Overexpression and mutation as a genetic mechanism of fluconazole resistance in *Candida albicans* isolated from human immunodeficiency virus patients in Indonesia

Yeva Rosana, Andi Yasmon and Delly Chipta Lestari

Fluconazole is the standard treatment for oropharyngeal candidiasis, which is the third most common opportunistic infection in human immunodeficiency virus (HIV)/AIDS patients in Indonesia. Overuse of this drug could lead to the emergence of resistance. The objective of this study was to analyse the role of *ERG11*, *CDR1*, *CDR2* and *MDR1* gene overexpression and mutations in the *ERG11* gene as a genetic mechanism of fluconazole resistance in *Candida albicans* isolated from HIV patients in Indonesia. Overexpression of *ERG11*, *CDR1*, *CDR2* and *MDR1* was analysed by real-time reverse transcription PCR, while *ERG11* gene mutation analysis was performed using sequencing methods. Seventeen isolates out of 92 strains of *C. albicans* isolated from 108 HIV patients were found to be resistant to azole antifungals. The highest gene overexpression of *ERG11* was found in *C. albicans* resistant to single fluconazole, while the highest gene overexpression of *CDR2* was detected in all isolates of *C. albicans* resistant to multiple azoles. Amino acid substitutions were observed at six positions, i.e. D116E, D153E, I261V, E266D, V437I and V488I. The amino acid substitution I261V was identified in this study and was probably associated with fluconazole resistance. The combination of overexpression of *CDR2* and *ERG11* and mutation in the *ERG11* gene was found to be a genetic mechanism of fluconazole resistance in *C. albicans* isolated from HIV patients in Indonesia.

INTRODUCTION

Candidiasis, which is caused by *Candida albicans*, is the most common *Candida* infection found in immunocompromised patients, particularly in human immunodeficiency virus (HIV)-infected patients (Pohan, 2006; de Repentigny *et al.*, 2004; Wingeter *et al.*, 2007). The antifungal fluconazole has been widely used as first-line therapy for antifungal prophylaxis or empirical treatment for *Candida* infections (Pappas *et al.*, 2009). This extensive use of fluconazole has the potential to lead to emergence of resistant *Candida* strains, which have been detected in many countries (Orrù *et al.*, 2008; Morio *et al.*, 2010; Perea *et al.*, 2001).

Four molecular mechanisms have been reported to confer fluconazole resistance in *C. albicans* (Perea *et al.*, 2001; Marichal *et al.*, 1999; White *et al.*, 2002). The first is the reduction of intracellular fluconazole due to the overexpression of genes encoding efflux transporters belonging to the ATP-binding cassette superfamily (*CDR1* and *CDR2*) or major facilitator superfamily (*MDR1*). A second mechanism is mutation of the *ERG11* gene encoding lanosterol 14α-demethylase (CYP51), the primary target of fluconazole. The mutation causes a structural change in lanosterol 14α-demethylase; consequently, it changes the affinity of fluconazole for this enzyme. The third is an intracellular concentration of fluconazole insufficient to inhibit the function of CYP51 due to overexpression of the *ERG11* gene. The last mechanism involves alterations in the ergosterol biosynthetic pathway.

In clinical isolates, several resistance mechanisms often act synergistically (White *et al.*, 2002; Wang *et al.*, 2009; Sanglard & Odds, 2002). One study reported that 85% of...
isolates overexpressed efflux pumps, 65 % of isolates had mutations in ERG11, and 35 % of isolates overexpressed ERG11 (Perea et al., 2001). Another study showed that most of the amino acid changes in CYP51 were clustered into three hotspot regions: aa 105–165, 266–287 and 405–488 (Marichal et al., 1999). Of these amino acid changes, A61V, K143R, S405F, F449S, G464S, R467K and I471T were associated with fluconazole resistance (Orru et al., 2008; Morio et al., 2010; Perea et al., 2001; Marichal et al., 1999; White et al., 2002).

There are no data available about the molecular mechanism of fluconazole resistance in C. albicans isolated in Indonesia. However, fluconazole is the standard treatment for oropharyngeal candidiasis (OPC), which is the third most common opportunistic infection in HIV/AIDS (acquired immunodeficiency syndrome) patients in Indonesia. Data about the molecular mechanisms of fluconazole resistance in C. albicans were therefore required for treatment guidelines and the utilization of laboratory testing for early detection. The molecular mechanisms of fluconazole resistance (mutation and overexpression of the ERG11 gene and overexpression of the CDR1, CDR2 and MDR1 genes) in C. albicans isolated from the oropharynx of HIV-infected patients in Indonesia were analysed in this study.

**METHODS**

**Isolation and culture.** C. albicans isolates were obtained from 108 HIV-infected patients in the Cipto Mangunkusumo Hospital, Jakarta, Indonesia from May 2009 to September 2010. All patients were included in this study except those exposed to antifungals up to 1 month previously. This study was approved by the Ethical Committee of the hospital and Faculty of Medicine, University of Indonesia (no. 162/PT02.FK/ETIK/2009) and all patients gave their written consent before enrolment in the study. C. albicans was isolated from the oral cavity by the concentrated oral rinse technique, i.e. an oral rinse procedure using 10 ml sterile PBS (0.1 M, pH 7.2), which was held in the mouth for 1 min, then collected in a sterile container. Each rinse was centrifuged (3000 g; 15 min), 7.5 ml of the supernatant was removed and 2.5 ml of the deposit was used for further testing. The presence of any budding yeast and pseudohyphae was detected by Gram staining. To test for growth, a portion (100 µl) of the concentrate was inoculated onto mycobiotic agar plates containing chloramphenicol and cycloheximide, and suspected *Candida* colonies were subcultured onto plates containing Sabouraud dextrose agar, which were incubated at 35 °C for 48 h.

**Identification and susceptibility testing.** Yeast identification was conducted using the commercial API 20C AUX method (bioMérieux). After incubation at 30 °C for 24–48 h, the results were read visually and interpreted using the API database (Agha et al., 2012). The susceptibility was tested using the commercial ATB Fungus 3 method (ATB3; bioMérieux). An ATB F3 strip is composed of 32 wells, including a growth control and three azole antifungals at different concentrations: fluconazole from 1 to 128 mg l⁻¹, itraconazole from 0.125 to 4 mg l⁻¹ and voriconazole from 0.06 to 8 mg l⁻¹ (Torres-Rodriguez & Alvarado-Ramírez, 2007). After incubation at 35 °C for 24 h, the strips were read visually. MIC values for *Candida* spp. were interpreted using Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) as resistant if the MIC values were ≥4 mg l⁻¹ for fluconazole, ≥1 mg l⁻¹ for itraconazole and ≥4 mg l⁻¹ for voriconazole. *C. albicans* ATCC 10231 was used as the control strain, i.e. a strain of *C. albicans* susceptible to azoles.

**RNA extraction.** Azole-resistant *C. albicans* isolates in ATBF3 medium containing antifungal drugs were immediately propagated in physiological saline with constant shaking at room temperature until 1 McFarland unit was reached. The suspension (1 ml) was centrifuged at 500 g at 4 °C for 5 min, and the pellet was stored at −30 °C until use. Total RNA was extracted using the Pure Link RNA Mini kit (Invitrogen) with PureLink DNase (Invitrogen). The quality of RNA was checked using real-time PCR (Fast Start SYBR Green Master kit; Roche). The eluate was stored at −80 °C for not more than 1 week before use.

**One-step real-time reverse transcriptase PCR (rRT-PCR) for mRNA overexpression analysis.** Primers for ERG11, CDR1, CDR2, MDR1 and the housekeeping gene (18S rRNA) were used as reported previously (Chen et al., 2010). One-step rRT-PCR was performed using 25 µl of the following reaction mixture: 1 × reaction mix, 0.6 µM each primer, 2 µl Superscript III Platinum SYBR Green One-Step qRT-PCR (Invitrogen) and 12.5 µl RNA. The reaction was performed under the following conditions (IQ5; Bio-Rad): 50 °C for 5 min; 95 °C for 10 min; 32 cycles of 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 1 min. When the amplification was complete, 8 µl amplification product was separated using 0.8 % agarose gel electrophoresis with the Fermentas 100 bp molecular mass standard. The expression levels of ERG11, CDR1, CDR2 and MDR1 were calculated by the 2⁻ΔΔCt method, where Ct is the mean of the number of threshold cycles from two independent experiments. The housekeeping (18S rRNA) gene was used to normalize the fold change in gene expression.

**Amplification of the full-length ERG11 gene.** The full-length ERG11 gene was amplified by using specific primers designed by Primer Designer Version 2, Scientific & Educational Software.

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence (5ʿ→3ʿ)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-F</td>
<td>ATGGCTATTTGTTGAAACTG</td>
<td>PCR</td>
</tr>
<tr>
<td>CA-R</td>
<td>TTAAACCATACAGTCTCTTCCT</td>
<td>PCR</td>
</tr>
<tr>
<td>CA8F</td>
<td>TTTTGGAACCTGCTTTGAT</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA398F</td>
<td>ATTTGCAATTCGAGATTTA</td>
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</tr>
<tr>
<td>CA803F</td>
<td>GTGATATTGATCCAAATCG</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA1210F</td>
<td>TATTTTTGATGTTCTTCCAG</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA501R</td>
<td>CAAAAATTTCTTCCTTACCTT</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA897R</td>
<td>ATTAGCAATTTCCTTGATCACG</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA1310R</td>
<td>ATAGAATTAGCTTTTGCAG</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>CA1579R</td>
<td>TACAAGTTCTTTTTTCC</td>
<td>DNA sequencing</td>
</tr>
</tbody>
</table>

*CA, Candida albicans; F, forward; R, reverse. Positions of the primers CA-F and CA-R were nt 1 and 1587. The position of the DNA primer for sequencing is indicated in the primer name.*

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DNA sequencing and overlapping DNA sequence editing. The purified DNA was subjected to DNA sequencing reactions by using eight overlapping primers (Table 1). Sequencing reactions were performed with BigDye Terminator Cycle Sequencing using 8 μl DNA template (10–40 ng) and 1 μl primers (10 μM). PCR was performed on 20 μl mixture for 25 cycles of 96 °C for 10 s, 53 °C for 5 s, 60 °C for 4 min and 4 °C for 10 min. After the reaction, the PCR product was purified with ethanol, EDTA and sodium acetate. The DNA pellets were dissolved in Hi-Di formamide for DNA sequencing. The DNA sequences were derived using an automated sequencer (3130/Genetic Analyzers; Applied Biosystems). Overlapping DNA sequence editing was performed by using the GeneJET PCR Purification kit (Genecraft).

RESULTS

Identification and susceptibility testing

The most common OPC aetiology found in HIV-infected patients in the Cipto Mangunkusumo Hospital, Jakarta, Indonesia, was C. albicans (85.2 %), whereas 5.5 % were Candida non-albicans, including C. dubliensis, C. guillermondii, C. famata, C. tropicalis and C. parapsilosis. Of 92 C. albicans isolates, 17 (18.5 %) were resistant to azole antifungals and 12 (13.0 %) were resistant to fluconazole, as seen in Table 2. Of the 17 C. albicans isolates resistant to azoles, three isolates (17.6 %) were single-resistant to fluconazole, two isolates (11.8 %) were single-resistant to itraconazole and two isolates (11.8 %) were single-resistant to voriconazole. C. albicans isolates that were multi-azole-resistant (fluconazole, itraconazole and/or voriconazole) constituted between 5.9 and 29.4 % of all isolates.

The MIC values used in this study were in accordance with CLSI methodology (CLSI, 2008), because this study was started on May 2009. All isolates had MICs exceeding the upper limit value, except for isolate number 36 (fluconazole MIC about 64 μg ml⁻¹) (Table 2). Other isolates had high MICs for fluconazole (>128 μg ml⁻¹), itraconazole (>4 μg ml⁻¹) and voriconazole (>8 μg ml⁻¹).

Overexpression of the CDR1, CDR2, MDR1 and ERG11 genes

Primer validity for CDR1, CDR2, MDR1, ERG11 and the housekeeping gene 18S rRNA were evaluated by the melting curves and confirmed by electrophoresis on 0.8 % agarose gels. A specific DNA band was detected for all gene amplifications (data not shown). Mean transcript levels of ERG11, CDR1, CDR2 and MDR1 were recorded relative to the susceptible control strain C. albicans ATCC 10231 (Fig. 1). Compared with control strain C. albicans ATCC 10231, the overexpression of ERG11, CDR1, CDR2 and MDR1 in 17 azole-resistant isolates was 1.7–133.4-, 1.03–2.8-, 1.4–121.1-, 1.02–50.9-fold, respectively. Five isolates showed no overexpression of CDR1 and MDR1. Overexpression of the CDR2 gene was observed in all C. albicans isolates resistant to single fluconazole and also in those resistant to multi-azoles (fluconazole and/or itraconazole, voriconazole). The highest overexpression of ERG11 individually was found in isolates that were resistant to single fluconazole (Table 3).

Molecular characteristics of lanosterol 14α-demethylase (CYP51) encoded by the ERG11 gene

The amino acid sequences of the CYP51 proteins of the 17 C. albicans isolates that were resistant to fluconazole were compared with those of C. albicans ATCC 10231, which is sensitive to fluconazole. Overall, six amino acid changes (D116E, D153E, I261V, E266D, V437I, V488I; Table 4) were detected; isolates 62, 82 and 88 showed no amino acid changes. Of these amino acid changes, five had been reported previously: D116E (Perea et al., 2001; Marichal et al., 1999; White et al., 2002; Xu et al., 2008; Chau et al., 2004), D153E (Marichal et al., 1999), E266D (Marichal et al., 1999; White et al., 2002; Chau et al., 2004; Goldman et al., 2004), V437I (Perea et al., 2001; White et al., 2002; Goldman et al., 2004) and V488I (Xu et al., 2008; Chau et al., 2004; Goldman et al., 2004). Amino acid substitution of CYP51 at E266D was most frequently found in multiresistant C. albicans isolates (fluconazole, itraconazole and voriconazole), while an amino acid
substitution V437I was frequently found in C. albicans resistant to single fluconazole, as seen in Fig. 2. To the best of our knowledge, one amino acid change (I261V) detected in isolate 73 has not been reported previously and was a novel substitution found in this study. Isolate 73 with I261V was resistant to fluconazole and voriconazole. Three other isolates (isolates 36, 45 and 96) that had the same resistance pattern as isolate 73 showed no I261V substitution. The role of the I261V substitution in fluconazole and voriconazole resistance in isolate 73 was, therefore, questionable. The issue should be addressed in future studies.

Combinations of resistance mechanisms, including overexpression of the CDR1, CDR2, MDR1 and ERG11 genes and an amino acid change in the CYP51 protein inazole-resistant C. albicans, were also analysed in this study (Table 3). A V437I substitution in the CYP51 protein and overexpression of ERG11 and CDR2 was a predominant combination factor conferring fluconazole resistance to C. albicans. An E266D substitution in the CYP51 protein and overexpression of CDR2 was another predominant combination factor, conferring multi-azole resistance to C. albicans (fluconazole, itraconazole and/or voriconazole). The highest overexpression of ERG11 was found in the resistant isolates without amino acid substitutions in CYP51: more than 100-fold greater than in the susceptible control strain C. albicans ATCC 10231 (Table 3). The resistant isolates with amino acid substitutions in CYP51 in at least in two positions showed overexpression in the CDR2 gene that was more than twofold greater than in the susceptible control strain C. albicans ATCC 10231.

**DISCUSSION**

The major cause of OPC in HIV-infected patients in the Cipto Mangunkusumo Hospital, Jakarta, Indonesia, was C. albicans (85.2 %), while Candida non-albicans,

![Fig. 1. Mean relative overexpression of CDR1, CDR2, MDR1 and ERG11 genes in C. albicans resistant to azoles. F, Fluconazole; I, itraconazole; V, voriconazole; F/I/V, multi-azole resistance (fluconazole, itraconazole, voriconazole).](image)

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Azole resistance pattern</th>
<th>Amino acid substitution</th>
<th>Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDRI</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>Azole-sensitive</td>
<td>D D I E V V</td>
<td>1.00</td>
</tr>
<tr>
<td>74</td>
<td>Res: Flu</td>
<td>D I</td>
<td>1.03</td>
</tr>
<tr>
<td>82</td>
<td>Res: Flu</td>
<td>I</td>
<td>2.95</td>
</tr>
<tr>
<td>102</td>
<td>Res: Flu</td>
<td>I</td>
<td>0.88</td>
</tr>
<tr>
<td>59</td>
<td>Res: Itra</td>
<td>E D</td>
<td>21.68</td>
</tr>
<tr>
<td>62</td>
<td>Res: Itra</td>
<td>E D</td>
<td>14.35</td>
</tr>
<tr>
<td>41</td>
<td>Res: Vori</td>
<td>E D I</td>
<td>2.93</td>
</tr>
<tr>
<td>88</td>
<td>Res: Vori</td>
<td>E D</td>
<td>1.66</td>
</tr>
<tr>
<td>36</td>
<td>Res: Flu, Itra</td>
<td>D I</td>
<td>3.35</td>
</tr>
<tr>
<td>45</td>
<td>Res: Flu, Itra</td>
<td>E D</td>
<td>4.34</td>
</tr>
<tr>
<td>96</td>
<td>Res: Flu, Itra</td>
<td>D I</td>
<td>0.59</td>
</tr>
<tr>
<td>73</td>
<td>Res: Flu, Vori</td>
<td>E V D I</td>
<td>32.75</td>
</tr>
<tr>
<td>86</td>
<td>Res: Itra, Vori</td>
<td>D I</td>
<td>14.50</td>
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<tr>
<td>33</td>
<td>Res: Flu, Itra, Vori</td>
<td>E D</td>
<td>8.68</td>
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<td>54</td>
<td>Res: Flu, Itra, Vori</td>
<td>D I</td>
<td>32.63</td>
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<tr>
<td>91</td>
<td>Res: Flu, Itra, Vori</td>
<td>I</td>
<td>1.61</td>
</tr>
<tr>
<td>92</td>
<td>Res: Flu, Itra, Vori</td>
<td>D I</td>
<td>19.27</td>
</tr>
<tr>
<td>94</td>
<td>Res: Flu, Itra, Vori</td>
<td>E I</td>
<td>3.85</td>
</tr>
</tbody>
</table>

*Res,Resistance; Flu, fluconazole; Itra, itraconazole; Vori, voriconazole; D, aspartic acid; E, glutamic acid; I, isoleucine; V, valine.
including \textit{C. dubliniensis}, \textit{C. guillermondii}, \textit{C. famata}, \textit{C. tropicalis} and \textit{C. parapsilosis}, was responsible for 5.5\% of infections. In this study, \textit{C. glabrata} was not found, although it was considered to be the second most prevalent yeast species after \textit{C. albicans}. This result indicates that \textit{C. glabrata} was not frequently the cause of OPC in HIV patients in Jakarta, Indonesia. \textit{C. albicans} is an opportunistic yeast that has been increasingly implicated in OPC in HIV-infected patients in Indonesia, as reported by Winge-ter et al. (2007) and Bandar et al. (2006) (93 and 75\% of patients, respectively).

Both single- and multidrug resistance were found in this study. The most common \textit{C. albicans} resistance to a single drug was toward fluconazole (17.6\%), which is recommended as a standard treatment for OPC in HIV-infected patients. The majority of the fluconazole-resistant isolates also showed decreased levels of susceptibility to the other azole compounds tested, itraconazole and voriconazole, suggesting that they are multiresistant isolates. This can be explained by the similarity in azole compounds; these are substrates for multidrug efflux transporters that respond to \textit{ERG11} mutations (Andes et al., 2006; Song et al., 2004). Multiple resistance to azole antifungals was mainly associated with specific point mutations in the \textit{ERG11} gene (Marichal et al., 1999; Wang et al., 2009; Goldman et al., 2004), and was also associated with overexpression of the \textit{ERG11} gene and efflux pumps encoded by the \textit{CDR1}, \textit{CDR2} and \textit{MDR1} genes (Hoot et al., 2008; Oliver et al., 2007).

Overexpression of \textit{ERG11}, \textit{CDR1}, \textit{CDR2} and \textit{MDR1} was evaluated using the $2^{-\Delta\Delta C_t}$ method (Chen et al., 2010), where $C_t$ is the mean threshold cycle number from two independent experiments. Data are presented as the fold change in gene expression normalized to the 18S rRNA

\begin{table}
\centering
\begin{tabular}{|l|l|c|c|c|c|c|}
\hline
Isolate & Azole resistance pattern & MIC (mg l$^{-1}$) & Amino acid positions in CYP51 \\
\hline
ATCC & Azole-sensitive & F <1, I <0.125, V <0.06 & D & D & I & E & V & V \\
74 & Res: Flu & >128 & D(1) & I(1) \\
82 & Res: Flu & >128 & D(1) & I(1) \\
102 & Res: Flu & >128 & I(1) \\
59 & Res: Itra & >4 & E(1) & D(1) \\
62 & Res: Itra & >4 & E(1) & I(1) \\
41 & Res: Vori & >8 & E(1) & D(1) & I(1) \\
88 & Res: Vori & >8 & I(1) \\
36 & Res: Flu, Itra & 64, >4 & D(2) & I(1) \\
45 & Res: Flu, Itra & >128, >4 & E(2) & D(1) \\
96 & Res: Flu, Itra & >128, >4 & E(1) & D(1) & I(1) \\
73 & Res: Flu, Vor & >128, >8 & E(1) & V(1) & D(1) & I(1) \\
86 & Res: Itra, Vori & >4, >8 & D(2) & I(1) \\
33 & Res: Flu, Itra, Vori & >128, >4, >8 & E(1) & D(1) \\
54 & Res: Flu, Itra, Vori & >128, >4, >8 & E(1) & D(1) & I(1) \\
91 & Res: Flu, Itra, Vori & >128, >4, >8 & E(1) & D(1) & I(1) \\
92 & Res: Flu, Itra, Vori & >128, >4, >8 & D(1) & I(1) \\
94 & Res: Flu, Itra, Vori & >128, >4, >8 & E(1) & I(1) \\
\hline
\end{tabular}
\caption{Amino acid changes in the lanosterol 14\alpha-demethylase (CYP51) protein in azole-resistant \textit{C. albicans} isolates compared with \textit{C. albicans} ATCC 10231}
\end{table}
gene as a control (Chen et al., 2010; Dheda et al., 2004). Overexpression of the ERG11, CDR1, CDR2 and MDR1 genes was found to be 1.7–133.4, 1.03–2.8, 1.4–121.1 and 1.02–50.9-fold, respectively, compared with C. albicans ATCC 10231, which is sensitive to fluconazole. There were five isolates that showed no overexpression of the CDR1 and MDR1 genes, but amino acid substitutions were also found in these isolates.

The highest overexpression of CDR2 and ERG11 was found in C. albicans resistant to fluconazole isolated from HIV-infected patients in Jakarta, Indonesia. The highest overexpression of ERG11 was found in C. albicans resistant to single fluconazole, whereas the highest overexpression of CDR2 was found in C. albicans multiresistant to fluconazole, itraconazole and/or voriconazole. This result was slightly different from that of Chen et al. (2010), who reported that the efflux genes, CDR1 and CDR2, are likely to play a more important role in fluconazole resistance.

PCR amplification and sequence analysis of the ERG11 genes showed six different amino acid changes (D116E, D153E, I261V, E266D, V437I and V488I). Amino acid changes occurred commonly in one allele (heterozygous); however, five isolates showed amino acid changes in both alleles (homozygous) – one isolate with D116E, three isolates with E266D and one isolate with V437I. Previous studies reported that ERG11 gene mutations that cause homozygous amino acid changes will increase the potential for resistance (Sanglard & Odds, 2002). However, in this study, these types of allele mutations did not influence the level of resistance.

In this study, mutations were found to occur in 82% of all resistant isolates tested, which is higher than the level of 65% reported by Perea et al. (2001). Of the six amino acid substitutions, four hotspots of ERG11 gene mutation were found: D116E, E266D, V437I and V488I. These hotspots are located in the three hotspot regions that were reported by Marichal et al. (1999), i.e. aa 105–165, 266–287 and 405–488. Mutations in the ERG11 gene resulting in the amino acid substitution E266D were found in isolates with multiresistance to fluconazole, itraconazole and voriconazole, while the amino acid substitution V437I was most frequently found in C. albicans resistant to single fluconazole. Based on previous studies in other countries, five substitutions (D116E, D153E, E266D, V437I and V488I) are not directly related to resistance to fluconazole. In this study, only D116E amino acid substitution was found in some sensitive isolates (Supplementary Fig. S1, available in the online Supplementary Material). This shows that genetic polymorphisms of CYP51 are highly permissive to structural changes (Lee et al., 2004; Martinez et al., 2002).

A novel amino acid substitution (I261V) was found in C. albicans resistant to fluconazole and voriconazole. The amino acid substitution I261V, resulting from the ERG11 gene mutation identified in this study, is probably associated with fluconazole resistance, since the substitution from the large isoleucine to the smaller valine probably hinders the entry of fluconazole.

It appeared that mutations in the ERG11 gene, combined with overexpression of ERG11, CDR1, CDR2 and MDR1, played an important role in the molecular mechanism of C. albicans resistance to single fluconazole or multiresistance to fluconazole, itraconazole and voriconazole. The combination of amino acid substitutions due to mutations in the ERG11 gene and overexpression of CDR2 and ERG11 may occur together in C. albicans isolates resistant to fluconazole. The highest overexpression in the ERG11 gene was found in isolates that had no amino acid substitutions. The highest overexpression was observed in the CDR2 gene, in which amino acid substitutions were associated with C. albicans multiresistance to fluconazole, itraconazole and/or voriconazole.

In summary, a combination of amino acid substitutions due to mutations in the ERG11 gene with overexpression of CDR2 and ERG11 may occur together in C. albicans isolates resistant to single fluconazole and multiresistant to fluconazole, itraconazole and/or voriconazole. The highest overexpression of ERG11 was found in isolates that had no amino acid substitutions. In isolates in which amino acid substitutions were found, the highest overexpression was observed in the CDR2 gene. To the best of our knowledge, studies that combine expression levels of drug-resistance-related genes and mutations in the ERG11 gene in individual C. albicans strains have not been reported previously. Thus, this study might provide novel and useful information for the therapeutic treatment of candidiasis patients. Moreover, a novel amino acid substitution (I261V) was found in this study; further testing is required to determine whether or not it is associated with fluconazole resistance.

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