BRIEF REPORTS

Amino acid substitution in Cryptococcus neoformans lanosterol 14-α-demethylase involved in fluconazole resistance in clinical isolates

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KEYWORDS
Cryptococcus neoformans; Fluconazole resistance; ERG11 gene; Amino acid substitution; Mutation

Abstract The molecular basis of fluconazole resistance in Cryptococcus neoformans has been poorly studied. A common azole resistance mechanism in Candida species is the acquisition of point mutations in the ERG11 gene encoding the enzyme lanosterol 14-α-demethylase, target of the azole class of drugs. In C. neoformans only two mutations were described in this gene. In order to evaluate other mutations that could be implicated in fluconazole resistance in C. neoformans we studied the genomic sequence of the ERG11 gene in 11 clinical isolates with minimal inhibitory concentration (MIC) values to fluconazole of ≥16 μg/ml. The sequencing revealed the G1855A mutation in 3 isolates, resulting in the enzyme amino acid substitution G484S. These strains were isolated from two fluconazole-treated patients. This mutation would not intervene in the susceptibility to itraconazole and voriconazole.

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PALABRAS CLAVE
Cryptococcus neoformans; Resistencia al fluconazol; Gen ERG11; Sustitución aminoacídica; Mutación

Sustitución aminoacídica en la enzima lanosterol 14 α-demetilasa de Cryptococcus neoformans involucrada en la resistencia al fluconazol de aislamientos clínicos

Resumen Las bases moleculares de la resistencia al fluconazol en Cryptococcus neoformans han sido poco estudiadas. Un mecanismo de resistencia a los azoles en Candida albicans es la adquisición de mutaciones puntuales en el gen ERG11, que codifica la enzima lanosterol 14 α-demetilasa, blanco de las drogas azólicas. En C. neoformans solo 2 mutaciones en este gen han sido descritas. Con el objetivo de estudiar otras mutaciones que podrían estar implicadas en la resistencia al fluconazol en C. neoformans, realizamos la secuenciación del gen ERG11.

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Cryptococcosis is a life-threatening infection caused by the encapsulated basidiomycetous yeast *Candida neoformans* that affects mainly immunocompromised patients, especially those suffering from AIDS. The most common manifestation is cryptococcal meningitis, which is fatal unless treated. *C. neoformans* is found worldwide and is responsible for approximately one million cases/year which result in over 600,000 deaths annually.

Fluconazole (FLC), a triazole antifungal drug, is the drug of choice for consolidation and maintenance therapy due to its efficacy, excellent central nervous system penetration and minor toxic effects. Due to the use of FLC in long-term therapies, there is concern about the emergence of antifungal resistance in *C. neoformans*. Several authors have associated in vitro resistance with treatment failure and infection relapse.

The molecular basis of resistance to azole antifungals is the acquisition of point mutations in the ERG11 gene sequence, which encodes for lanosterol 14-α-demethylase, and AFR1, which encodes for an ABC transporter. It has been demonstrated that upregulation of the AFR1 gene is involved in the resistance to FLC in this yeast.

A common FLC resistance mechanism in *Candida* species is the acquisition of point mutations in the ERG11 gene resulting in an altered target with reduced affinity for or inability to bind azoles. Only two mutations in this gene have been associated with resistance to FLC in *C. neoformans*. Furthermore, one of them caused resistance to both FLC and voriconazole (VRC) and increased susceptibility to itraconazole (ITC) and posaconazole (PSC); this mutation was identified in an isolate with a lower MIC value was incorporated because it came from a patient who had presented an isolate with a high MIC value. The isolates and the patients’ clinical data are described in Table 1. All the isolates included in this study were *C. neoformans var grubii* genotype VNI determined by PCR-RFLP of the URA5 gene.

The minimal inhibitory concentration (MIC) was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.Def 7.2 reference document. Amphotericin B (AMB) and ITC (Sigma–Aldrich Quimica, Argentina); FLC and VRC (Pfizer S.A., Argentina) were the drugs tested and were provided as standard powders of known potency. The susceptibility tests were repeated from 4 to 10 times for each isolate.

DNA was extracted according to the method reported by Möller et al. To obtain the complete sequence of the ERG11 gene, four PCRs were performed according to Rodero et al. PCR products were purified using the PureLink purification kit (Invitrogen) and were sequenced on both strands using the initial amplification primers with an automated DNA sequencer ABI Genetic Analyzer 3500 (Applied Biosystems, CA). Sequences were edited using the BioEdit Version 7.0.0 (Tom May, North Carolina State University). All ERG11 gene sequences were deposited in the GenBank database (Table 2).

The FLC susceptibility testing confirmed that the strains selected for this study presented high MIC values (≥16 μg/ml) (Table 2). All the isolates exhibited similar in vitro susceptibility patterns toward AMB, ITC and VRC as the FLC susceptible isolate included in this study and other isolates of our collection with FLC MIC values <16 μg/ml (data not shown). Only one isolate (patient B, isolate no. DMic 021206) exhibited higher MIC values to ITC and VRC (0.5 and 1 μg/ml respectively).

The Genbank accession numbers of the ERG11 gene sequences obtained are listed in Table 2. Eight of the eleven isolates studied contained nucleotide variations compared to the wild type published sequences of *ERG11* (GenBank accession No. AY263535 and JQ044790). Only one of these nucleotide variations resulted in an amino acid substitution, the G1855A mutation producing substitution G484S. Two isolates recovered from the same episode of patient D and the isolate recovered from the third episode of patient A presented this mutation. We include in this study an isolate recovered from the initial episode of patient A, which did not contain this nucleotide variation and was susceptible to FLC. The other five isolates, obtained from three patients, contained different combinations of five nucleotide variations that did not result in any amino acid substitution: C233T present in an intron, and the silent nucleotide changes A1032G, C1659T, A1779G. These isolates did not exhibit nucleotide variation compared to the published wild type sequence of the *ERG11* gene.
The study of specific C. neoformans physiological responses and the possible resistance mechanisms to drugs used in the treatment of cryptococcosis are important both to identify potential new treatments for the infection and to enhance the inhibitory effects of existing drugs.

The ERG11 gene encodes the lanosterol 14-α-demethylase involved in ergosterol biosynthesis and the primary target for theazole class of antifungals. Several point mutations in this gene leading to different amino acid substitutions have been shown to decrease the target affinity for FLC resulting in drug resistance in C. albicans. Two of them have been described and related to FLC resistance in C. neoformans: substitutions G484S and Y145F. In this study, three clinical isolates with high MIC values presented the G484S substitution. These isolates were recovered from two patients who had had previous cryptococcosis episodes with a history of treatment with FLC. Moreover, we were able to study the isolate obtained from one of these patients' first episode, where cryptococcosis was the hallmark of HIV and the patient had not received any treatment. This initial isolate presented a lower MIC value and did not carry the amino acid substitution. These results reinforce the hypothesis that relates the G484S substitution to FLC resistance in C. neoformans. This relationship was proposed previously by Rodero et al. as a result of the study of a resistant isolate recovered from a patient suffering four episodes of relapse.

According to the 3-dimensional model of Lanosterol 14-α-demethylase from C. neoformans, the amino acid G484 is located in the heme environment into the active site of the enzyme. It is proposed that this amino acid substitution...
### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Episode</th>
<th>Isolate no.</th>
<th>MIC (µg/ml)</th>
<th>Nucleotide mutations</th>
<th>Amino acid substitution</th>
<th>Genbank accession no.</th>
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<td>FLC</td>
<td>ITC</td>
<td>VRC</td>
<td>AMB</td>
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<td>A</td>
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<td>4</td>
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<td>Third episode</td>
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<td>0.03</td>
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<td>0.25</td>
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<td>B</td>
<td>Second episode</td>
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<td>0.5</td>
<td>1</td>
<td>0.25</td>
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<tr>
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<td>0.03</td>
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<td>0.13</td>
</tr>
<tr>
<td>D</td>
<td>First isolate</td>
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<td>0.06</td>
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<tr>
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<td>DMic 052504</td>
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<td>0.5</td>
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<tr>
<td>H</td>
<td>Third episode</td>
<td>DMic 031631</td>
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<tr>
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<td>Fifth episode</td>
<td>DMic 021146</td>
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<td>Sixth episode</td>
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<td>0.13</td>
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</tbody>
</table>

MIC, minimal inhibitory concentration; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B.

- The values expressed represent the mode MIC values obtained for each isolate.
- The base numbers are with respect to the first ATG codon of the ERG11 gene.
Mutations in the ERG11 gene of *C. neoformans*

might decrease the flexibility required for binding with the substrate and the azole antifungal agents.

Mutation G1885A leading to amino acid G484S substitution was found independently in isolates from different patients and may represent a "hot spot" for the development of azole resistance; furthermore this substitution correlates with substitution G464S in *C. albicans* also proposed as a "hot spot" for that species.

The structure of VRC is very similar to FLC and in accordance with the three-dimensional models in *C. neoformans*, VRC might show higher affinity with the enzyme than FLC.

On the other hand ITC and PSC have very long side chains and might present the lowest interaction with the enzyme. With one exception, the isolates included in the present study exhibited low MIC values for VRC and ITC; moreover, we found no differences in the VRC and ITC MIC values between the isolates with the G484S substitution and others in our collection susceptible to FLC (data not shown), suggesting that the G484S substitution would not intervene in the enzyme interaction with ITC and VRC. In contrast, the other amino acid substitution, the Y145F, described in *C. neoformans*, afforded resistance to VRC but increased susceptibility to ITC and posaconazole.

We also found different combinations of five nucleotide variations that did not result in any amino acid substitution, which may indicate allelic differences present in the *ERG11* gene, and heterogeneity in the *C. neoformans* population. These allelic differences were also observed in *C. albicans* ERG11 gene. It is worthy of note that all the isolates included in this study were *C. neoformans* var. *grubii* genotype VNI in line with the worldwide distribution since this genotype is the most ubiquitous and prevalent and causes most of the cryptococcal infections.

Xu et al. proposed that mutation to FLC resistance in *C. neoformans* is a dynamic and heterogeneous process involving multiple simultaneous mechanisms. Overexpression of efflux transporters and chromosome duplication may occur in the isolates without any amino acid substitution and may also be acting together with the G484S amino acid substitution. It remains to be determined how this mutation individually contributes to FLC resistance.

In summary, the results showed that FCZ resistance in *C. neoformans* may result from the presence of the G1885A point mutation in the *ERG11* gene responsible for the amino acid substitution G484S. This mutation would not change susceptibility to ITC and VRC.

**Ethical disclosures**

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

**References**


