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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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Five sequential *Cryptococcus neoformans* isolates recovered from an AIDS patient with recurrent meningitis were analyzed. Four isolates were fluconazole susceptible, while the fifth isolate developed fluconazole resistance. Analysis of the 14-α lanosterol demethylase gene (*ERG11*) showed a point mutation in the resistant strain responsible for the amino acid substitution G484S.

Mechanisms of azole resistance already described for yeasts include altered affinity of lanosterol 14-α 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 (5, 13). Changes in the azole affinity of the lanosterol 14-α demethylase have already been related to low-level fluconazole resistance in *Cryptococcus neoformans* isolates (15). In addition, the decreased affinity of lanosterol 14-α demethylase for azole derivatives due to mutations that contributes to the increase in the MICs of fluconazole has been described for sequential clinical isolates of *Candida albicans* (5, 6). To elucidate if this mechanism could also be implicated in the resistance of *C. neoformans* to azole, we compared the *ERG11* genomic sequence in five sequential isolates recovered from recurrent episodes of cryptococcal meningitis.

Clinical case. The five strains of *C. neoformans* were isolated from a 33-year-old male patient who had been positive for human immunodeficiency virus since 1990 and was presenting advanced AIDS ($CD4^+$ count, <100 cells/mm^3) and recurrent cryptococcosis. The first episode of cryptococcosis was diagnosed in August 1997 at Hospital Fernandez, Buenos Aires, Argentina. During the following 15 months, four more episodes of cryptococcal meningitis were detected and documented by cultures recovered from cerebrospinal fluid. In the first episode the patient was treated with amphotericin B (AMB), and for the rest of the episodes a fluconazole therapy at different doses was always established, reaching a cumulative dose of 336 g at the moment of the fifth episode. 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 was performed by microdilution and E-test methods. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains throughout the experiments (7).

Microdilution method. The susceptibility testing followed the NCCLS recommendations (7) but included some modifications as previously described (11). Briefly, the susceptibility testing included RPMI medium supplemented with 2% glucose as the assay medium (RPMI–2% glucose), an inoculum size of 10^5 CFU/ml, flat-bottomed trays, spectrophotometric reading at 530 nm, incubation at 30°C, and shaking at 350 rpm for 48 h (11). The antifungal agents used in the study were as follows: 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.). 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, while for flucytosine and azole drugs the MIC endpoint was defined as an inhibition of 50%.

E-test method. Tests were performed according to the manufacturer's instructions. Plates containing RPMI 1640 medium without sodium bicarbonate and with L-glutamine (Sigma Aldrich Quimica), supplemented with 2% glucose and buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma Aldrich Quimica), were prepared. Bacto agar (Difco, Soria Melguizo, Madrid, Spain) was added at a final concentration of 1.5 g/100 ml (Etest technical guide 4b: antifungal susceptibility testing of yeasts, AB BIODISK, Piscataway, N.J.).

Strain typing. The five strains were genotyped by using restriction fragment length polymorphisms generated by digesting total DNA samples to completion with the restriction enzyme *Sac*I (Promega, Madison, Wis.) followed by hybridization with the CNRE-1 probe (kindly provided by S. Spitzer, New York, N.Y.) as previously described (1, 14). Three other *C. neoformans* isolates recovered from different patients were included as control strains. Hybridization patterns were analyzed

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TABLE 1. Development of in vitro fluconazole (FCZ) resistance in the five sequential clinical isolates of *C. neoformans*

Episode (strain)	Time of follow-up (mo)	Cumulative dose of FCZ (g)	MIC (μg/ml)					
			FCZ[a]	FCZ[b]	ITZ[a]	VOR[a]	5FC[a]	AMB[a]
Initial (CN-1)	0	0	2	2	0.06	0.5	16	0.12
First relapse (CN-2)	2	0	1.5	2	0.12	0.06	8	0.25
Second relapse (CN-3)	3	24	2	1	0.06	0.06	16	0.12
Third relapse (CN-4)	9	180	1.5	4	0.12	0.12	32	0.25
Fourth relapse (CN-5)	15	336	32	16	0.06	0.25	32	0.12

[a] Determined by microdilution.
[b] Determined by E-TEST.

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 sterol 14-α demethylase gene (*ERG11*) amplification, primers were designed on the basis of the sequence of the *ERG11* gene from *C. neoformans* (GenBank accession no. AF225914). Primer CnERG11A (5′ TCG TCGAACCATCTTTCG 3′) was designed 83 bp upstream of the ATG initiation codon, and CnERG11B (5′ CGTCTATG ACTTCATGACC 3′) was designed 73 bp downstream of the termination codon. The rest of the primers were designed to complete 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 with a 50-μl volume containing 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 2 mM $MgSO_4$, 10 ng of bovine serum albumin, 0.1% Triton X-100, a 250 μM concentration (each) of dATP, dGTP, dCTP, and dTTP (Applied Biosystem, Madrid, Spain), a 0.5 μM concentration of each primer, 2.5 U of *Taq* DNA polymerase (Applied Biosystem), and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystem) for 1 cycle of 5 min at 94°C, and then 30 cycles of 30 s at 94°C, 45 s at 48°C, and 2 min at 72°C, followed by one final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 0.8% agarose gels and visualized by transillumination after being stained with ethidium bromide.

Cloning and plasmids. *C. neoformans* isolates were grown at 30°C in YPD-Na (2% glucose, 1% yeast extract, 2% peptone, 3% NaCl), during 24 h in an orbital shaker at 150 rpm. DNA was extracted as previously described (14). *Escherichia coli* JM109 was grown in Luria-Bertani medium (12), supplemented with ampicillin (100 μg/ml), for propagation of plasmids for DNA extraction. PCR products were purified by Spin columns-200 (Clontech, Madrid, Spain) and cloned into pGEM-T easy vector system (Promega, Madrid, Spain). Insert 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) by 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 sterol 14-α demethylase were deduced from nucleotide sequences and analyzed by using the MegAlign software package (DNAstar, Inc., Lasergene, Madison, Wis.). The multiple amino acids alignments were derived by CLUSTAL analysis (3).

Development of resistance to FCZ. Recurrent episodes of cryptococcal meningitis are frequent events among patients with AIDS. Several authors have described strains of *C. neoformans* exhibiting resistance in vitro to FCZ (2, 8). Herein, we describe a case of cryptococcal meningitis in which resistance to FCZ is developed.

Table 1 shows the results of antifungal susceptibility testing. For isolates 1 to 4, the MICs were between 1 and 2 μg/ml but for isolate 5 the MICs were 32 μg/ml. No variations in the MICs of ITZ and VOR were detected in the sequential isolates. The strain exhibiting decreased susceptibility to FCZ (MIC, 16 μg/ml) was isolated after 15 months of treatment (after having received a 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 (6). In addition, Friese et al. reported a cryptococcal meningitis case in which the emergence of an FCZ-resistant strain (FCZ

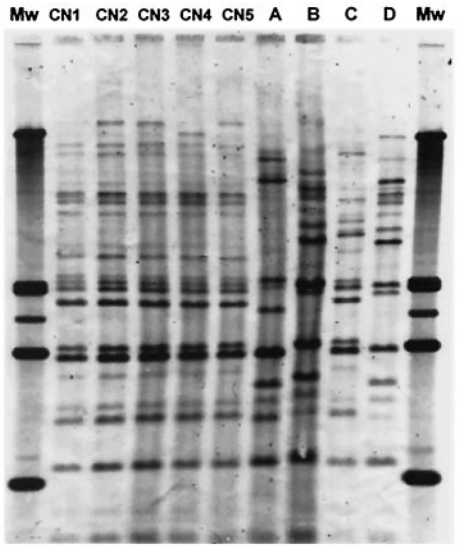

FIG. 1. Southern hybridization analysis of *C. neoformans* total cellular DNAs digested with *Sac*I and hybridized with the CNRE-1 probe. Lanes CN-1 to CN-5 show successive strains of recurrent episodes of cryptococcal meningitis. Lanes A, B, and C show DNAs of *C. neoformans* strains isolated from three patients from the same hospital, and lane D corresponds to *C. neoformans* ATCC 90112. Mw, molecular weight.

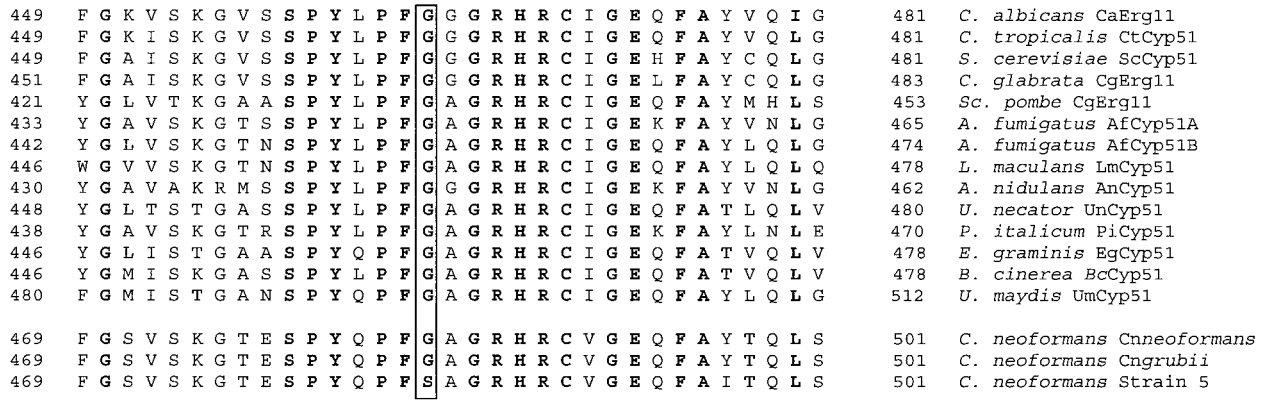

FIG. 2. Alignment of the 33 amino acid residues of ERG11/Cyp51p sequences from *C. neoformans* (CnCyp51; GenBank accession no. AAF35366), *C. neoformans* var. *grubii* (CnN1Cyp51; GenBank accession no. AY265353), *Aspergillus fumigatus* (AfCyp51A; GenBank accession no. AAK73659; and AfCyp51B; GenBank accession no. AAK73660), *Leptosphaeria maculans* (GenBank accession no. AAN28927), *Aspergillus nidulans* (AnCyp51; GenBank accession no. AAF79204), *Penicillium italicum* (PiCyp51; GenBank accession no. CAA89824), *Botryotinia fuckeliana* (BcCyp51; GenBank accession no. AAF85983), *Erysiphe graminis* (EgCyp51; GenBank accession no. AAC97606), *Uncinula necator* (UnCyp51; GenBank accession no. AAC49812), *Saccharomyces cerevisiae* (ScCyp51; GenBank accession no. AAA34546), *Candida glabrata* (CgErg11; GenBank accession no. AAB02329), *Candida tropicalis* (CtCyp51; GenBank accession no. AAA53284), *C. albicans* (CaErg11; GenBank accession no. AAF00598), and *Ustilago maydis* (UmCyp51; GenBank accession no. CAA88176). Residues that are identical among all the filamentous fungi and yeast are bolded. The same 33 amino acids from the FCZ-resistant strain of *C. neoformans* (CN-5) are at the bottom. The residue at position 484 is boxed.

MIC, 64 μg/ml) was documented after three episodes of meningitis (2). The authors concluded that the development of resistance to FCZ was probably due to the FCZ maintenance therapy. It is interesting that our isolates and those studied by Friese et al. remained susceptible to other triazole agents (Table 1).

The genotypic characterization of all strains by hybridization with CNRE-1 (Fig. 1) and DNA fingerprinting using primers aimed to microsatellites (data not shown) demonstrated that all isolates were isogenic and therefore clonal.

Little is known about mechanisms of azole resistance in *C. neoformans* (9, 15). Only recently, up-regulation of an ABC transporter-encoding gene (CnAFR1) causing an active drug efflux mechanism has been shown to be directly related to azole resistance in a *C. neoformans* strain (10). In this study, the sequences of the *ERG11* genes of all isolates were analyzed in order to find any *ERG11* point mutations (4, 6, 13) responsible for resistance to FCZ, as has been previously described for *C. albicans* strains (5).

Fragments of 2,147 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. The experiments were repeated a second time to confirm this result. 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 (Fig. 2). The amino acid substitution present in the fifth isolate, G484S, corresponds with the G464S of *C. albicans* ERG11 (Fig. 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 (4, 13). In sequential *C. albicans* strains recovered from oropharyngeal candidiasis, it has been demonstrated that this substitution appeared in isolates for which the MICs of FCZ were between 32 and 64 μg/ml (6). Similarly, for the *C. neoformans* isolates from the presented patient it was also clear that the increase of the MIC of FCZ (from 2 to 16 μg/ml) was matched to the G484S amino acid substitution. However, the possibility of involvement of another concomitant molecular mechanism of resistance cannot be disregarded.

Nucleotide sequence accession number. 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.

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L.R. and E.M. contributed equally to this work.

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