI-2%-glucose), inoculum size of 10^5 CFU/ml, flat-bottomed trays, and spectrophotometric reading. All microplates were wrapped with film sealer to prevent the medium from evaporating, they were attached to an electrically driven wheel inside the incubator at 30ºC, and agitated at 350 rpm for 48 h (14). The antifungal agents used in the study were as follows: Amphotericin B (AMB) (Sigma Aldrich Quimica S.A., Madrid, Spain), 5-flucytosine (5FC) (Sigma Aldrich Quimica), fluconazole (FCZ) (Pfizer S.A:, Madrid, Spain), itraconazole (ITZ) (Janssen S.A., Madrid, Spain) and voriconazole (VOR) (Pfizer S.A.). The MICs were determined at 24 and 48 h spectrophotometrically. MICs were obtained measuring the absorbance at 530 nm with a MRXII reader (Dynatech, Cultek, Madrid, Spain). For AMB the MIC endpoints were defined as the lowest drug concentration exhibiting reduction in growth of 90% or more compared with that of the

control growth. For flucytosine and azole drugs the MIC endpoint was defined as the lowest drug concentration exhibiting a reduction in growth of 50% or more compared with that of the control growth.

E-test method: Tests were performed according to the manufacturers instructions. Inoculum density was adjusted to 1 McFarland standard and 240 μl was dispensed onto the centre of a 90 mm-diameter plate containing RPMI 1640 medium without sodium bicarbonate and with L-glutamine (Sigma Aldrich Quimica, S.A., Madrid, Spain), supplemented with 2% of glucose, and buffered to pH 7.0 with 0.165 M MOPS (Sigma Aldrich Quimica). Bacto Agar (Difco, Soria Melguizo, Madrid, Spain) was added at a final concentration of 1.5 g/100 ml. Plates were incubated at 30ºC and MICs were interpreted at 48 and 72 h as previously described (1).

Strain typing: The five strains isolated from CM episodes were genotyped using RFLPs generated by digesting total DNA samples to completion with the restriction enzyme *Sac*I (Promega corporation, Wi, USA) followed by hybridization with the CNRE-1 probe (kindly provided by Dr S. Spitzer, New York, USA) as previously described (3;19). Other three *C. neoformans* isolates recovered from different patients were included as control strains. Hybridizations patterns were analyzed visually. DNA fingerprinting using primers aimed to microsatellites, M13 and $(GACA)_4$, applied to all five strains, was performed to confirm their clonal origin.

Mutation detection: For the 14-α sterol demethylase (*ERG11*) gene amplification, primers were designed on the basis of the sequence of *ERG11* gene from *C. neoformans* (GenBank AF225914). Primer CnERG11A (5´ TCGTCGAACCATCTTTCG 3´) was designed 83 bp upstream of the ATG initiation codon, and CnERG11B (5´ CGTCTATGACTTCATGACC 3´) was designed 73 bp downstream the termination codon. The rest of the primers were designed to complite the full sequence of the gene. All the primers used in the present work were synthesized by Pharmacia (Madrid, Spain). The PCRs were carried out in a 50 μl volume, containing 10 mM $(NH_4)_2$ SO_4, 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 2 mM $MgSO_4$, 10 ng BSA, 0.1% Triton X-100, 250 μM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystem, Madrid, Spain), 0.5 μM of each primer, 2.5 units of *Taq* DNA polymerase (Applied Biosystem) and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystem) for one cycle of 5 min at 94º C, then 30 cycles of 30 s at 94º C, 45 s at 48º C and 2 min at 72º C, followed for one final cycle of 10 min at 72º C. The PCR products were analyzed by

electrophoresis on 0.8 % agarose gels and visualized by transillumination after staining them with ethidium bromide.

Cloning and plasmids: *C. neoformans* isolates were grown at 30ºC in YPD-Na (2% glucose, 1 % yeast extract, 2% peptone, 3% ClNa), during 24 h at 150 rpm. DNA was extracted as previously described (19). *Escherichia coli* JM109 was grown in Luria Bertani (LB) medium (16), supplemented with ampicillin (100 μg/ml), for propagation of plasmids for DNA extraction. PCR products were purified by Spin cholumns-200 (Clontech, Madrid, Spain), and cloned into pGEM-T easy vector system (Promega, Madrid, Spain). Inserts DNAs of recombinant plasmids were sequenced by the BigDye terminator cycle sequencing ready reaction system (Applied Biosystem) according to the manufacturer's instructions. All the clones were sequenced on both strands. For each *Cryptococcus* strain at least two inserts were analyzed. Sequence analysis was performed on an ABI prism 377 DNA sequencer (Applied Biosystem) using the sequencing facilities available at the Biopolymers Unit at Instituto de Salud Carlos III, Majadahonda, Madrid (Spain).

Sequence analysis: The amino acid sequences of 14-α sterol demethylase were deduced from nucleotide sequences and analyzed using the MegAlign software package (DNAstar, Inc., Lasergene, Madison, USA) run on a PC computer. The multiple amino acids alignments were derived by CLUSTAL analysis (5). The full nucleotide sequences of the *ERG11* gene from *C. neoformans* var. *grubii* determined in this work appear in the GenBank nucleotide sequence database under accession number: AY265353.

Results and Discussion: Recurrent episodes of cryptococcal meningitis is a frequent event among patients with AIDS. Several authors have described strains of *C. neoformans* exhibiting resistance in vitro to FCZ. Usually, these isolates were recovered from patients with recurrent episodes of cryptococcosis (4;11). However, the development of resistance to azoles in this species remains infrequent (2). Herein, we describe a case of cryptococcal meningitis in which FCZ resistance is developed. Susceptibility testing of the five sequential isolates to antifungal agents is displayed in Table 1. Regarding fluconazole MICs, isolates one to four were susceptible (MIC range 1-2 μg/ml) and a significant increase of the MIC was observed for the fifth isolate. However, no variations in MICs of the other azole drugs tested (ITZ and VOR) were detected in the sequential isolates. The strain exhibiting decreased susceptibility to fluconazole (MIC 16 μg/ml) was isolated after 15 months of treatment, being the

cumulative dose of fluconazole of 336 g. A similar event has been described by Martinez *et* al. with *C. albicans* in a recurrent oropharyngeal candidiasis (OPC) (9). In addition, Friese *et* al. reported a cryptococcal meningitis case in which the emergence of a FCZ resistant strain (MIC of 64 μg/ml) was documented after three episodes of meningitis (4). The authors concluded that the development detection of FCZ resistance isolate was probably due to the FCZ maintenance therapy. It is interesting to note that our isolates and those studied by Friese *et* al. remained susceptible to other triazole agents (Table 1).

All five strains isolated from the patient were genetically characterized by restriction fragment length polymorphisms (RFLPs), followed by hybridization using the CNRE-1 probe. Hybridization patterns of strains CN-1 to CN-5 showed that these isolates were clonally related (Figure 1). DNA fingerprinting using primers aimed to microsatellites, M13 and $(GACA)_{4,}$ applied to all five strains demonstrated that the susceptible strains and the resistant isolate were isogenic and it ratified their clonal origin (data not shown).

In cases of OPC, the emergence of *Candida* isolates with decreased susceptibility to azole agents is related to prolonged exposure of these antifungal agents (8;9;17). The molecular mechanisms of resistance studied in *C. albicans* strains included: (i) enhanced expression of efflux multidrug transporters genes causing decrease intracellular antifungal drugs content, and (ii) changes in the interaction between the azole antifungal agent and the 14-α lanosterol demethylase (ERG11) enzyme due to ERG11 modifications or its overproduction (8;9;17). In *C. neoformans* little is known about mechanisms of azole resistance (12;20). Only recently, up-regulation of an ABC transporter-encoding gene (CnAFR1) causing an active drug efflux mechanism has been shown directly related to azole resistance in a *C*. *neoformans* strain (13). In this study, the sequence of the *ERG11* gene of sequential clinical isolates was analyzed in order to know if we could find any *ERG11* point mutations (6,9;18) responsible for FCZ resistance, as has been previously described in *C. albicans* strains isolated from OPC (8).

Fragments of 2147 bp containing the full *ERG11* genomic sequence from all five isolates were obtained by PCR amplification. A point mutation (g1855t) in the *ERG11* gene was detected in the FCZ resistant isolate (CN-5) only. In order to verify that this point mutation was not due to errors introduce by the PCR amplification, the *ERG11* gene from all five isolates was newly amplified and sequenced a second time confirming the single point mutation in the CN-5 isolate. This mutation is responsible for

the amino acid substitution glycine 484 for serine (G484S), in the ERG11 deduced protein sequence of *C. neoformans.* The G484 is a residue that forms part of the conserved hemo-binding domain and is conserved in all cytochrome P450 ERG11/Cyp51 of yeasts and filamentous fungi (Figure 2). Several amino acid substitution in ERG11 has been previously described in *C. albicans* (6, 8, 9, 18). Most of these substitutions were present in enzyme domains highly conserved across yeasts and filamentous fungi. The amino acid substitution present in the fifth isolate G484S corresponds with the G464S of *C. albicans* ERG11 (Figure 2). Several studies have demonstrated that this amino acid substitution detected in *C. albicans* ERG11 confers a change in the orientation of the P450 hemo binding domain, leading to a decrease of azole binding and a decrease of the catalytic activity of the enzyme (6, 18). In sequential *C. albicans* strains recovered from OPC, has been demonstrated that this substitution appeared in isolates with MIC of FCZ between 32-64 μg/ml (9). Similarly, in the *C. neoformans* isolates from the presented patient it was also clear that the increase of FCZ MICs (from 2 to 16 μg/ml) was matched to the G484S amino acid substitution. However, the possibility of involvement of another concomitant molecular mechanisms of resistance can not be disregarded.

We thank the Biopolymers Unit at Instituto de Salud Carlos III, Majadahonda, Madrid (Spain) for DNA sequencing and to Dr. Sylvia Spitzer for providing CNRE-1 repetitive probe. E. M. held a contract Ramon y Cajal from Ministry of Science and Technology. L.R. held a grant from PAHO and ISCIII.

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FIGURE LEGENDS:

Figure 1.- Southern hybridization analysis of *C. neoformans* total cellular DNAs digested with *Sac*I and hybridized with the CNRE-1 probe. Lane CN-1 to CN-5 show successive strains of recurrent episodes of cryptococcal meningitis. Lanes A, B and C show DNAs of *C. neoformans* strains isolated from 3 different patients from the same hospital and lane D corresponds to *C. neoformans* ATCC 90112. MW: Molecular Weight (Kb).

Figure 2.- Alignment of the 33 amino acid residues of ERG11/Cyp51p sequences from *C. neoformans* (CnCyp51-GenBank AAF35366), *C. neoformans* var. *grubii* (CnN1Cyp51-GenBank AY265353), *A. fumigatus* (AfCyp51A-GenBank AAK73659 and AfCyp51B-GenBank AAK73660), *Leptosphaeria maculans* (GenBank AAN28927) *Aspergillus nidulans* (AnCyp51.-GeeBank AAF79204), *Penicillium. italicum* (PiCyp51.-GenBank. CAA89824), *Botryotinia fuckeliana* (BcCyp51-GenBank AAF85983), *Erysiphe graminis* (EgCyp51.-GeneBank AAC97606), *Uncinula necator* (UnCyp51-GenBank AAC49812), *Saccharomyces. cerevisiae* (ScCyp51-GenBank AAA34546), *Candida glabrata* (CgErg11-GenBank AAB02329), *C. tropicalis* (CtCyp51-GenBank AAA53284), *C. albicans* (CaErg11.-GenBank AAF00598), and *Ustilago maydis* (UmCyp51.-GenBank CAA88176). Identical residues between all the filamentous fungi and yeast are bolded. The same 33 amino acid from the FCZ-resistant strain of *C. neoformans* (CN-5) is at the bottom. Residue at position 484 is boxed.

TABLE 1. Development of in vitro fluconazole (FCZ) resistance in the five sequential clinical isolates of *Cryptococcus neoformans*. Table displays the time of follow-up at the hospital, the cumulative dose of FCZ and the FCZ (ETEST and microdilution), itraconazole (ITZ), voriconazole (VOR), flucytosine (FC) and amphotericin B (AMB) MICs of the five recurrent *Cryptococcus neoformans* isolates

Episode (Strain)	Follow-up (Months)	Cumulative dose of FCZ (g)	MIC (µg/ml)					
			FCZ[(1)]	FCZ[(2)]	ITZ[(1)]	VOR[(1)]	5FC[(1)]	AMB[(1)]
Initial (CN-1)	0	0	2	2	0.06	0.5	16	0.12
1st Relapse(CN-2)	2	0	1.5	2	0.12	0.06	8	0.25
2nd Relapse (CN-3)	3	24	2	1	0.06	0.06	16	0.12
3rd Relapse (CN-4)	9	180	1.5	4	0.12	0.12	32	0.25
4th Relapse (CN-5)	15	336	32	16	0.06	0.25	32	0.12

(1) Microdilution (2) E-TEST