New evidence that *Candida albicans* possesses additional ATP-binding cassette MDR-like genes: implications for antifungal azole resistance

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Emergence of resistance of *Candida albicans* to antifungal triazoles is increasingly recognized as an important cause of refractory mucosal candidiasis in HIV-infected patients. Recently, CDR1, which is thought to be analogous to the human MDR-1 P-glycoprotein, has been cloned in *C. albicans*. It has been proposed that its expression is partially responsible for fluconazole resistance in *C. albicans*. This gene is characterized by the presence of an ATP binding cassette (ABC) region and is distinct from the BENr gene which does not encode such a functional domain. As the molecular basis for fluconazole resistance appears to be multifactorial, we considered that there may be other ATP binding cassette-containing MDR genes that may potentially contribute to antifungal azole resistance in *C. albicans*. We therefore sought to identify potential target sequences that may be derived from candidate genes that share homology with the ATP binding cassette region of the human MDR-1 P-glycoprotein. Degenerate oligonucleotide primers based on the known sequence from the ATP binding cassette region of the human MDR-1 P-glycoprotein were used to amplify PCR products within the range of 100 bp in length from *C. albicans* isolates (3 fluconazole-susceptible and 3 fluconazole-resistant). Sequence analysis of individually subcloned PCR products, derived from the six isolates revealed 34 sequences in total. The results of our study identified 14 clones (with at least one per isolate) with a high degree of homology to the ATP binding cassette of the human MDR-1 P-glycoprotein. The BLAST search did not disclose homology of these new sequences to the *C. albicans* CDR1 gene, suggesting that *C. albicans* may possess more than one MDR-like gene. We conclude that *C. albicans* may possess one or more additional genes encoding ATP binding cassette MDR-like proteins that are distinct from CDR1 and which could participate in the development of fluconazole resistance.

**Keywords** ATP binding cassette, *C. albicans*, MDR-like proteins

**Introduction**

Oropharyngeal and oesophageal candidiasis (OPEC) is one of the most common opportunistic infections in HIV-infected patients. Fluconazole-resistant *C. albicans* is emerging as an increasingly important cause of refractory OPEC [1,2]. Similar trends are being reported for *C. albicans* and non-albicans species of *Candida* in leukaemia and marrow transplant recipients [3,4]. The emergence of resistance of *C. albicans* to fluconazole and other antifungal azoles threatens the utility of this valuable class of antifungal compounds. Understanding the mechanisms of antifungalazole resistance is fundamentally important to developing strategies to prevent or reverse the molecular and biochemical events of this process. Recent studies indicate that impaired uptake or increased efflux is an important mechanism of resistance.
of fluconazole in *C. albicans* [5,6]. This mechanism in *C. albicans* is mediated by a multidrug resistant protein (MDR) which serves as an efflux pump, resembling the human MDR-1 P-glycoprotein. This class of proteins contains an ATP binding cassette region and is distinct from the protein encoded by BENr gene. The BENr protein is a facultative pump which also is implicated in impaired uptake of antifungal azoles but which does not contain an ATP binding cassette domain [7]. In considering that several proteins and mechanisms appear to mediate fluconazole resistance, we hypothesized that other ABC-containing MDR proteins may mediate fluconazole resistance in *C. albicans*. We therefore sought to determine if *C. albicans* possesses genes homologous to the human MDR-1 P-glycoprotein.

To further characterize the molecular basis for this MDR-like resistance to fluconazole by *C. albicans*, we searched for short, homologous sequences that could indicate the presence of a family of genes in *C. albicans* that contain the signature ATP-binding cassette motif. Degenerate PCR oligonucleotide primers derived from the human MDR-1 gene were used in this strategy to identify segments of a possible family of genes that share homology with the human MDR-1 gene.

**Materials and methods**

**Organisms**

Isolates of *C. albicans*, each from the oral cavity of a different HIV-infected child with symptomatic oropharyngeal candidiasis were collected at the NCI Pediatric Branch of NIH in the Warren Grant Magnuson Clinical Center in Bethesda, MD. Each isolate was identified as *C. albicans* by the Clinical Microbiology Laboratory at the NIH using standard methods [8] and stored on potato dextrose agar slants at -70 °C. Susceptibility to ketoconazole, fluconazole, and itraconazole was determined by NCCLS methods [9]. The minimum inhibitory concentration (MIC) for an azole drug was the lowest concentration that inhibits at least 80% of the control growth. For the purposes of this study, organisms were considered susceptible to fluconazole if the MIC was ≤ 0.5 μg ml⁻¹ and resistant to fluconazole if the MIC was > 64 μg ml⁻¹. For the purpose of assessing cross-resistance, organisms were considered resistant to ketoconazole and itraconazole, if MICs were > 6 μg ml⁻¹, respectively. Three fluconazole-susceptible isolates (CA-180, 195 and 198) and three fluconazole-resistant isolates (CA-103, 126 and 156) were studied. The three fluconazole-resistant isolates also were cross-resistant to ketoconazole and/or itraconazole.

**DNA extraction**

*Candida* DNA was extracted using a modification of methods described by Magee et al. [10]. For DNA extraction, 45 ml of 2% Sabouraud glucose medium in a 250 ml Erlenmeyer flask was inoculated with three colonies of the isolate from a culture grown overnight. *C. albicans* was pelleted and washed in normal saline prior to preparation of spheroplasts. Pellets were resuspended in 8 ml of Spheroplast Buffer (10 m sorbitol, 50 mM sodium phosphate monobasic, 0.1% 2-mercaptoethanol, 10 μg ml⁻¹ lyticase (Sigma L-8137), vortexed, and incubated for 45 min at 30 °C. Spheroplast pellets were resuspended in lysis buffer (50 mM EDTA, pH 8.0, 0.3% SDS) and incubated for 30 min at 65 °C, prior to extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation. Resuspended DNA samples were treated with 10 units of ribonuclease A (Sigma, R-9134) for 1 h at 37 °C. The quality of DNA was verified on 1% agarose gel.

**Polymerase chain reaction**

Stock DNA was diluted 1:10 in double distilled H₂O and used as template for PCR. The following degenerate oligonucleotides modified from Allikmets et al. [11] were used as primers: MDR5 GG(ACGT)CA(GA)(AC)A (AG) CA (GA)(CA)G(ACGT) (AC) T (ACGT) G C and MDR3 CA (GA) A (GA)C (AT)(GT)(AT) (ACGT)GT (ACGT) (GC)C(TC)TCATC. The final concentrations for the 50 μl PCR reactions were 1.5 mM MgC1₂, 500 mM KCl, 100 mM Tris-HCl, gelatin 0.01% (w:v), 0.32 mM dNTP mixture, 0.4 ng ml⁻¹ of each primer, and 0.5 μl Taq polymerase (2-5 units). The PCR reaction was run under the following conditions (Perkin-Elmer Cetus, Emeryville, CA): 94 °C for 1 min, 45/50 °C for 1 min (depending on isolate), 72 °C for 1 min, for 29 cycles; 94 °C for 1 min, 45/5055 °C for 1 min (depending on isolate), 72 °C for 7 min, for 1 cycle; 4 °C soak. Negative controls, which were performed with each run, consisted of a normal reaction containing the PCR master mix plus 1 μl of water instead of DNA. PCR products were confirmed by gel electrophoresis and determined to be approximately 100 bp in length. Large quantities of PCR products were generated in seven separate 50 μl reaction tubes and run simultaneously. The bands of interest were excised and purified from agarose with QIAEX Gel Extraction Kit (Qiagen).

**Sequence analysis of PCR products**

The agarose purified gene segments of interest were directly subcloned into the pCRH vector (Invitrogen, Cat. no. K2000-01). Sequence analysis of the subcloned PCR fragments was performed by the Sanger dideoxy chain
Table 1 summarizes significant nucleotide base pair homologies between MDR-like genes from C. albicans and genes encoding the human MDR-1 P-glycoprotein gene (P = 3.3 x 10^-7) and Plasmodium falciparum MDR-1 P-glycoprotein gene (P = 2.2 x 10^-5). Significant homologies between MDR-like genes from C. albicans and genes encoding the MDR-1 P-glycoproteins from other species including Leishmania donovani and Saccharomyces cerevisiae.

Table 1. Significant homologies between new ATP binding cassette-containing MDR-like genes from Candida albicans and genes encoding other MDR proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clone no.</th>
<th>P value</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MDR P-glycoprotein</td>
<td>M14758</td>
<td>3.3 x 10^-7</td>
<td>74</td>
</tr>
<tr>
<td>MDR P-glycoprotein (Plasmodium falciparum)</td>
<td>M24850</td>
<td>2.2 x 10^-5</td>
<td>73</td>
</tr>
<tr>
<td>ATM 1 (Saccharomyces cerevisiae)</td>
<td>EMBL X82612</td>
<td>9.8 x 10^-7</td>
<td>69</td>
</tr>
<tr>
<td>MDR Protein (ldmdr1) (Leishmania donovani)</td>
<td>01572</td>
<td>6 x 10^-9</td>
<td>71</td>
</tr>
</tbody>
</table>

Results

Thirty-four separate clones were characterized by sequence analysis from six separate clinical isolates of C. albicans, three sensitive and three resistant to fluconazole. Each of the six isolates yielded five or six clones. Using the BLASTN and BLASTX programs, 14 sequences shared significant homology with MDR-like sequences in GenBank. The remaining 20 sequences shared significant homology with non-MDR-like sequences. Even using a less stringent P value (≤0.05) did not reveal any significant homology with the CDR1 gene.

Discussion

This study demonstrated that C. albicans possesses novel gene fragments homologous to the MDR-1 P-glycoprotein of humans, protozoa, and Saccharomyces cerevisiae. Further suggesting the uniqueness of these gene segments was the absence of significant sequence homology of these new sequences to the C. albicans CDR1 gene at the significance level of P ≤ 0.01.

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homology between the previously reported CDR1 gene and the MDR-like gene fragments found in this study. As the BENr gene does not encode an ATP binding cassette domain, one would not anticipate that any homology with this gene would be identified with this search strategy. It is notable that short segments of MDR-like genes are found in fluconazole-susceptible and fluconazole-resistant strains of *C. albicans*.

The mechanisms of resistance to fluconazole in *C. albicans*, appear to be multifactorial, including impaired uptake or increased efflux, and amplification of target protein. An efflux pump analogous to that of human MDR-1 P-glycoprotein in neoplastic cells has been identified as contributing to the mechanisms of resistance to fluconazole [5,7,15,16]. This study further establishes that *C. albicans* may possess additional genes analogous to this class of human MDR proteins. That several genes may mediate azole resistance is also feasible given their structural heterogeneity, ranging from relatively hydrophilic to hydrophobic. Both the amino acid sequence and nucleotide sequence homology studies in this report reveal several candidate genes. As these genes have no homology with the recently described BENr and CDR1 genes related to multidrug resistance in *C. albicans*, there are apparently other genes likely encoding MDR-like proteins that may contribute to azole resistance [7,17,18].

That a family of signature sequences that encode for ATP-binding cassettes homologous with human MDR-1 in *C. albicans* is not unexpected. For example, two isoforms of the murine MDR gene (MDR-1 and MDR-3), which were cloned and overexpressed in Chinese hamster ovary cells, were found to confer different profiles of drug resistance [19]. By analogy different MDR-like genes in *C. albicans* may encode proteins mediating resistance to different groups of antifungal compounds.

As demonstrated in Fig. 1, there is a highly significant homology between amino acid sequences encoded by the human MDR1 gene and the uppermost group of sequences. Of note, analysis of the middle group, which possesses intermediate homology, reveals that a single base pair change would lead to a higher degree of amino acid sequence homology with that of the human MDR1. While PCR artifact is a possible explanation for these differences in homology, another possibility exists that we have identified one or more potential pseudogenes.

Others have reported several MDR-like genes, MDL1 and MDL2 in *Saccharomyces* which contain an ATP-binding domain and show considerable similarity to P-glycoprotein (MDR) and peptidetransporter (TAP) genes [20]. In our study, we did not identify target sequences with a high homology to MDL1 and MDL2, supporting the concept of diversity within organisms and between different species. Homology also exists between the genes found in this study and the genes encoding MDR proteins found in other eukaryotic cells, including *P. falciparum* and *L. donovani* [21,22].

In summary, new evidence reveals that *C. albicans* possesses several candidate genes that share homology with the genes for human MDR P-glycoprotein. Such genes may encode ATP binding cassette-containing proteins that contribute to fluconazole resistance.

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**References**

C. albicans possesses ATP-binding cassette MDR-like genes


