Functional analysis of selected deletion mutants in *Candida glabrata* under hypoxia

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Abstract Increased drug resistance in *Candida glabrata* (a model non-*albicans* *Candida*) calls for the identification of potential molecular targets for the development of effective drugs. Hypoxia (a state of low oxygen) is an important host factor, which affects the virulence of the pathogen and efficacy of drugs. In the present study, in vitro characterization of 13 null mutants of *C. glabrata* were done under hypoxic condition (1% O₂). These mutants have a major role to play in cellular pathways, viability and pathogenesis (cell wall biosynthesis, ergosterol synthesis, calcium–calcineurin, etc.). The in vitro growth, biofilm formation and susceptibility of biofilm to antifungal drugs of these mutants were compared with the control. Hypoxia reduced the susceptibility of planktonic cells to fluconazole. The mutants *ecm33Δ*, *kre1Δ*, *rox1Δ*, and *kre2Δ* showed maximum reductions in their biofilm activities (>20%). The selected mutants (*upc2BΔ*, *kre2Δ*, *ecm7Δ*, *rox1Δ*, *mid1Δ*, *ecm33Δ*, *cch1Δ*, *kre1Δ*) showed reduced biofilm activities (>30%) in the presence of 16 µg ml⁻¹ fluconazole under hypoxia. Functional analysis revealed that Kre1, Ecm33, Upc2B, Kre2, Ecm7, Cch1, Mid1 and Rox1 can be explored as a potential drug target for developing novel antifungal drugs.

Keywords Hypoxia · *Candida glabrata* · Deletion mutants · Biofilm · Drug targets

Introduction

Fungal infections often contribute to high morbidity and mortality, which require the immediate attention of researchers to explore the host of factors and molecular determinants that are required for their virulence and pathogenesis. Candidiasis, a condition resulting from the infection of *Candida*, ranges from superficial to deeply invasive and systemic infections. Oral thrush and vulvovaginitis are the most common superficial forms of candidiasis, while candidemia (bloodstream infection) is the most common invasive infection (Giri and Kindo 2012). In the USA, candidemia infection is the fourth most widely hospital-acquired blood stream infection (BSI), while in Europe it stands between the sixth and tenth position of BSI (Wisplinghoff et al. 2004; Mean et al. 2008). Immune-suppressed state and implantation devices such as catheter, parenteral nutrition and the overuse of antibiotics are common factors that facilitate *Candida* infection (Rodrigues et al. 2014).

The trend of causatives of candidiasis is now shifting from *albicans* to non-*albicans* *Candida* (NAC) species worldwide (Guinea 2014; Yapar 2014). *C. glabrata* is one of the most pathogenic non-*albicans* *Candida* species after *C. albicans*, which is inherently resistant to azole drugs (Kaur et al. 2005; Caggiano et al. 2015). Among NAC species, *C. glabrata* is the major cause of *Candida* infection in Northern Europe and the USA, while most reported cases in Spain and Brazil are of *C. parapsilosis* (Guinea 2014). Many studies from different parts of India have also reported the shift in *Candida* epidemiology from *albicans*

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to drug-resistant NAC species, such as *C. glabrata*, *C. parapsilosis* and *C. tropicalis* (Chander et al. 2013; Juyal et al. 2013; Pahwa et al. 2014; Tak et al. 2014).

The pathogenic behavior of microbes is affected by several host factors, such as pH, temperature, hypoxia and nutritional availability. Hypoxia is a low oxygen state, which modulates the transcription of a subset of genes that ultimately affects pathogenicity (Synnott et al. 2010; Gleason et al. 2011). Inside the human body, 2.5–9.0% oxygen level is reported in normal tissues, while wounds and tumors have much lower oxygen levels ($\leq 1.0\%$) (Dewhirst 1998; Nizet and Johnson 2009). Adaptation to hypoxia is required for the survival and virulence of pathogenic fungi, e.g., *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Grahl et al. 2012). In general, hypoxic response of fungal pathogens includes upregulation of genes involved in heme biosynthesis, fatty acid synthesis, ergosterol biosynthesis, glycolysis, adhesins and hyphal transition (Setiadi et al. 2006; Askew et al. 2009; Mundy and Cormack 2009; Synnott et al. 2010; Gleason et al. 2011).

The threat of upcoming pathogenic NAC species and their rising resistance to existing antifungals have encouraged us to characterize the deletion mutants of *C. glabrata* with an aim to identify the molecular drug targets in *C. glabrata* (a model NAC species). An effective drug target must be crucial for survival and virulence of pathogen and must not share similarity with any of the host proteins (Spitzer et al. 2011; Pierce and Lopez-Ribot 2013). It is well proven that drugs screened under normoxic condition did not show in vivo efficacy because of the hypoxic environment of the tissue (Pellegrini et al. 2015). Therefore, we aimed to characterize the molecular drug targets under hypoxia, a metabolic stress present inside the host tissue. In the present study, an approach of reverse genetics was applied to characterize 13 selected deletion mutants of *C. glabrata*, which were prepared earlier in a multi-centric study, under hypoxic as well as normoxic conditions, (Schwarzmueller et al. 2014). The deletion mutants were selected from different pathways on the basis of their requirement for viability and virulence of *C. glabrata*, e.g., cell wall biosynthesis (*ecm33Δ*, *kre1Δ*, *kre2Δ*), DNA checkpoint pathway (*dun1Δ*), ergosterol pathway (*erg5Δ*), calcium–calcineurin pathway (*cch1Δ*, *mid1Δ*, *ecm7Δ*), transporters (*atm1Δ*, *cdr1Δ*) and selected transcription factors (*pdr1Δ*, *rox1Δ*, *upc2BΔ*).

Materials and methods

Cultures, culture conditions and chemicals

Strain and deletion mutants used in this study (Table 1) were prepared by Schwarzmueller et al. (2014) and

thankfully provided by Dr. Rupinder Kaur, Staff Scientist and Group Leader, Laboratory of Fungal Pathogenesis, Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India. Strains were routinely maintained in YPD media at 37 °C (1% yeast extract, 2% Bacto-Peptone and 2% dextrose; Difco). Biofilm assays were performed in RPMI-1640 medium supplemented with 50 mM HEPES (HiMedia) and L-Glutamine, pH 7.0 (referred as to RPMI henceforth) and SD medium (0.67% yeast nitrogen base w/o amino acids with ammonium sulfate supplemented with 2% dextrose; Difco) in sterile 96-well polystyrene cell culture plates with flat bottom (HiMedia). All other routine chemicals of molecular grade were procured from Merck and plastic wares from Tarson. Fluconazole (FLU), menadione and 2,3-bis(2-methoxy-4-nitro-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from HiMedia. To monitor cell growth under hypoxia, a mixture of O₂: 1%; CO₂: 5% and N₂: 94% was maintained inside a hypoxia incubator (Make: New Brunswick, Model: Galaxy 170R).

Antifungal susceptibility assay

Antifungal susceptibility was studied in 96-well round-bottom plate following M27-A2 guidelines of broth microdilution (NCCLS 2002). Overnight-grown culture of *C. glabrata* in YPD was re-inoculated in fresh YPD broth and incubated for 2 h at 37 °C. Log-phase cells were diluted to 2.5×10^3 cells ml⁻¹ in RPMI and 100 µl of this suspension was added to wells of microtiter plate, containing 100 µl of fluconazole (FLU) at log₂ concentration range of 0.5–16 µg ml⁻¹, with a drug-free control in RPMI. Plates were incubated at 35 °C for 48 h under normoxic or hypoxic conditions. After incubation, the absorbance of culture in wells was measured at 600 nm in an ELISA reader (Multiskan Go, Thermo Scientific). The concentrations of FLU required for 50% inhibition of growth of different strains (IC₅₀) were determined for comparison.

Biofilm formation assay

A biofilm was developed in 96-well flat-bottom microtiter plates (Riera et al. 2012). The cell suspension was prepared in PBS at a concentration of 10^7 cells ml⁻¹ and 100 µl of suspension was added to each well. The plates were incubated for 90 min at 37 °C on normoxic condition for the adhesion phase. Adhesion was visualized under inverted light microscope. Wells were washed twice with 200 µl of PBS to remove non-adhered cells. In each well, 200 µl of culture media were added and plates were incubated at 37 °C under hypoxic or normoxic conditions for 48 h with shaking. Afterward, wells were washed twice with 200 µl

Table 1 *C. glabrata* strains used in this study

Sr. no.	Strain/mutants (systematic name of ORF from Candida Genome Database, CGD)	Genotype
1	ATCC 2001	<i>C. glabrata</i> wild-type strain
2	<i>pdr1</i> Δ (CAGLOA00451 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>pdr1</i> Δ::NAT1
3	<i>cch1</i> Δ (CAGLOB02211 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>cch1</i> Δ::NAT1
4	<i>upc2B</i> Δ (CAGLOF07865 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>upc2B</i> Δ::NAT1
5	<i>cdr1</i> Δ (CAGLOM01760 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>cdr1</i> Δ::NAT1
6	<i>ecm33</i> Δ (CAGLOM01826 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>ecm33</i> Δ::NAT1
7	<i>mid1</i> Δ (CAGLOM03597 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>mid1</i> Δ::NAT1
8	<i>kre1</i> Δ (CAGLOM04169 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>kre1</i> Δ::NAT1
9	<i>atm1</i> Δ (CAGLOM13739 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>atm1</i> Δ::NAT1
10	<i>rox1</i> Δ (CAGLOD05434 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>rox1</i> Δ::NAT1
11	<i>kre2</i> Δ (CAGLOH07403 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>kre2</i> Δ::NAT1
12	<i>ecm7</i> Δ (CAGLOM00748 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>ecm7</i> Δ::NAT1
13	<i>dun1</i> Δ (CAGLOL07326 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>dun1</i> Δ::NAT1
14	<i>erg5</i> Δ (CAGLOM07656 g)	<i>C. glabrata his3</i> Δ::FRT <i>leu2</i> Δ::FRT <i>trp1</i> Δ::FRT <i>erg5</i> Δ::NAT1

of PBS to remove non-adhered cells and the biofilm was quantified by the XTT reduction assay by reading the absorbance at 492 nm through an ELISA Reader (Multiskan Go, Thermo Scientific).

XTT reduction assay

The XTT reduction assay was performed as described previously by Tsang et al. (2012) for the quantification of the biofilm. The XTT solution was prepared by mixing 1 mg of XTT salt in 1 ml of PBS; the solution was syringe filtered (pore size 0.22 μm) and finally stored at −20 °C. Menadione solution (0.4 mM) was prepared freshly in acetone. After washing wells with PBS, 158 μl of PBS, 40 μl XTT and 2 μl menadione was added to each well and the plates were incubated for 2 h in the dark at 37 °C. After incubation, 100 μl of solution was transferred to a new plate and the absorbance was measured at 492 nm using an ELISA Reader (Multiskan Go, Thermo Scientific). The biofilm formed by each deletion mutant was compared with the control under hypoxic and normoxic conditions. The percentage decrease in the mean biofilm activity of the mutant with respect to its control (at the respective condition) was calculated and shown as percent relative reduction in metabolic activity (RRMA).

Antifungal susceptibility of the biofilm

In vitro biofilm was developed, in the presence of FLU (at a log₂ concentration range of 0.5–16 μg ml^{−1}), with a drug-free control under hypoxic or normoxic conditions for 48 h at 37 °C (Riera et al. 2012; Tsang et al. 2012). The biofilm development was quantified by an XTT reduction

assay and represented in terms of relative reduction in metabolic activity (RRMA).

Statistical analysis

Experiments were performed in triplicate and the averages of the values are shown in the tables and figures along with standard deviations. Student's *t* test was applied for analyzing the significant differences between the values. *p* value <0.05 was considered as significant and represented by “*” in the figures.

Results

Deletion mutants of *C. glabrata*, used in this study, were prepared in an earlier study by Schwarzmuller et al. (2014). The use of *URA3* marker was avoided, since it is known to alter the virulence properties of *Candida* (Lay et al. 1998; Brand et al. 2004). The HTL strain (*his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT; isogenic to ATCC2001) has displayed similar growth properties and rates as the parental wild-type strain (ATCC2001), on both minimal and rich media, under hypoxic as well as normoxic conditions (data not shown). The auxotrophic markers of the mutant (*HIS3*, *LEU2* and *TRP1*) did not influence the in vitro growth and survival of the mutants in immunocompetent mice when compared to the parental wild type strain (ATCC2001) (Jacobsen et al. 2010).

Antifungal susceptibility assay

The IC₅₀ values of FLU for the strains under hypoxia and normoxia are shown in Table 2. No deletion was found to

increase the susceptibility to FLU above the control strain under hypoxic condition. Some deletions such as *pdr1Δ*, *kre1Δ* and *atm1Δ* showed IC_{50} values similar to the control under hypoxia. For each mutant, a line graph was plotted between the mean absorbance (O.D._{600nm}) of cultures and the presence of different concentrations of FLU (Fig. 1a–m).

It is obvious that the mutants behaved differently in broth microdilution assay under hypoxic and normoxic conditions (Table 2). The susceptibility of mutants to FLU was reduced under hypoxia, resulting in increased growth. On the basis of IC_{50} values, *kre1Δ*, *atm1Δ* and *pdr1Δ* did not show further drop in susceptibility over the respective control strain under hypoxic conditions. It indicated that Kre1, Atm1 and Pdr1 may be good drug targets for the planktonic cells of *C. glabrata* under hypoxic conditions.

Biofilm development

C. glabrata mutants, which reduced the biofilm formation under hypoxic and normoxic conditions in RPMI medium, were selected in this study. Data showed that the control strain did not exhibit any significant difference in biofilm development under hypoxic and normoxic conditions (p value= 0.373118), while the mutants exhibited significant differences in their biofilm activities (Supplementary figure, Fig. S1; Table 3). Most of the mutants showed that hypoxic exposure increased the biofilm formation, when compared with the respective biofilm at normoxia. The percent relative reduction in metabolic activities (RRMA) of the mutants under normoxic and hypoxic conditions ranged between 10.39–50.65% and 0–35.62%, respectively (Table 3).

Table 2 IC_{50} of fluconazole for the strains under normoxia and hypoxia

Sr. no.	Strain/mutant	IC_{50} value ($\mu\text{g ml}^{-1}$) under normoxia	IC_{50} value ($\mu\text{g ml}^{-1}$) under hypoxia
1	ATCC2001	2–4	4–8
2	<i>pdr1Δ</i>	2–4	4–8
3	<i>cch1Δ</i>	4–8	>16
4	<i>upc2BΔ</i>	4–8	8–16
5	<i>cdr1Δ</i>	2–4	>16
6	<i>ecm33Δ</i>	4–8	>16
7	<i>mid1Δ</i>	4–8	8–16
8	<i>kre1Δ</i>	2–4	4–8
9	<i>atm1Δ</i>	2–4	4–8
10	<i>rox1 Δ</i>	4–8	>16
11	<i>kre2 Δ</i>	4–8	>16
12	<i>ecm7Δ</i>	>16	>16
13	<i>dun1Δ</i>	4–8	8–16
14	<i>erg5Δ</i>	8–16	>16

As shown in Table 3, mutants (*pdr1Δ*, *cch1Δ*, *upc2BΔ*, *ecm33Δ*, *mid1Δ*, *kre1Δ*, *atm1Δ*, *rox1Δ*, *kre2Δ*, and *ecm7Δ*) showed high reductions in the biofilm activities (>20%) under normoxic condition, whereas under hypoxic condition only few mutants (*ecm33Δ*, *kre1Δ*, *rox1Δ*, and *kre2Δ*) showed high reduction in their biofilm activities (>20%). *C. glabrata* mutants, *kre1Δ* and *ecm33Δ* showed maximum decrease in biofilm activities (35.62 and 26.03%, respectively) upon hypoxic exposure, indicating them to be the most promising drug targets under hypoxia.

Antifungal susceptibility of biofilm

Antifungal resistance of *Candida* biofilm is about thousand times more than planktonic cells (LaFleur et al. 2006). Antifungal susceptibility of the biofilm assay showed that all mutants exhibited reduced biofilm in the presence of FLU under normoxia and hypoxia. The percent relative metabolic activities of biofilm of deletion mutants in the presence of FLU is shown as bar graphs (Supplementary figure, Fig. S2). Interestingly, no change in antifungal susceptibility of biofilm was observed for *cch1Δ*, *atm1Δ* and *upc2BΔ* (Table 4).

When compared with the normoxic results, hypoxia highly increased the biofilm susceptibility to FLU for *pdr1Δ*, *mid1Δ*, *kre1Δ*, *rox1Δ*, *kre2Δ*, *ecm7Δ*, *dun1Δ* and *erg5Δ* deletion mutants. Some mutants (*cch1Δ*, *upc2BΔ* and *ecm33Δ*) did not show much differences in percent RRMA at hypoxia and normoxia, but showed large reductions in biofilm activities and so can be considered as potential drug targets. The deletion mutants, *dun1Δ*, *erg5Δ*, *pdr1Δ* and *cdr1Δ*, showed percent RRMA >20% under hypoxic condition, whereas all mutants except *atm1Δ* showed percent RRMA > 30%, under hypoxic condition. Under hypoxic condition, Upc2B, Kre2, Ecm7, Rox1, Mid1, Ecm33, Cch1 and Kre1, appeared to be the most potential drug targets (percent RRMA of deletion mutants >30%) to sensitize the biofilm in the presence of FLU (Table 4).

Discussion

Adaptations to hypoxia are required for the survival and enhanced virulence of pathogenic fungi such as *C. albicans*, *C. glabrata*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Grahl et al. 2012; Gupta et al. 2014, 2015). The function of most of the genes analyzed in this study is still unknown in *C. glabrata*. This study has also revealed the functions of the selected genes in virulence.

Yeast Atm1 is an ATP binding cassette (ABC) transporter found in mitochondrial inner membrane with ABC

Fig. 1 Antifungal susceptibility assay of *C. glabrata* mutants by broth microdilution assay. Different dilutions of FLU were tested in RPMI against the mutants. The growth was measured by taking O. D. at 600 nm after 48 h incubation at 35 °C

Table 3 Mean value of O.D._{492nm} for biofilm of deletion mutants

Strain	Normoxic condition		Hypoxic condition	
	Mean of O.D. _{492nm} ± SD	Percent RRMA	Mean of O.D. _{492nm} ± SD	Percent RRMA*
ATCC 2001	0.077 ± 0.0050	—	0.073 ± 0.0020	—
<i>pdr1Δ</i>	0.050 ± 0.0036	35.06	0.068 ± 0.0035	06.85
<i>cch1Δ</i>	0.054 ± 0.0020	29.87	0.080 ± 0.0020	Nil
<i>upc2BΔ</i>	0.052 ± 0.0026	32.47	0.072 ± 0.0005	01.37
<i>cdr1Δ</i>	0.068 ± 0.0020	11.69	0.063 ± 0.0015	13.70
<i>ecm33Δ</i>	0.053 ± 0.0050	31.17	0.054 ± 0.0041	26.03
<i>mid1Δ</i>	0.053 ± 0.0049	31.17	0.072 ± 0.0050	01.37
<i>kre1Δ</i>	0.038 ± 0.0055	50.65	0.047 ± 0.003	35.62
<i>atm1Δ</i>	0.048 ± 0.0047	37.66	0.060 ± 0.0049	17.81
<i>rox1Δ</i>	0.057 ± 0.0026	25.97	0.057 ± 0.0011	21.92
<i>kre2Δ</i>	0.057 ± 0.0051	25.97	0.058 ± 0.0005	20.55
<i>ecm7Δ</i>	0.058 ± 0.0045	24.68	0.066 ± 0.0035	09.59
<i>dun1Δ</i>	0.069 ± 0.0065	10.39	0.064 ± 0.0047	12.33
<i>erg5Δ</i>	0.062 ± 0.0035	19.48	0.060 ± 0.0056	17.81

* Percent RRMA Relative reduction in the mean metabolic activity of biofilm in percent, with reference to the mean metabolic activity of wild-type strain, ATCC 2001

domain-facing mitochondrial metrics (Leighton and Schatz 1995). It has a role in the generation of cytosolic Fe/S proteins, which catalyzes several metabolic reactions like isomerization, dehydration and electron transport in the redox reaction (Cammack 1992; Johnson 1998). Earlier, it has been shown that the absence of *ATM1* in yeast results in defective mitochondrial respiration and increases intracellular oxidative stress (Kispal et al. 1999). In *S. cerevisiae*, the deletion mutant of *ATM1* is inviable in aerobic condition, but exhibits slow growth in anaerobic condition (Leighton and Schatz 1995). Similarly, we observed that the biofilm of *atm1Δ* was severely compromised under normoxic condition, whereas the decrease was relatively lesser under hypoxic conditions (Table 3). This effect may be due to less dependency on mitochondrial respiration under hypoxic conditions.

In biofilm formation and antifungal susceptibility assays, Kre1 and Ecm33 came out as the most promising drug target under hypoxic conditions (Tables 3, 4). Yeast Kre1 and Ecm33 are key components of cell wall organization and synthesis. Their deletion weakens the cell wall and increases sensitivity (Terashima et al. 2003; Breining et al. 2004). It plays an important role in β -glucan synthesis, a component of fungal cell wall (Boone et al. 1990; Roemer and Bussey 1995). Kre1 is required for the synthesis of β -1,3 glucan, which is a crucial element of the extracellular matrix and has a role in the sequestration of the antifungal molecules, causing drug resistance of the *Candida* biofilm (Nett et al. 2007, 2010). Ecm33 is a GPI protein and has an important role in establishing and maintaining the cell wall integrity (Groot et al. 2013). In

addition to the defective cell wall matrix in *kre1Δ* and *ecm33Δ*, hypoxic exposure may lead to the reduced ergosterol and fatty acid synthesis, resulting in increased sensitivity. Therefore, the biofilm of *C. glabrata kre1Δ* and *ecm33Δ* strain is reduced and the mutant appeared more susceptible in the presence of azole.

C. glabrata Kre2 is uncharacterized and its ortholog in *C. albicans* is alpha-1,2-mannosyl transferase, which is predicted as type II Golgi membrane protein that adds the second mannose during cell wall mannoprotein biosynthesis. It is required for wild-type virulence and adherence to epithelial cells (Singh et al. 2011). Therefore, in the present study, the *kre2Δ* mutant of *C. glabrata* has shown reduction in the biofilm and biofilm susceptibility to FLU (probably due to reduction in ergosterol level at hypoxia) (Tables 3, 4).

Hypoxic response in *S. cerevisiae* is mostly mediated through Rox1, a transcription factor which represses hypoxic genes under normoxic conditions (Zitomer et al. 1997). Rfg1 of *C. albicans* is the closest homolog of yeast Rox1, which represses filamentous growth (Kadosh and Johnson 2001). CgROX1 is uncharacterized and this report has revealed for the first time the role of CgRox1 in biofilm formation and in biofilm resistance to azole under hypoxic condition (Tables 2, 3). As shown in supplementary figure S1, the *rox1Δ* mutant did not show any difference in biofilm activities under normoxic and hypoxic conditions; it suggested that like *S. cerevisiae*, Cg Rox1 is required for survival in hypoxia.

The highest biofilm susceptibility to FLU ($16 \mu\text{g ml}^{-1}$) was shown by *upc2BΔ* (percent RRMA 45.15% in normoxia and 49.66% in hypoxia; Table 4). Therefore, CgUpc2B appeared to be a very potent drug target to

Table 4 Reduction in metabolic activities of biofilm of deletion mutants when compared with the control in the presence of 16 $\mu\text{g ml}^{-1}$ fluconazole

Sr. no.	Strain	Percent RRMA* under normoxia	Percent RRMA* under hypoxia
1	<i>pdr1</i> Δ	11.00	23.48
2	<i>cch1</i> Δ	39.00	38.19
3	<i>upc2B</i> Δ	45.15	49.66
4	<i>cdr1</i> Δ	27.64	20.18
5	<i>ecm33</i> Δ	26.96	32.20
6	<i>mid1</i> Δ	Nil	32.92
7	<i>kre1</i> Δ	04.52	31.32
8	<i>atm1</i> Δ	15.58	14.02
9	<i>rox1</i> Δ	11.01	34.59
10	<i>kre2</i> Δ	20.36	46.14
11	<i>ecm7</i> Δ	18.14	46.35
12	<i>dun1</i> Δ	12.62	21.02
13	<i>erg5</i> Δ	03.14	23.11

* Percent RRMA relative reduction in the mean metabolic activity of biofilm in percent, with reference to the mean metabolic activity of wild-type strain, ATCC 2001

sensitize the biofilm in the presence of azole. CgUpc2B was also required for the biofilm synthesis under normoxic condition. Fungal Upc2B is a functional homolog of *S. cerevisiae* Ecm22 and a study has shown that it is not the main transcriptional regulator of the genes of sterol homeostasis and sterol susceptibility (Nagi et al. 2011). CgUpc2B is not characterized, but this study has indicated that Upc2B regulated biofilm formation and biofilm resistance to FLU.

The results of the present study indicated that Cch1, Mid1 and Ecm7 have a role in biofilm formation under normoxic condition (Table 3). Cch1 is required in biofilm resistance to FLU under normoxic as well as hypoxic conditions, whereas Mid1 and Ecm7 were required for biofilm resistance to FLU under hypoxic condition only (Table 4).

The proteins, Cch1, Mid1 and Ecm7, are involved in Ca^{2+} transport across the membrane (Teng et al. 2013). It has been previously reported that a drop in intracellular Ca^{2+} level results in increased susceptibility to FLU (Kaur et al. 2004). Earlier, it has been shown that amlodipine besylate, an inhibitor of mammalian Ca^{2+} channel, reduced the virulence of *C. albicans* and *C. glabrata* clinical isolates, in vitro (Gupta et al. 2016).

Though the other mutants (*pdr1* Δ , *dun1* Δ , *erg5* Δ) slightly regulated the virulence properties, they are required to be addressed for functional characterization. CgPdr1 belongs to the family of zinc finger transcription factors. It is functionally similar to Pdr1 and Pdr3 of *S. cerevisiae*. CgPdr1 is the transcriptional regulator of multi-

drug transporters belonging to the ABC family, such as CgCdr1 and CgCdr2. Deletion of *CgPDR1* results in loss of major drug transporters and decreases resistance to azoles (Tsai et al. 2006; Vermitcky et al. 2006). This study has also shown that loss of *PDR1* decreased biofilm resistance to FLU (Table 4). The biofilm activity of *pdr1* Δ was reduced by 35.06% in normoxia; whereas hypoxia partially rescued the loss of biofilm (Table 3).

CgDun1 is uncharacterized and its role in biofilm formation and increase in biofilm resistance to FLU under hypoxia has been reported in this study. In yeast, Dun1 is downstream to Rad53 in the DNA checkpoint pathway and gets activated by phosphorylation at its FHA domain through activated Rad53. Dun1 on activation promotes the synthesis of ribonucleotide reductase through multiple mechanisms (Sanvisen et al. 2013).

CgErg5 is also uncharacterized and appears to play an important role in biofilm formation under normoxic as well as hypoxic conditions, whereas it is required for biofilm resistance to FLU under hypoxic condition. Erg5 is a C-22 sterol desaturase enzyme required for sterol synthesis in yeast. *ERG5* deletion is viable in *S. cerevisiae* and is reported to upregulate the *ERG3* expression (Arthington-Skaggs et al. 1996; Skagg et al. 1996).

Conclusion

The present study was undertaken to identify the molecular drug targets under hypoxic condition to develop an effective drug against *C. glabrata*. The results indicated that Kre1, Ecm33, Upc2B, Kre2, Ecm7, Cch1, Mid1 and Rox1 are potential drug alone or in combination with azoles. None of these proteins has human homologs (except Cch1), hence the risk of cross-reactivity of the drug with host proteins will be the least, resulting in minimal side effects. Kre1 and Ecm33 have emerged as the most potential candidates as drug targets against growth, biofilm and biofilm resistance in the pathogen. These targets may be further characterized by site-directed mutagenesis for the identification of the target domain for the synthesis of inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest regarding any issue related to this manuscript and the data presented.

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