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Identification and Functional Characterization of a *Cryptococcus neoformans UPC2* Homolog

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Azoles are currently the most widely used class of antifungal drugs clinically, and are effective for treating fungal infections. Target site of azoles is ergosterol biosynthesis in fungal cell membrane, which is absent in the mammalian host. However, the development of resistance to azole treatments in the fungal pathogen has become a significant challenge. Here, we report the identification and functional characterization of a *UPC2* homolog in the human pathogen *Cryptococcus neoformans*. *UPC2* plays roles in ergosterol biosynthesis, which is also affected by the availability of iron in *Saccharomyces cerevisiae* and *Candida albicans*. *C. neoformans* mutants lacking *UPC2* were constructed, and a number of phenotypic characteristics, including antifungal susceptibility and iron utilization, were analyzed. No differences were found between the mutant phenotypes and wild type, suggesting that the role of *C. neoformans UPC2* homolog may be different from those in *S. cerevisiae* and *C. albicans*, and that the gene may have a yet unknown function.

KEYWORDS : Antifungal drugs, *Cryptococcus neoformans*, Ergosterol biosynthesis, Fungal pathogenesis, Iron

Fungal infections have increased dramatically during the last two decades and in most cases, the infections are associated with immunocompromised individuals such as AIDS patients and organ transplant recipients. In order to treat emerging fungal infections, azole (e.g. fluconazole) and polyene (e.g. amphotericin B) antifungal drugs have been developed and widely used. Among them, azoles are the most widely used clinically and are effective against fungal infections. The mode of action of azoles is the disruption of ergosterol synthesis in the fungal cell membrane, which is absent in mammalian and plant counterparts [1]. Azole compounds that have been developed include clotrimazole, miconazole, econazole, isoconazole, ketoconazole and fluconazole. Since their clinical introduction, various azole derivatives have become widely used antifungal drugs due to their safety [2]. Azole derivatives bind to and inhibit Erg11, 14α-methyl sterol demethylase, which cocatalyses cytochrome P-450-dependent 14α-demethylation of lanosterol in ergosterol biosynthetic pathwaysis. *ERG3*, which encodes $\Delta^{5,6}$ sterol desaturase, is also known to be associated with fluconazole susceptibility of fungi [3].

The human pathogen *Cryptococcus neoformans* is a basidiomycetes yeast that causes life-threatening diseases, such as pulmonary cryptococcosis and cryptococcal meningitis, mainly in HIV-positive patients [4]. Indeed, *C. neoformans* has caused diseases in about 5~10% of all AIDS patients over the past few decades [4]. *C. neoformans* expresses several virulence factors, such as a polysaccharide capsule and melanin, and its ability to grow at the host body temperature of 37°C [5-8]. The capsule is induced by environmental conditions such as iron depletion, CO_2 levels and lack of nutrients. Mutants that lack the ability to synthesize capsule have been reported to be avirulent [6, 7, 9]. Melanin is a polymer of exogenous dihydroxyphenolic compounds that plays a role in virulence by mediating resistance to oxidative stress [10-13]. The formation of melanin is catalyzed by phenoloxidase laccase, the expression of which is known to be influenced by glucose and also by iron [14-18]. The calcium/calcineurin pathway was identified in *C. neoformans* and was found to contribute to the expression of virulence factors including the ability of the fungus to tolerate the host body temperature [19, 20].

Due to its clinical significance, much attention has been paid to understand the mechanisms of drug susceptibility and resistance in the fungal pathogens. A number of studies using a model fungus *Saccharomyces cerevisiae* have revealed regulatory mechanisms of expression of genes involved in ergosterol synthesis. Among them are the *S. cerevisiae UPC2* gene and its paralog *ECM22*, which belong to the fungal Zn(2)-Cyc(6) binuclear cluster family of transcription factors. These transcription factors regulate the expression of *ERG2* and *ERG3* by binding to sterol response elements in the promoters of these genes [21]. The same protein was identified in *Candida albicans*. *C. albicans UPC2* (*CaUPC2*) is the homolog of *S. cerevisiae UPC2*/*ECM22*, and the mutant lacking *CaUPC2* displayed significantly increased sensitivity to the azole antifungals ketoconazole and fluconazole, and overexpression of *CaUPC2* increased resistance to these drugs [22].

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Genome-wide analysis using a ChIP-chip technique was performed to define downstream target promoters of *UPC2* in *C. albicans*. This study identified 202 promoters including those for *ERG11* and the genes encoding drug transporters such as *CDR1* and *MDR1* [22]. These findings clearly indicated that *UPC2* encodes a key regulator that controls the expression of the *ERG* genes in the ergosterol synthesis pathway and that influences resistance to azole antifungal drugs in some fungi. In this study, we therefore aimed to identify the *UPC2*/*ECM22* homolog in *C. neoformans* and to characterize its function with regard to drug susceptibility to currently available antifungal drugs including fluconazole. A *C. neoformans* mutant lacking the *UPC2* homolog was constructed by homologous recombination. A number of phenotypic analysis including drug sensitivity and iron utilization were carried out to identify its roles in *C. neoformans*.

Materials and Methods

Strains and growth conditions. *C. neoformans* var. *grubii* H99 (serotype A) was used throughout the study. Strains were maintained in yeast extract and bacto-peptone medium with 2.0% glucose (YPD; Difco, Detroit, MI, USA), or yeast nitrogen base (Difco) with 2.0% glucose. Defined low-iron medium was prepared as described elsewhere [23, 24]. Iron-replete media were prepared by adding $FeCl_3$ as an iron source. Hemin was added to low-iron media to investigate phenotypes in relation to heme utilization.

Construction of the mutants. Sequence information was obtained from the *C. neoformans* var. *grubii* serotype A genome database (http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans). The mutants were constructed as follows. A *UPC2* disruption cassette was constructed by overlapping PCR using the primers UPC2-KO1, UPC2-KO2, UPC2-KO3, UPC2-KO4, UPC2-KO5 and UPC2-KO6 (Table 1), with genomic DNA and the plasmid pCH233 as templates [25, 26]. The amplified disruption cassette was biolistically transformed into the wild-type strain as previously described [27]. The 2,524 bp genomic region containing the entire coding sequence of *UPC2* was replaced with the nourseothricin acetyltransferase gene (*NAT*) using the 5' and 3' flanking sequences of *UPC2*. Positive transformants were identified by PCR. Four independent mutants were selected and used throughout the study.

Phenotypic analysis. To analyze antifungal susceptibility, 10-fold serial dilutions of cells were spotted onto YPD plates containing fluconazole or amphotericin B. Plates were incubated at 30°C for two days. To investigate phenotypes related to iron utilization, strains were first grown in low-iron medium at 30°C overnight in order to deplete intracellular iron. Cells were serially diluted 10-fold and spotted onto low- or high-iron media.

Results and Discussion

Identification of the Upc2 homolog in *C. neoformans* and construction of the mutants. The sequence of *S. cerevisiae* Upc2 was used to search for its ortholog in *C. neoformans*. The search resulted in the identification of a gene, CNAG_03115.2 on chromosome 8, which contains a fungal Zn(2)-Cys(6) binuclear cluster domain. To determine gene function, mutants lacking *UPC2* were constructed by homologous recombination (see Materials and Methods). Positive transformants were selected and confirmed by PCR (data not shown). Four independent mutants were used throughout the experiments.

Roles of Upc2 in antifungal susceptibility. In *S. cerevisiae*, Upc2 influences ergosterol synthesis and susceptibility to antifungal drugs. Moreover, *C. albicans* mutants lacking *CaUPC2* displayed significantly increased sensitivity to the azole antifungal drugs ketoconazole and fluconazole, and overexpression of CaUpc2 increased resistance to these drugs [22]. In this study, we investigated the susceptibility of the *C. neoformans upc2* mutants to antifungal drugs. Growth of the mutants was analyzed on media containing fluconazole or polyene antifungal drug amphotericin B. The mutants grew equally well and showed no significant difference compared to the wild type (Fig. 1). This suggests that Upc2 played no role in relation to susceptibility to fluconazole or amphotericin B. Furthermore, unlike *S. cerevisiae* and *C. albicans*, *C. neoformans* may possess different regulatory mechanisms to regulate the genes involved in ergosterol synthesis.

Table 1. Primers used for construction of mutant strains

Name	Sequence
UPC2-KO1	GTATTCTAACCCCGACTCACCACCATC
UPC2-KO2	AATTCTGCAGATATCCATCACACTGGCGGCGACAATTTCGTCTCGGATTACTCGCCA
UPC2-KO3	AATTCCAGCACACTGGCGGCCGTTACTAGTCAGGGAGATTCAATAATCAGACCATACA
UPC2-KO4	CACACTTCACCATCGCCAAAGCCACC
UPC2-KO5	CAAGAAGACCTCCTCTTCTGATGAGAG
UPC2-KO6	GAGCAAGTTGAGAGGGAGGTAATTATCG

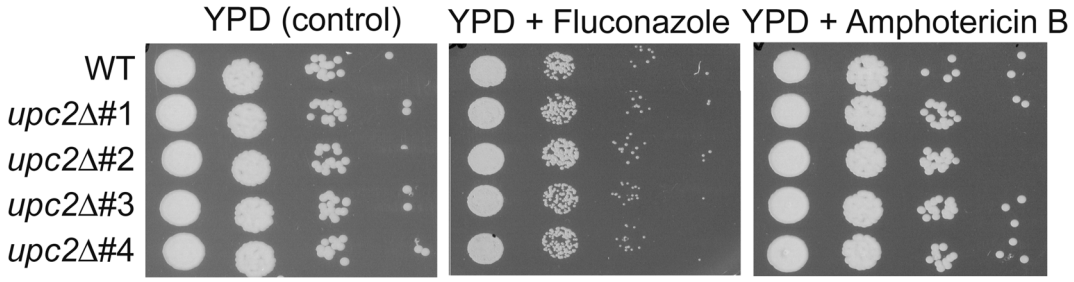

Fig. 1. Antifungal susceptibility of *Cryptococcus neoformans upc2* mutants. Ten-fold serial dilutions of cells (starting at 10^4 cells) were spotted onto solid yeast extract and bacto-peptone medium with 2.0% glucose (YPD) plates without or with antifungal drug at the concentrations indicated. Fluconazole and amphotericin B were added at final concentrations of 10 μg/mL and 0.5 μg/mL, respectively. Plates were incubated at 30°C for two days.

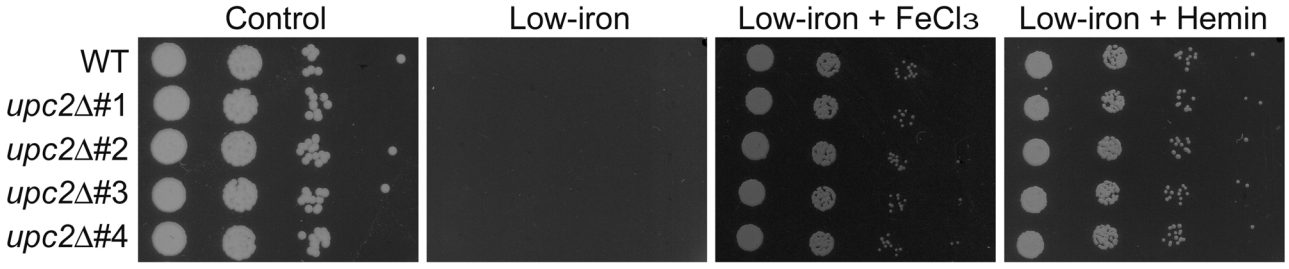

Fig. 2. Phenotypic analysis of *Cryptococcus neoformans upc2* mutants in relation to iron uptake. Ten-fold serial dilutions of cells (starting at 10^4 cells) were spotted onto solid yeast nitrogen base (YNB) medium (control), low-iron medium (YNB + 100 μM Bathophenanthroline disulfonate (BPS)), low-iron medium containing 100 μM $FeCl_3$ (YNB + 100 μM BPS + 100 mM $FeCl_3$) and low-iron medium containing 10 μM heme (YNB + 100 μM BPS + 10 μM Heme). Plates were incubated at 30°C for three days.

Roles of Upc2 in iron utilization. Iron utilization and metabolism are known to be related to ergosterol synthesis in *S. cerevisiae* and *C. albicans*. A number of studies have suggested that ergosterol synthesis relies on iron metabolism in *S. cerevisiae* [28, 29]. Examples include the influence of cytosolic iron levels on Erg25, which is a C-4 methyl sterol oxidase (Erg25) in ergosterol synthesis [30]. Similarly, susceptibility of *C. albicans* to antifungal drugs has also been shown to be influenced by the availability of iron. Moreover, *C. albicans* lacking iron permease Ftr1 displayed increased sensitivity to fluconazole [31]. Recently, we also found that *C. neoformans* mutants lacking the reductive iron uptake pathway show increased susceptibility to antifungal drugs [23, 32]. In this study, we tested whether the *C. neoformans upc2* mutants would show a deficiency in iron uptake. Unlike *S. cerevisiae* and *C. albicans*, the *C. neoformans upc2* mutants displayed no change in growth in both low- and high-iron media (Fig. 2). These results suggested that the Upc2 ortholog in *C. neoformans* plays distinct roles compared to *S. cerevisiae* and *C. albicans*.

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