Genomic analysis of a *Candida glabrata* clinical isolate resistant to antifungals unveils novel features of drug resistance in this pathogenic yeast

Sara Barbosa Salazar

*Under supervision of Nuno Gonçalo Pereira Mira*

IST, Lisbon, Portugal

June, 2015

**Abstract**

An alarming increase in the incidence of infections caused by *C. glabrata* has been reported in the last years, in part, due to the emergence of strains resistant to azoles. Although some knowledge has been gathered on the elucidation of the molecular mechanisms of resistance to antifungals in *C. glabrata*, little is known on the genetic adaptive responses that occur at the genomic level. In this work the genome sequences of a *C. glabrata* clinical isolate (named FFUL887) resistant to voriconazole, fluconazole and caspofungin was compared with the genome of the reference strain CBS138, which was found to be susceptible to all the above-referred antifungals. The genomic sequence determined for the FFUL887 isolate includes 12.29 Mb, corresponding to 99.1% of the total genome size estimated by flow cytometry. Around 80,000 genomic variations were identified, 10,000 of them corresponding to missense mutations occurring in the coding sequence of 3,200 genes (60% of the predicted *C. glabrata* ORFeome). Around 100 proteins previously associated with drug resistance in *C. glabrata* were found to harbour mutations in the FFUL887 genome including the transcription factor CgPdr1, a key player in the control of drug resistance in *C. glabrata*. Using a transcriptomic analysis it was found that the FFUL887 isolates over-expresses several described targets of CgPdr1 including the drug efflux pumps CgCDR1, CgPDH1 and CgQDR2, all previously demonstrated to contribute for azole resistance in *C. glabrata*. These observations, together with phenotypic data, demonstrate that the CgPdr1 encoded by FFUL887 has a new gain-of-function mutation.

**Key words:** Acquired antifungal resistance; *C. glabrata*; comparative genomics
Introduction

Invasive infections caused by species of the Candida genus, generally known as candidemia or invasive candidiasis, are responsible for more fatalities than any other systemic mycosis having an associated mortality of around 40% \[1\]. Among Candida spp., C. albicans is the more common causative agent of invasive and superficial fungal infections; however, in the recent years the number of infections caused by non-albicans Candida species (NCAC) has been raising significantly \[2\]. C. glabrata is now the second major cause worldwide of invasive fungal infections \[3\]. One of the reasons underlying this emergence of infections caused by NCAC and, in particular, by C. glabrata, relates with the high resistance of this yeast species to fluconazole, the frontline drug used for both active and prophylactic treatments of candidiasis \[2\]. To overcome this problem of azole-resistance, drugs alternative to fluconazole had been developed including new azoles (e.g. voriconazole) and echinocandins\[4\].

The mechanism of action of azoles involves binding to the iron atom located in the heme group present in the active site of the P450 demethylase, one of the enzymes of the ergosterol biosynthetic pathway \[5\]. This binding inhibits enzyme activity consequently leading to the accumulation of toxic sterols in the membrane. Echinocandins inhibit β-1,3-D-glucan synthase which consequently perturbs cell wall synthesis \[6\]. The inhibition of β-1,3-D-glucan synthase impairs proper cell wall formation leading to osmotic instability and ultimately promoting an apoptotic or necrotic cell death \[6\].

Although the majority of strains resistant to azoles are susceptible to echinocandins \[7\] the number of strains resistant to these drugs is increasing \[8\] thereby rendering clear that a thorough understanding of the mechanisms underlying the development of acquired resistance to azoles and echinocandins is urgent. In particular, the identification of new biological targets is essential. Despite some genome-wide approaches had been used, especially in the latter years, to characterize global responses of C. glabrata isolates resistant to azoles \[9-11\], there is still scarce information on the adaptive responses that occur at the level of the genome sequence. In fact, only a very limited number of studies had been performed examining the genomic alterations of azole-resistant isolates.

Materials and Methods

Strains and growth media

A cohort of 58 C. glabrata clinical isolates was used in this work. These clinical isolates were recovered from patients attending three major Hospitals of the Lisbon area through the years 2000 and 2008 (Annex A) and were kindly provided by Prof. Maria Manuel Lopes, Faculdade de Farmácia da Universidade de Lisboa and Dr. Rosa Barros, Head of the Microbiology Laboratory of “Centro Hospitalar de Lisboa Central”. The reference strain Candida glabrata CBS138 strain was also used.

C. glabrata cells were batch-cultured at 30°C, with orbital agitation of 250rpm in the rich growth mediums Yeast Peptone Dextrose (YPD) or RPMI (from Roswell Park Memorial Institute Medium). YPD contains, per liter, 20g glucose (Merck Millipore), 10g yeast extract (HiMedia Laboratories, Mumbai, India) and 20g Peptone (HiMedia Laboratories). RPMI, contains, per liter, 20.8g RPMI-1640 synthetic medium (Sigma), 36g glucose (Merck Millipore), 0.3g of L-glutamine (Sigmaa) and 0.165mol/L of MOPS (3-(N-morpholino) propanesulfonic acid, Sigma). Components of RPMI-1640 medium are discriminated at EUCAST E.Dis 7.1 \[16\]. The pH of the media was adjusted to 7.0 with NaOH as base,
as recommended by EUCAST. Different C. glabrata isolates were maintained at -80°C in YPD medium supplemented with 30% glycerol (v/v) (Merck). All media were prepared in deionized water. YPD medium was sterilized by autoclave for 15 minutes at 121°C and 1 atm. RPMI medium was filtered with a 0.22-µm pore size filter and preserved at 4°C until further use.

Quantification of MIC50 using the micro dilution method

To assess resistance levels of the C. glabrata clinical isolates or of the reference strain CBS138 to azoles and echinocandins the micro-dilution method recommended by EUCAST \[12\] was used. To prepare the 96-multiwell plates required for the microdilution assay, 300µl of the stock solution of each antifungal was transferred for a new tube and 1:2 dilutions (in DMSO) were performed in a final volume of 300µl, yielding concentrations ranging from 12800 mg/L to 25 mg/L for FLC and from 1600 mg/L to 3 mg/L for the other drugs. Afterwards, a second set of diluted solutions was prepared by adding 100µl of the dilutions solutions to 9.9mL of RPMI 2% glucose medium 2x concentrated. 100µl of these diluted solutions 2 were used to inoculate the 96-multiwell plates. 100µL of a diluted cell suspension was used to inoculate the 96-multiwell plates, yielding a the final test range of concentration of 64 mg/L to 0.125 mg/L for FLC and 8 mg/L to 0.015 mg/L for other drugs. These cell suspensions were prepared from a pre-culture that was cultivated for 18h in 5mL of YPD at 30ºC and 250rpm orbital agitation. The initial OD of the cultures was approximately 0.025 corresponding to around 1.25x10^5 CFU/mL which is within the range of 0.5x10^5 – 2.5x10^5 CFU/mL recommended by the EUCAST protocol \[12\]. In column 11 the cells were diluted in 100µl sterile drug-free medium to assess their growth performance in the absence of the antifungal and in column 12 only the sterile drug-free growth medium was added (diluted 1:2 in sterile water) to serve as blank. After inoculation, the 96-multiwell plates were incubated without agitation at 37°C for 24h. After that time, cells were resuspended and the recommended OD_{530nm} of the cultures was read in a microplate reader. The value of the blank was subtracted from readings of the rest of the wells. Resistance of each isolate was assessed based on results of the analysis of two independent experiments in which each isolate was assayed twice (which means that four MIC values were determined for each drug and for each isolate). The minimum inhibitory concentration (MIC50) value was calculated, comparing the ratio of the OD in each of the wells with the OD attained in control conditions, and compared to the EUCAST (www.eucast.org) recommended breakpoints for the antifungal agents.

Caspofungin time-kill assays

The viability of FFUL887 and CBS138 when exposed to 0.25 mg/L of caspofungin was quantified according to the method described by Klepser, M., et al., 1998 \[13\]. A pre-culture of the two strains was performed in YPD. This suspension was used to inoculate 10 mL of fresh RPMI growth medium (at an initial cell concentration of 10^6 CFU/mL) either or not supplemented with caspofungin. The cultures were incubated at 30°C and 250rpm for 12h. Cell viability, based on the number of CFU’s formed onto the surface of YPD plates, was measured at designated times.

Genomic DNA extraction

FFUL887 and CBS138 cells were grown in YPD until an OD_{600nm} above 3.0. Afterwards cells centrifuged at 5000rpm, during 5min at 4°C and the supernatant discarded. Pellet was resuspended in 1ml Sorbitol 1M (Sigma) and EDTA (tetrasodium salt dehydrate, Sigma-Aldrich) 0.1M at pH 7.5 solution
and transferred to an eppendorf. Afterwards, 10mg/ml zymolease (Zymo research) was added and the solution was incubated at 37°C until protoplast formation. The sheroplasts were centrifuged at 5000rpm for 5min, and the pellet resuspended in 1mL Tris-HCL with pH 7.4 50mM (Sigma-Aldrich), and EDTA 20mM solution. After this step, 30µl of SDS 10% was added to the mixture. After an incubation step of 30min at 65°C, 250µl of Potassium Acetate (5M, Merck) was added to induce protein precipitation, this being followed by a 1h incubation on ice. Afterwards the solution was centrifuged at 10000rpm for 10min and the supernatant transferred to 2 new eppendorfs. 1 volume of cold isopropanol was used to wash the pellet followed by centrifugation at 5000rpm for 15min. Supernatant was discarded and the resulting pellet was incubated in 1mL ethanol 70% during 5min, and wash with ethanol 70% twice. The pellet was dried in speed vacuum and resuspended in 200µl TE (pH 7.4). The final step, addition of 0.5µl of RNase (10 mg/ml) followed by 1h incubation at 37°C followed. Mixture was centrifuged at 10000rpm during 15min and the supernatant was preserved at 4°C till further use.

**Genome Sequencing and Annotation**

The genome of FFUL887 isolate was obtained at the next-generation sequencing (NGS) laboratory of Stab Vida, using Ion PGM sequencing technology. Two rounds of deep sequencing were performed and the reads (5 920 417) obtained were analysed using the software CLC Genomics Workbench. The reads obtained in the sequencing step were trimmed based on quality, as detailed below in Table 6. The trimmed reads were assembled using ‘de novo assembly’ and mapped ‘against the reference genome of CBS138. Variation detection was performed from the mapped reads using both probabilistic and quality-based variant detection tools and InDels and Structural Variants tool.

**Microarray profiling of FFUL887 and CBS138 strains**

The transcriptome of FFUL887 and CBS138 cells was compared in mid-log phase during growth in RPMI growth medium. For this the two strains were cultivated in 25mL of YPD at 30°C with orbital agitation (250rpm) until mid-exponential phase and then re-inoculated in 150mL of RPMI at 30°C and 250rpm. When the OD_{600nm} of the cultures achieved 2 cells were harvested by centrifugation (8000xg, 7min, 4.°C – Beckman J2.21 Centrifuge, rotor JA.10) and immediately frozen at -80°C until further use. RNA extraction was performed using RiboPure™ RNA Isolation Kit (Ambion, Life Technologies, California, USA). The resulting cell pellet was resuspended in 480µl of lysis buffer, 48µl of SDS and 480µl of a mixture of Phenol:Chloroform:IAA. The suspension was then added to new tubes containing about 750µL cold Zirconia Beads. Cell disruption was performed by position the tubes horizontally on the vortex and agitated at maximum speed for 10min. Separation of the aqueous phase, containing the RNA, from the organic phase was obtained by centrifugation for 5min at 16.100xg at room temp. 1.90mL of Binding Buffer and 1.25mL of 100% Ethanol was added and the total volume was centrifuged through a filter cartridge. Filter cartridge was washed with 700µl of Wash Solution 1 and washed two times with 500µl of Wash Solution 2/3 followed 1min centrifugation and an extra minute to ensure the complete removal of Wash solution. Total RNA obtained was eluted in two times with 50µl of Elution Solution and washed two times with 500µl of Wash Solution 2/3 followed 1min centrifugation and an extra minute to ensure the complete removal of Wash solution. Total RNA obtained was eluted in two times with 50µl of Elution Solution, previously heated at 95°C. DNase treatment was perform by adding to the 100µl-RNA sample 8 U of DNase I and 10µl of 10X DNase I Buffer. The mixture was incubated at 37°C during 30min. After incubation period 10µL of DNase Inactivation Reagent were added to the mixture,
which was then vortexed and left for 5min at room temperature. The purified RNA (in the supernatant fraction) was collected by centrifugation at >10000rpm for 3min and supernatant fraction was added to fresh tubes. RNA extracted was quantified and integrity verified using Bioanalyser. The microarray analysis was performed according with the methodology described by Rossignol, T., et al., 2007 [14].

Adherence to biotic and abiotic surfaces

Adhesion of FFUL887 and CBS138 strains to polystyrene, fibronectin and vitronectin of the different clinical isolates was measured in 96-mutiwell plates using the crystal violet staining method. When vitronectin and fibronectin were used, a pre-coating step of the plate was required. This coating step was performed by adding 200µl of fibronectin (Sigma) and vitronectin (Sigma) 10µg/ml diluted in sterile PBS and leaving the plate rest overnight at 4ºC. After 4h and 8h of cultivation of the two strains in the plates containing RPMI medium, the plates were washed two times with sterile PBS and non-specific binding sites were blocked with 200µl sterile PBS with 2% of bovine serum albumin (BSA, Sigma) during 1h at room temperature. Two washing steps with sterile PBS, strains were cultivated under the same experimental setup described above for the micro dilution method. After 4h and 8h of growth in the presence or absence of the antifungals the cells were washed in PBS 1x (NaCl 8 g/L, KCL 0.2 g/L, Na2HPO4 1.44 g/L and KH2PO4 0.24 g/L) and fixed with 200µl of methanol for 15min. After drying, 200µl of crystal violet (Merck) was added to each cell suspension for staining. After 15 minutes, the biofilm was washed with deionized water, resuspended in ethanol and the OD570nm was measured in a microplate reader.

Results

Profiling of antifungal resistance within a cohort of clinical C. glabrata isolates

Under the experimental conditions used, the MIC value of the reference strain CBS138 was of 16 mg/L for fluconazole and 0.25 mg/L for voriconazole. Nine isolates were found to be resistant to fluconazole, while seven were found to be resistant to voriconazole. These numbers correspond to an incidence of resistance of about 16% and 14% of the total number of isolates tested. Seven isolates were resistant to both azoles. None of the C. glabrata isolates tested could be considered susceptible to fluconazole as the MIC values were always above 0,002 mg/L. Consequently, the majority of the isolates were clustered in the intermediate resistant class.

Regarding echinocandins, the MIC value of the reference strain CBS138 was of 0.06 mg/L for anidulafungin and 0.125 mg/L for caspofungin. None of the isolates tested were able to grow in concentrations of anidulafungin above 0.06 mg/L, the defined breakpoint concentration. Consequently, none of the isolates tested could be considered resistant to anidulafungin. Interestingly, CBS138 reference strain was found to be considerably more tolerant to anidulafungin than the remaining isolates as it exhibited some growth in the presence of 0.06 mg/L of the drug. Although the identification of isolates resistant to caspofungin is not possible due to the absence of a defined breakpoint and MIC distributions, a closer look into growth of the isolates in the presence of this antifungal renders clear that isolate FFUL887 is considerably more tolerant than the remaining isolates being able to grow in the presence of 0.25 mg/L of this drug. Notably, FFUL887 was one of the isolates that was also found to be resistant to fluconazole and voriconazole (fluconazole and voriconazole MICs of 2 mg/L and
>64 mg/L). The FFUL887 isolate was further selected to proceed in this work considering its interesting traits of resistance to fluconazole, voriconazole and caspofungin.

**Resistance to echinocandin-induced death**

Echinocandins have a reported fungicidal action against *C. glabrata* [15]. Taking this into consideration, cell viability of FFU887 and CBS138 cells was compared upon sudden exposure to 0.25 mg/L of caspofungin, a concentration that was found to induce loss of cell viability. Remarkably, FFUL887 cells were found to more resistant to the killing effect exerted by caspofungin than the CBS138 strain (Figure 1).

![Figure 1 - Resistance of FFUL887 and CBS138 cells to killing induced by 0.25 mg/L of caspofungin. Cells of the two strains were cultivated in RPMI 1640 2% G growth medium in control supplemented with 0.25 mg/L caspofungin (■ and ○, respectively) or 0 mg/L (□ and □, respectively) for 12h during which cell viability of the two cultures was at designated times. The viability results shown are representative of three independent experiments.](image)

**Genome Sequencing and Annotation**

To better understand the genetic adaptive responses underlying the increased resistance of the FFUL887 isolate to voriconazole, fluconazole and caspofungin the genome sequence of this isolate was obtained and then compared with the publicly available genome sequence of the CBS138 strain. The genome sequence of the FFUL887 isolate was obtained after two rounds of sequencing in a PGM sequencer from Life Technologies (Ion Torrent technology) in the NGS laboratory of StabVida. The total number of assembled bases, 12.29 Mb, corresponds to 99.1% of the estimated genome size of the FFUL887 strain, 12.4 Mb, as determined by flow cytometry. A total of 79 076 mutations (78 865 corresponding to SNPs and 211 to InDels) had been identified in the genomes of CBS138 and FFUL887. A total of 3198 gene coding sequences were found to differ between FFUL887 and CBS138, 25 342 of these differences corresponding to synonymous mutations and 10 065 to non-synonymous ones. The statistics of genome sequencing, assembly and variant call performed are summarized in Table 1.

**Table 1 - Results of FFUL887 genome sequence, assembly and variations detection, in comparison with the genome of the reference strain CBS138. These results were obtained using the software CLC Genomics Workbench.**

<table>
<thead>
<tr>
<th>Genotype Sequencing Statistics</th>
<th>Reads</th>
<th>Assembly</th>
<th>Variant calling</th>
<th>InDels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of sequenced bases</td>
<td>1 182 Mb</td>
<td>1 160 Mb</td>
<td>Total</td>
<td>78 865</td>
</tr>
<tr>
<td>Number of reads</td>
<td>5 928 417</td>
<td>769</td>
<td>Synonymous SNPs</td>
<td>Coding region</td>
</tr>
<tr>
<td>Read average size</td>
<td>199.75 bp</td>
<td>15 984 bp</td>
<td>Non-synonymous SNPs</td>
<td>In non-coding region</td>
</tr>
<tr>
<td>Total number of matched bases</td>
<td></td>
<td>Contig length Sum 12 291 887 bp</td>
<td>25 942</td>
<td>58</td>
</tr>
<tr>
<td>Total of contigs</td>
<td></td>
<td>Coverage 95.8x</td>
<td>10 065</td>
<td>153</td>
</tr>
<tr>
<td>Average Contig length</td>
<td></td>
<td></td>
<td>42 858</td>
<td></td>
</tr>
</tbody>
</table>
harbored 5 non-synonymous SNPs or less, but up to 100 mutations per gene is reported. A closer look into the biological function of the genes that harbored more than 16 mutations clearly revealed the enrichment of proteins involved in adhesion. 51% of adhesin-related proteins in *C. glabrata* [16] were found to have non-synonymous mutations in FFUL887. It is possible that these heavily mutated genes are those under a stronger selective pressure [17]. It was interesting to observe that besides extensive number of SNPs other mutations were identified in the set of adhesins encoded by the FFUL887 genome: including disruptive mutations being predicted to eliminate the glycosylphosphatidylinositol (GPI)-anchor; variable the number of repeated sequences found in the coding sequence of the adhesin genes; increase in size of the protein.

**Ability of CBS138 and FFUL887 cells to adhere to biotic and abiotic surfaces**

The significant number of differences found in the coding sequences of genes related with adhesion in CBS138 and in FFUL887 suggested that these isolates could have different adherence capacities. Both strains adhered rapidly to polystyrene and vitronectin, with the FFUL887 cells reaching higher biomass values, especially for vitronectin (Figure 2). Adherence to fibronectin was considerably slower and reached higher values for the CBS138 strain (Figure 2). Altogether, the results confirm the suggestion that CBS138 and the FFUL887 isolate have differences in their adherence properties.

![Figure 2 - Adherence of FFUL887 and CBS138 cells to biotic and abiotic surfaces. Cells of the two strains were cultivated in polystyrene 96-microwell plates or in these same plates pre-coated with fibronectin (10 µg/ml) or vitronectin (10 µg/ml) for 4 and 8h. After incubation, the amount of biomass present was quantified using the crystal violet staining method.](image)

Mutations occurring in genes associated with antifungal resistance in *C. glabrata*

It is hard, if not impossible, to predict the consequences for protein activity of the mutations that were identified throughout the FFUL887 proteome and to assess how these modifications impact the overall physiology of CBS138 and FFUL887 cells. However, in the case of mutations leading to premature STOP codons such analysis is possible since this alteration may lead to protein inactivation, depending on the region where the protein is prematurely truncated. The deletion of any of the genes that were found to be truncated in the FFUL887 has not been found to increase *C. glabrata* resistance to azoles or echinocandins, indicating that the premature interruptions of the proteins is not contributing for tolerance to antifungals in the FFUL887 strain. Despite this, four genes found to be interrupted in the FFUL887 strain, CAGL0A03872g, CAGL0H08217g, CAGL0I09746g (truncated at 28%), CAGL0K11396g (truncated at 59%) are also found to encode proteins whose deletion in other species led to azole resistance.

106 of the “antifungal-resistance” genes were found to harbor non-synonymous mutations in the FFUL887 isolate, 45 of these genes being associated with resistance to fluconazole and/or voriconazole, 55 involved in resistance to caspofungin and 6 affecting *C. glabrata* resistance to both classes of antifungals. Among the genes that were found to have mutations in FFUL887 were the FKS genes, the biological targets of echinocandins (Figure 3). The Gly14Ser mutation occurring in the coding sequence of FFUL887 CgFKS1 occurs in the C-terminal region of the protein and creates a
potential phosphorylation site, according to the NetPhosYeast algorithm \cite{18}. Phosphorylation of FKS1 has been described to serve as an activating mechanism for this enzyme \cite{19}. In the case of CgFKS2 one of the mutations identified in the FFUL887, Thr926Pro, is located in the catalytic domain of the protein (Figure 3).

6 genes described to confer resistance to echinocandins and azoles in C. glabrata were found to harbour mutations in the genome of the FFUL887 isolate (Table 2), this set including two proteins involved in chromatin remodelling and several proteins involved in the high-affinity calcium uptake system (HACS) \cite{10,11,20}.

Table 2. Genes described to mediate resistance to azoles and echinocandins in C. glabrata that were found to harbour mutant variations in the FFUL887 isolate.

<table>
<thead>
<tr>
<th>Gene/ORF name</th>
<th>Amino acid modification found in FFUL887</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgSIN3</td>
<td>Asn50Lys; Lys288Thr</td>
</tr>
<tr>
<td>CgSWI4</td>
<td>Asn300Ile; Asn669Ser; Val324Ala; Arg715Lys</td>
</tr>
<tr>
<td>CgCCH1</td>
<td>Met1Ile; Arg51Pro; Glu1912Lys</td>
</tr>
<tr>
<td>CgMID1</td>
<td>Gly77Asp</td>
</tr>
</tbody>
</table>

Around 70% of the genes associated with resistance to azoles in C. glabrata were found to harbor non-synonymous mutations in FFUL887. Functional clustering of these proteins reveals an enrichment of proteins involved in chromatin remodeling and in multidrug resistance, including several drug efflux pumps and various transcriptional regulators (Table 3).

Table 3. Genes described to mediate azole resistance and/or associated to multidrug resistance in C. glabrata that were found to harbour mutant variations in the FFUL887 isolate.

<table>
<thead>
<tr>
<th>Gene/ORF name</th>
<th>Amino acid modification found in FFUL887</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgPDH1</td>
<td>Lys438Gln; Glu839Asp</td>
</tr>
</tbody>
</table>

A transcription factor found to harbor mutations in the FFUL887 strain was CgPDR1. Notably, the X mutation has never been described \cite{21}, although this mutation is mapped in a region where other GOF mutation had been identified. Figure 4. Analysis of the secondary structure of the CgPdr1 protein encoded by FFUL887 and CBS138, performed using the algorithm Phyre2 \cite{22}, also showed that A mutation in the FFUL887 strain may induce a change in the protein structure.

Figure 4 - Alterations in the amino acid sequence of the CgPdr1 transcription factor encoded by FFUL887, when compared to its counter-partners encoded by the CBS138 strain. Neutral mutations are represented in grey.

To fully elucidate if the FFUL887 CgPdr1 indeed corresponded to a GOF mutant the transcriptomes of the FFUL887 and CBS138 strains were compared using DNA microarrays (in collaboration with the group of Professor Geraldine Butler, from University College of Dublin). Around 544 genes were found to be differently expressed (above or below a 1.5-fold threshold level) between
the CBS138 and FFUL887 strains. Significantly, 21 genes found to be more expressed in the FFUL887 isolate correspond to documented targets of CgPdr1. Among these set of well-described CgPdr1-regulated genes are the CgPDR1 gene itself and the drug-efflux pumps CgPHD1 and CgCDR1.

55 genes found to contribute for resistance of C. glabrata to echinocandins were found to harbor non-synonymous mutations in FFUL887, a selected set of these being shown in Table 3. A substantial amount of these echinocandin-resistance genes that have mutations in FFUL887 are involved in cell wall biosynthesis and assembly and PKC pathway signalling pathway genes.

**Table 16.** Genes described to mediate resistance to echinocandins in C. glabrata found to harbour mutant variations in the FFUL887 isolate.

<table>
<thead>
<tr>
<th>Gene/ORF name</th>
<th>Amino acid modification found in FFUL887</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAGL0B01441g (RPD3)</td>
<td>Thr414Ala</td>
</tr>
<tr>
<td>CAGL0L03520g (BCK1)</td>
<td>Ile22Met; Ala550Val</td>
</tr>
<tr>
<td>CgRLM1</td>
<td>Ala103Thr; Pro233Leu; Gln309Arg</td>
</tr>
<tr>
<td>CgSWi6</td>
<td>Glu100Asp</td>
</tr>
<tr>
<td>CgSTE20</td>
<td>Val279Ile; Arg352Lys</td>
</tr>
<tr>
<td>CAGL0B00858g (STE50)</td>
<td>Glu40Asp</td>
</tr>
<tr>
<td>CAGL0L05632g (PBS2)</td>
<td>Leu178Pro</td>
</tr>
<tr>
<td>CAGL0G03597g (SHO1)</td>
<td>Thr224Ala</td>
</tr>
</tbody>
</table>

**Discussion**

The goal of this work was to analyse the increasing problem of acquired resistance to antifungals in the human pathogen Candida glabrata using a comparative genomics approach. For that the genome sequence of a clinical isolate FFUL887 found to be resistance to fluconazole and voriconazole and showing increased tolerance to caspofungin, was compared with the genome of the susceptible reference strain CBS138. A very high mutational rate of proteins involved in adhesion, that play a key role in shaping C. glabrata recognition, adhesion and further adaptation in the host [16], was found in FFUL887. Differences in the ability of FFUL887 and CBS138 strains to adhere in vitro to different biotic and abiotic surfaces were registered. It was particularly prominent the higher capacity of the FFUL887 strain to adhere to vitronectin. FFUL887 was recovered from an urine sample and therefore it is possible that is increased adherence to vitronectin could result from an adaptive response considering that vitronectin is relevant in the human urinary tract were it is involved in anchoring basal urothelial cells to the underlining basement membrane against constant bladder cyclin [23].

FFUL887 cells were also found to have a lower adherence to fibronectin than the CBS138 strain. Interestingly, studies have shown that decreased adherence to fibronectin could help Candida spp. to evade the action of the host immune system [24, 25].

Several genes were found to harbour frameshift mutations in the FFUL887 that result in protein inactivation. None of the genes found to be prematurely truncated in the FFUL887 isolate has been reported to increase C. glabrata resistance to antifungals. Nevertheless, the deletion of 4 genes interrupted in FFUL887 strain, CAGL0A03872g, CAGL0H08217g, CAGL0I09746g and CAGL0K11396g led to increased resistance to azoles in S. cerevisiae. It will thus be interesting to examine if the deletion of these genes also augments tolerance to antifungals in C. glabrata.

A new gain-of-function mutation had been identified in the coding sequence of the transcriptional regulator CgPdr1 encoded by the FFUL887 strain. This mutation, corresponding to a modification in the lysine residue at position 274 to a glutamine, is the only mutation identified in the coding sequence of FFUL887 CgPdr1 that has not
been described to occur in susceptible *C. glabrata* clinical isolates. Transcriptomic analysis of CBS138 and FFUL887 revealed the over-expression in the later isolate of a significant number of documented targets of CgPdr1 thereby sustaining the idea that the X mutation identified is indeed a GOF mutation. Since the X mutation occurs in the transactivation domain of the protein it can be hypothesized that the mutation might lead to an enhanced ability of the transcription factor to recruit transcriptional machinery, an idea that needs to be further demonstrated. Among the CgPdr1-targets that were found to be up-regulated in FFUL887 are the CgPDR1 gene itself and also the drug-efflux pumps CgPDH1 and CgCDR1 previously found to contribute for resistance to fluconazole and/or voriconazole [9-11, 26-28].

Besides azoles, the FFUL887 strain was also found to be more tolerant than CBS138 to caspofungin. Several mutations were identified in genes encoding proteins that are known to contribute for tolerance to caspofungin in *C. glabrata*. In particular, several mutations in components of the PKC and of the HOG1 signalling pathway had been identified which suggest that the activity of these two signalling systems may differ in the FFUL887 and in the CBS138 strain. Indeed, inspection of the transcriptomes of FFUL887 and CBS138 revealed that several genes that are documented to be regulated by Rlm1 and SFB complex (Swi4/Swi6) of *C. albicans* and *S. cerevisiae*, two transcription factors that are the final effectors of the PKC pathway, were found to be up-regulated in the FFUL887 strain (data not shown) which might indicate a higher activity of this pathway in this strain. Nevertheless, the killing effect exerted by caspofungin was significantly higher in CBS138 than in FFUL887, which is necessarily an important contributing factor to improve tolerance in this later strain. The molecular mechanisms by which *C. glabrata* cells lose viability upon caspofungin exposure are not known although, in *C. albicans* this has been demonstrated to involve both apoptotic and necrotic-induced death [29]. The genes mediating these mechanisms of programmed cell death induced by caspofungin have not been identified and therefore it is not possible to know if the CBS138 and the FFUL887 might have differences that could justify the observed resistance of the later strain to caspofungin-induced killing, although this an hypothesis that deserves further investigation.

References


