Genetic and phenotypic characterization of *Candida albicans* strains isolated from infectious disease patients in Shanghai

Lvyin Hu, Xin Du, Tianming Li, Yan Song, Shubei Zai, Xiangnan Hu, Xiaonan Zhang and Min Li

1Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, 12 Central Urumqi Road, Shanghai, PR China
2Department of Clinical Laboratory, Shanghai Public Health Clinical Center, Fudan University, No. 2901 Cao Lang Rd, Shanghai, PR China
3Department of Laboratory Medicine, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, PR China
4Research Unit, Shanghai Public Health Clinical Center, Fudan University, No. 2901 Cao Lang Rd, Shanghai, PR China

*Candida albicans*, as an opportunistic pathogen, can cause superficial and life-threatening candidiasis in immunocompromised individuals. The formation of surface-associated biofilms and the appearance of drug resistance pose a significant challenge for clinical intervention. In this study, a total of 104 hospital-acquired *C. albicans* clinical isolates were collected from sterile sites and mucosal lesions of 92 infectious disease patients in the Shanghai Public Health Clinical Center and analysed. The resistance rates to fluconazole, itraconazole and voriconazole were 12.5%, 15.4% and 11.5% respectively. Multilocus sequence typing (MLST) analysis identified 63 diploid sequence types (DSTs) with a decentralized phylogeny, of which 37 DSTs (58.7%) had not been reported in the online MLST database. Loss of heterozygosity was observed in *ACC1* and *ADP1* sequences obtained from six sequential isolates from a patient receiving antifungal treatment, which exemplified the effect of microevolution on *C. albicans* genetic alterations. Biofilm formation capability, an important virulence trait of *C. albicans*, was variable among strains isolated from different anatomical sites (*P* = 0.0302) and affected by genotypes (*P* = 0.0185). The mRNA levels of the azole antifungal target *ERG11* gene and efflux pump genes (*CDR1*, *CDR2* and *MDR1*) were detected in 9–18.1% of azole-resistant and susceptible-dose dependent (S-DD) isolates. Twelve mutations encoding distinct amino acid substitutions in *ERG11* were found in azole-resistant and S-DD isolates. Among them, A114S, Y132H and Y257H substitution in the *ERG11* gene may be primarily related to azole resistance. Taken together, we observed a high level of diversity within *C. albicans* isolates. Multiple inter-related underlying mechanisms, including genetic and environmental factors, may account for high surface adhesion or azole resistance in clinical *C. albicans* infections.

### Abbreviations

- AAT1, aspartate aminotransferase
- ACC1, Acetyl-coenzyme A carboxylase
- ACT1, actin
- ADP1, ATP-dependent permease
- ALS, hypha-specific surface protein
- BSI, nosocomial bloodstream infection
- CDR1, CDR2, ATP-dependent efflux pumps
- CLSI, Clinical and Laboratory Standard Institute
- DST, diploid sequence type
- ERG11, cytochrome P450 14α-demethylase
- HWP1, hypha-specific cell wall protein
- MDR1, major facilitator pump
- MLST, multilocus sequence typing
- MPI, mannose phosphate isomerase
- SAP, secreted aspartyl protease
- S-DD, susceptible-dose dependent
- SYA1, alanyl-RNA synthetase
- VPS13, vacuolar protein sorting protein
- XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-2H-tetrazolium-5-carboxanilide
- YPD, yeast extract, peptone, dextrose medium
- ZWF1b, glucose-6-phosphate dehydrogenase

The GenBank accession numbers for the *ERG11* sequences are KM052652, KM052653, KM052654 and KM052655.

The GenBank/EMBL/DDBJ accession numbers for the *ERG11* sequences are KM052652, KM052653, KM052654 and KM052655.

Two supplementary tables are available with the online Supplementary Material.
INTRODUCTION

*Candida albicans* is a common opportunistic pathogen in immunocompromised hosts, causing infections that range from superficial mucosal lesions to invasive, life-threatening diseases (Guo et al., 2013; Johnson & Cobb, 2010; Kim & Sudbery, 2011). Advancements in medical practice such as invasive procedures and widespread use of broad-spectrum antibiotics have paralleled the increase of *Candida* infections. A survey indicated that *Candida* species were the fourth leading cause of nosocomial bloodstream infection (BSI) in the United States, of which *C. albicans* accounts for 54% (Wisplinghoff et al., 2004).

*C. albicans* colonizes and forms biofilms on medical implants, causing device-associated disease (d’Enfert, 2006). These biofilms consist of matrix-enclosed micro-colonies of yeast, hyphae and pseudohyphae that are arranged in a complex structure. Cells within a biofilm structure display a reduced susceptibility to commonly used antifungals, such as fluconazole and amphotericin B, and host immune defences (Mathé & Van Dijk, 2013; Seneviratne et al., 2008). Several genes are reported to orchestrate the formation of biofilms, including ALS3 and HPW1 (Nailis et al., 2010).

The azole resistance of *C. albicans* had been increasingly reported over the past 30 years. For example, the fluconazole resistance rate, reported to be 3% before 1998 in the USA (Kontoyiannis & Lewis, 2002), recently rose to 5.6% (Fothergill et al., 2014). In China between 2009 and 2011, approximately 10% of *C. albicans* isolated from patients in intensive care units showed resistance to fluconazole (Guo et al., 2013). The cytochrome P450 lanosterol 14 alpha-demethylase (Erg11p) encoded by the *ERG11* gene serves as a key enzyme in the ergosterol biosynthesis pathway of *C. albicans*, and is the primary target for azole antifungals (Perea, 2000). Mutations of the *ERG11* gene and high transcriptional levels of *CDR1*, *CDR2*, and *MDR1* have been attributed to the emergence of resistance (Kontoyiannis & Lewis, 2002; Sanglard et al., 1995; Sanglard & Odds, 2002; Xiang et al., 2013). Indeed, White et al. (1998), in a comprehensive review, described multiple mechanisms leading to azole resistance. For instance, increased levels of *MDR1*, *CDR* and *ERG11* mRNA, loss of allelic variation and point mutation in the *ERG11* gene occurred at distinct stages of fluconazole therapy in an AIDS patient co-infected with *C. albicans* (White, 1997a,b; White et al., 1998). In this study, we sought to further clarify factors leading toazole resistance of *C. albicans* using clinical isolates of our patients.

First, we used the multilocus sequence typing (MLST) method to characterize the phylogenetic relationships among clinical isolates of *C. albicans*. MLST is a high-resolution method based on nucleotide polymorphisms in seven housekeeping genes (Bougnoux et al., 2003; Odds et al., 2006). The capability of biofilm formation was measured by 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. In addition, we sequenced the *ERG11* gene from azole-resistant, susceptible-dose dependent (S-DD) and sensitive isolates to identify mutated loci that were associated with resistance. Furthermore, mRNA expression of the *ALS3*, *HPW1*, *CDR1*, *CDR2*, *MDR1*, and *ERG11* genes was measured by quantitative real-time RT-PCR, and their roles in biofilm formation or azole resistance were investigated.

METHODS

Ethics statement. This study was approved by the ethics review board of the Shanghai Public Health Clinical Center on 10 May 2012. (no. 201205010). All patients from whom specimens were collected provided written informed consent.

*C. albicans* isolates and drug susceptibility. A total of 104 *C. albicans* isolates were collected from sterile sites and mucosal lesions of infectious disease patients from August 2006 to December 2011 in the Shanghai Public Health Clinical Center, Shanghai, China. These infectious disease patients were diagnosed with viral hepatitis (53.3%), tuberculosis (22.8%) or AIDS (15.2%). The clinical samples were obtained from which *C. albicans* isolates were obtained included blood (five isolates), ascitic fluid (nine isolates), bronchoalveolar lavage (six isolates), catheters (three isolates), abscesses (two isolates), pleural effusions (three isolates), stomach tissue (two isolates), urine (44 isolates), vaginal secretions (13 isolates) and mucosal lesions (17 isolates). The identification of all isolates was done with ATB ID32C Fungus test strips (bioMérieux). MICs of 5-fluorocytosine, amphotericin B, fluconazole, itraconazole and voriconazole were measured by ATB Fungus 3 test strips (bioMérieux) according to the Clinical and Laboratory Standard Institute (CLSI) standards (CLSI, 2002). The categorization of azole susceptibility was based on the interpretive breakpoints employed by Sanguinetti et al. (2005).

MLST analysis. After mechanical disruption of yeast cell walls, DNA was extracted with phenol/chloroform and precipitated with ethanol. MLST analysis and PCR amplification of the seven housekeeping genes *AAT1a*, *ACCI*, *ADP1*, *MPIb*, *SYA1*, *VP513* and *ZWF1b* were performed using the primer sequences and conditions described by Bougnoux et al. (2003) and Odds et al. (2007). After column purification, the PCR products were sequenced (Invitrogen) using forward and reverse primers. Sequence analysis of both strands was performed by Vector NTI 8 software (InforMax). The number of alleles and diploid sequence types (DSTs) was determined using the well-characterized online MLST database (http://c albicans.mlst.net/). Clades were defined and numbered as described previously (Odds et al., 2007). Isolates that did not cluster with a known clade, and clusters containing fewer than 10 isolates, were labelled as singletons. Phylogenetic studies of the 104 strains together with 1485 DSTs retrieved from the MLST database from well-defined clades were analysed by the unweighted-pair group method using average linkages (UPGMA) in MEGA6 software with 1000 bootstraps determined by p-distance. Clonal clusters of isolates that differed in sequence at only one of the seven loci were determined by eBURSTv3 (http://c albicans.mlst.net/eburst/). According to a method by Shin et al. (2011) defining the p-distance between 0.04 and 0.035 that should separate clades, the p-distance threshold of 0.038 was used to define clades.

Semi quantitative biofilm assay. *C. albicans* biofilms were formed on polystyrene, flat-bottom, 96-well microtitre plates (Corning) containing 1 × 10⁶ cells in 100 μl of RPMI 1640 per well. After incubation for 48 h at 37°C, the cells were washed three times with sterile PBS. Residual PBS was removed by blotting with paper towels and 100 μl XTT was added to each well (Pierce et al., 2009; Ramage et al., 2001). The plates were incubated in the dark at 37°C for an
additional 2 h, 80 μl of XTT solution was then transferred to a second microtitre plate and the XTT reduction was measured at 490 nm in a Bio-Tek ELX800 microtitre plate reader. Each sample was processed four times, and high biofilm formers were defined as isolates that exhibited XTT activity greater than the value of the arithmetic mean ± s.d. while low biofilm formers were defined as isolates that exhibited XTT activity below the arithmetic mean.

**Quantitative RT-PCR.** *C. albicans* isolates were grown to the exponential growth phase in yeast extract, peptone, dextrose medium (YPD), and their total RNA was extracted using the Qiagen Rneasy Mini kit (Qiagen) according to the manufacturer’s instructions. The total RNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo) and reverse transcribed using the Qiagen QuantiTect Reverse Transcription kit (Qiagen). The quantitative PCRs of *ERG11, CDR1, CDR2, MDR1, ALS3, HWP1*, and *ACT1* were performed in duplicate using the SYBR Premix Ex-PCR kit (Takara) with an ABI 7500 Real-Time PCR System (Applied Biosystems). To control for the duplicate using the SYBR Premix Ex-PCR kit (Qiagen). The quantitative PCRs of *Thermo* and reverse transcribed using the Qiagen QuantiTect Mini kit (Qiagen) according to the manufacturer’s instructions.

**RESULTS**

*C. albicans* isolates and their antimicrobial susceptibility

One hundred and four clinical isolates of *C. albicans* were isolated from 92 patients during the 6-year study period. Among them, 10 patients had been previously exposed to azoles. Among all isolates, three were 5-fluorocytosine-resistant (2.88 %, MIC \(>32 \mu g ml^{-1}\)), 13 were fluconazole-resistant (12.5 %, MIC \(>64 \mu g ml^{-1}\)), 16 were itraconazole-resistant (15.4 %, MIC \(\geq 1 \mu g ml^{-1}\)), and 12 were voriconazole-resistant (11.5 %, MIC \(\geq 4 \mu g ml^{-1}\); Table S1), no isolates were resistant to amphotericin B. Sixteen isolates were azole-resistant, six isolates were S-DD (fluconazole MIC=16–32 μg ml⁻¹), itraconazole MIC=0.25–0.5 μg ml⁻¹, voriconazole MIC=2 μg ml⁻¹), and 11 isolates (10.6 %) were pan-azole-resistant.

**Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG11a-F</td>
<td>ATGGCTATTGGTAAAACGTG</td>
</tr>
<tr>
<td>ERG11a-R</td>
<td>CATTAACTGGAAATTTGGAC</td>
</tr>
<tr>
<td>ERG11b-F</td>
<td>TTAATCTCCAGTTTTCCG</td>
</tr>
<tr>
<td>ERG11b-R</td>
<td>CCACTTTTTTTCTTAAACAA</td>
</tr>
<tr>
<td>ERG11c-F</td>
<td>GGTGAAAAACCTCATTTACA</td>
</tr>
<tr>
<td>ERG11c-R</td>
<td>CCAATATGTTTCTGTGTTTCAGT</td>
</tr>
<tr>
<td>ACT-F</td>
<td>GCCACGCTTTAGTTGATTGTGAGGA</td>
</tr>
<tr>
<td>ACT-R</td>
<td>CGCTATTTTCCTTTTCTAATCCTCAGT</td>
</tr>
<tr>
<td>CDR1-F</td>
<td>AAAGATGAc CTCGTCAGCAGGTTT</td>
</tr>
<tr>
<td>CDR1-R</td>
<td>CCAATTTCCAATTTGGAAGGT</td>
</tr>
<tr>
<td>CDR2-F</td>
<td>TGTGTTGACCTATTCTACATTTCTTGG</td>
</tr>
<tr>
<td>CDR2-R</td>
<td>AAGAGATTGCAATTTTGCCCATTA</td>
</tr>
<tr>
<td>MDR1-F</td>
<td>TGTTTTTACGAAAGGGCTTGTTG</td>
</tr>
<tr>
<td>MDR1-R</td>
<td>CATAATCCTACAATTTGGAAC</td>
</tr>
<tr>
<td>ERG11-F</td>
<td>TTACCTCATTAGTTGAAGCTATGAT</td>
</tr>
<tr>
<td>ERG11-R</td>
<td>CAGGTTCCTCCATTCTGAGTTAAATTTTCTTC</td>
</tr>
<tr>
<td>ALS3-F</td>
<td>CCGTTGTTCTCACTGAATCTTTAGT</td>
</tr>
<tr>
<td>ALS3-R</td>
<td>AGCAAGAAGTTGCAAGTTAATACATCT</td>
</tr>
<tr>
<td>HWP1-F</td>
<td>CAACTTATTTGCAATCAGTTATACATG</td>
</tr>
<tr>
<td>HWP1-R</td>
<td>GCTTCCTCTGTTACCTTTGA</td>
</tr>
</tbody>
</table>

These three overlapping fragments covered the entire *ERG11* ORF extending from 1 bp to 1569 bp. The PCR program was as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; followed by an extension step at 72 °C for 7 min. The PCR products were fully sequenced using forward and reverse primers and assembled. The heterozygosity of sequencing nucleotides was analysed by Vector NTI suite 8.0, and sequences were assembled using SeqMan II (DNASTAR) and aligned with *ERG11* sequence from whole genome sequence of *C. albicans* SC5314 (GenBank, NW_139483).

**MLST genotyping**

Sixty-three DSTs were found among 104 clinical *C. albicans* isolates. eBURST analysis of the MLST data assigned the 104 isolates to 14 clonal clusters of DSTs differing from each other by a single genotype and to 25 singleton DSTs. The common clone types found in our study included DST443 (76.9 %), DST365 (67.3 %), DST1867 (67.3 %) and DST79 (577 %), in which DSTs 79 and 1867 belonged to the same clade 1. Sixty-one isolates (58.6 %) belonged to 26 previously described DSTs, whereas 43 (40.4 %) belonged to 37 novel DSTs. The Single Locus Sequence Query from the *C. albicans* database identified new alleles including one *ACC1*, one *ADP1*, two *PMIB*, one *SY1*, one *VPS13* and two *ZWFIb* alleles in these isolates. These novel sequence types and DSTs have been deposited in the MLST database. In order to assign the Chinese isolates to the existing clades, we performed an analysis of these isolates together with 1485 DSTs retrieved from the MLST database from well-defined clades (61.5 % of all DSTs currently available in the MLST database) (Fig. 1) (Odds et al., 2007; Shin et al., 2011). Of 104 Chinese isolates, 98 (94.2 %) clustered within 14 of the 18 known clades (including...
clades 1, 3, 4, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17 and 18), while six (5.8%) of the isolates did not cluster within these clades. Clade 1 contained the greatest proportion of isolates (26.9%), followed by clade 17 (13.5%), clade 8 (12.5%) and clade 12 (8.6%). Of the 43 novel DSTs isolates, 37 (86.0%) clustered within 11 clades (1, 3, 6, 7, 8, 9, 11, 12, 14, 17, 18), in which clade 1 held 11 (25.6%), and six (9.5%) isolates defined as singletons. Interestingly, of seven patients who had two isolates in different anatomical sites, three patients (43%) presented two DSTs (SH19/19-1 and SH20/19-2; SH31/31-1 and SH32/31-2; SH128/128-1 and SH131/128-2). Furthermore, loss of heterozygosity was clearly observed in the ACC1 (nt 211, 317) and ADP1 (nt 40, 46, 109, 125, 166, 205, 255, 232) genes in six strains of C. albicans sequentially isolated from the urine of one patient on weeks 0, 9, 18, 26, 31 and 47. This patient was diagnosed with tuberculous meningitis combined with recurrent urinary C. albicans infection during 11 months of intermittent antifungal (fluconazole and/or 5-fluorocytosine) treatment (Tables 2 and S1).

Table 2. Mutations in the MLST analysis alleles of six isolates from one patient

<table>
<thead>
<tr>
<th>Isolate</th>
<th>ACC1</th>
<th>ADP1</th>
<th>MPIb</th>
<th>SYA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH71/71-1</td>
<td>T</td>
<td>M</td>
<td>C</td>
<td>Y</td>
</tr>
<tr>
<td>SH77/71-2</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>R</td>
</tr>
<tr>
<td>SH78/71-3</td>
<td></td>
<td></td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>SH83/71-4</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>SH86/71-5</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>SH100/71-6</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
</tbody>
</table>

Dots indicate the same nucleotide as SH71/71-1.

**Degree of biofilm formation and related gene expression**

Biofilm formation was variable among C. albicans strains that were isolated from different anatomical sites (Fig. 2a). We observed that the biofilm formation capabilities differed in strains isolated from different anatomical sites (P = 0.0302, Kruskal–Wallis test) (Fig. 2a). Biofilm formation by blood isolates was lower than that of catheter isolates (P < 0.001, Dunn’s post hoc test) (Fig. 2a).

Genotypes also affected the biofilm formation capability of our C. albicans isolates (P = 0.0185 Kruskal–Wallis test) (Fig. 2b), where seven isolates belonging to DST365 (clade 8) exhibited higher biofilm formation capability compared with eight isolates of DST443 (clade 17) (P < 0.05, Dunn’s post hoc test). Our study found 16 high biofilm formers from a total of 104 clinical isolates. Although there were two strains showing higher expression of HWP1 and four different strains showing higher expression of ALS3, their upregulation did not directly correlate with the ability to form abundant biofilms (P > 0.05, Mann–Whitney U test) (Table S2).

**Gene expression associated with azole resistance.**

The total RNA of 15 azole-susceptible, 16 azole-resistant and 6 S-DD isolates was analysed by RT-PCR (Table 3). The MDR1 gene was overexpressed in four of the 22 azole-resistant and six S-DD isolates (18.1%); three of which were pan-drug-resistant strains and all four were itraconazole-resistant (Table 3). Of the 22 isolates, two overexpressed CDR1 (9.0%), one of which was a pan-azole-resistant strain and one was itraconazole S-DD strain. Similarly, three isolates overexpressed CDR2 (13.6%), of which, two were pan-azole-resistant strains and one was an itraconazole S-DD strain. ERG11 was overexpressed in three isolates, two of which were pan-drug-resistant strains and one was sensitive to all three azoles. Overall, no statistically significant differences were observed in any of these genes among the azole-resistant, S-DD and susceptible strains.

**ERG11 mutations in azole-resistant, S-DD and sensitive isolates**

Sanger sequencing of the ERG11 genes of 16 azole-resistant isolates (13 were resistant to fluconazole), six azole S-DD isolates and 16 azole-susceptible isolates, identified 12 amino acid substitutions (A114S, D116E, K128T, Y132H, V143I, G448R, G448E, G465S, and G465A) among which V332L had not been previously reported. Four ERG11 sequences containing novel V332L mutations (KM052652, KM052653, KM052654 and KM052655) were submitted to GenBank. In agreement with previous studies, the majority of missense mutations were detected in a total of seven strains, two of which were itraconazole resistant, four were itraconazole S-DD, one was pan-azole-resistant and none was present in any of the azole-sensitive strains. In the sequences of azole-sensitive strains, point mutations in both alleles (homozygous for the mutation) D116E, E266D, V332L and V488I (Table 3), among which V332L had not been previously reported. Four ERG11 sequences containing novel V332L mutations (KM052652, KM052653, KM052654 and KM052655) were submitted to GenBank. In agreement with previous studies, the majority of missense mutations were located in three hot spot regions: amino acids 105–165, 266–287 and 405–488 (Marichal et al., 1999). The combination of A114S and Y257H mutations in the N-terminal regions was detected in a total of seven strains, two of which were itraconazole resistant, four were itraconazole S-DD, one was pan-azole-resistant and none was present in any of the azole-sensitive strains. In the sequences of azole-sensitive strains, point mutations in both alleles (homozygous for the mutation) D116E, E266D, V332L and mutations in only one allele (heterozygous for the mutation) D116E/D, K128T/K, G448R/G, V488I/V were found.
Fig. 1. Phylogenetic analysis of *C. albicans* isolates. UPGMA dendrogram showing p-distance for 1485 *C. albicans* isolates from MLST database and 104 *C. albicans* isolates from patients in this study. The numbers and vertical bar to the right of each clade indicate the clade designation and relative size. The p-distance threshold of 0.038 was used to define clades. The symbol ○ indicates isolates with known DSTs in the MLST database; ● indicates isolates with novel DSTs in the MLST database.
In the azole-susceptible isolates, values that fall outside the 2-SD range are shown in bold italics. Substitutions in Erg11p were compared to the entire sequence of C. albicans SC5314 (GenBank, NW_139483).

**Table 3. MIC, RT-PCR and ERG11 substitution data for azole-susceptible, S-DD and resistant Candida albicans isolates**

In this retrospective study, we analysed multiple genetic and phenotypic characteristics of C. albicans clinical isolates from infectious disease patients in Shanghai Public Health Clinical Center. Phylogenetic analysis of our 104 isolates, together with 1485 known DSTs, revealed that 94.2% of them clustered within 14 of 18 known clades, (clades 1, 3, 4, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17 and 18) and 5.8% did not cluster within known clades (Fig. 1) (Gong et al., 2012; Odds et al., 2007; Shin et al., 2011). Shin et al. (2011) first reported clade 18 as a novel group predominantly consisting of Asian isolates. Indeed, Gong...
et al. (2012) found that 66.7% of the strains collected in north China were within clade 18. In this study, however, only eight isolates (7.69%) were found to reside in clade 18. The difference in geographical location may be responsible for this discrepancy. In addition, it is surprising that none of the 104 isolates characterized in our study could be assigned to clade 2, one of the major clades identified by other studies (McManus & Coleman, 2014; Odds et al., 2007). The reason for this phenomenon may relate to the highly distinct global distribution of C. albicans clades, as 70% of isolates in clade 2 came from the UK (Odds et al., 2007). Another surprising finding in this study is that the clone types of C. albicans from sterile body sites and mucosal lesions of infectious disease patients presented a decentralized trend (see Fig. 1, Table S1). This is a striking difference from a previous report that indicated C. albicans isolates associated with genital candidiasis in China were principally concentrated in clade 1 (Ge et al., 2012). It is speculated that the much more diversified nature of body sites from which C. albicans isolates obtained might account for this difference. Indeed, in European C. albicans, Odds et al. (2007) found clade 1 included a higher proportion of isolates from superficial infections than blood and commensal isolates, while clade 4 manifested the opposite relative distribution.

In this study, sequencing of seven housekeeping genes amplified from six sequential isolates of the same patient during 11 months of intermittent antifungal therapy clearly indicated loss of heterozygosity in the acetyl-coenzyme A carboxylase (ACCl) and ATP-dependent permease (ADP1) genes. Loss of heterozygosity may result from chromosome deletion or loss, recombination, and/or gene conversion events and is important to C. albicans genetic polymorphism (Odds et al., 2006). For example, Marco et al. (1999) showed that urine and stool isolates from one third of patients (33%) underwent microevolution during colonization compared to blood isolates from candidaemia patients. Another study found that catheter strains from 36% of patients showed microevolution compared with the corresponding blood isolates (Shin et al., 2004).

C. albicans cells can form biofilms, which contributes to the pathogenesis of superficial and systemic candidiasis and reduced susceptibility to antifungal drugs (d’Enfert, 2006; Mathe & Van Dijck, 2013; Seneviratne et al., 2008). We found that isolates from different anatomical sites could exhibit varied biofilm formation capability. Isolates from blood samples showed low biofilm formation whereas isolates from catheters showed high adherence. Furthermore, genetic background could also influence biofilm formation capability. The four major DST groups detected in this study exhibited varied biofilm formation capability (Fig. 2b). These results suggest that host niches and genotype are both associated with a high biofilm-forming phenotype. However, further confirmatory research is needed since a limited number of isolates were analysed in the current study.

Apart from environmental and genetic factors, the expression level of key genes associated with biofilm formation may also be responsible for surface adherence in clinical isolates. For example, Als3p and Hwp1p play important roles in the surface adherence of hyphal-form cells during the biofilm formation process (Nobile et al., 2006, 2008). Als3p mediates attachment to epithelial cells, endothelial cells, and extracellular matrix proteins, which may assist the fungus in causing infection and disease (Liu & Filler, 2011). Hwp1p, a hypha-specific surface protein, serves as a substrate for mammalian transglutaminases,
mediates tight binding to oral epithelial cells and is important in the development of mucosal candidiasis (Staab et al., 1999). It was found that 18.75% of high biofilm formers had elevated HWP1 expression and 25% had elevated ALS3 expression, whereas only one in 15 (6.7%) was found in low biofilm formers. These observations suggest an association between biofilm-forming phenotypes and expression profiles although no statistically significant difference was found. Furthermore, a previous microarray study identified other genes involved in adherence (ALS1 and ALS4) and hypha formation (HWP2) that may also contribute to a biofilm phenotype in vivo (Nett et al., 2009).

Azole resistance in C. albicans isolates is believed to result from multiple molecular mechanisms (Kontoyiannis & Lewis, 2002; Perea, 2000; Prasad et al., 1995; Sanglard et al., 1995, 1996, 1997; Sanglard & Odds, 2002). These mechanisms include overexpression of ATP-dependent efflux pumps (Sanglard et al., 1997) (CDR1, CDR2), the major facilitator pump (MDR1) and drug target enzyme (ERG11); and lower affinity of the drug to the target caused by point mutations (Maebashi et al., 2003). In our study, a significant portion of resistant isolates acquired mutations in ERG11 or exhibited significant changes in efflux pump expression levels, which is consistent with previous reports (Chau et al., 2004; Perea et al., 2001). However, in contrast to a previous report that CDR2 overexpression occurred in the majority of the azole-resistant, S-DD isolates (Chau et al., 2004), and another report in which 85% of all resistant isolates overexpressed genes encoding efflux pumps (Perea et al., 2001), only three azole-resistant isolates (13.64%) exhibited overexpression of CDR1 or CDR2 in our study; the reason for this difference remains unknown.

In addition to upregulation of relevant gene expression, mutations in key interaction sites between antifungal drugs and cytochrome P450 14a-demethylase (ERG11) was demonstrated to be of importance for azole resistance. Y132H, the most well-characterized mutation associated with azole resistance, is situated in the B-B’ helix cluster, a region believed to play a role in the entrance of the substrate into the pocket (Kelly et al., 1999; Marichal et al., 1999; Park et al., 2011). The A114S substitution is near the substrate channel and Y257H, which is located in the G helix, and may be primarily related to azole resistance (Xiang et al., 2013). In our study, the combination of A114S and Y257H mutations was found in three itraconazole-resistant isolates and four itraconazole S-DD isolates, but none in susceptible isolates. G448E and G448R had been detected in fluconazole- and itraconazole-resistant isolates (Löffler et al., 1997; White et al., 2002). In our isolates, only one azole-resistant isolate (SH149) contained a G448E mutation, and no sensitive one did. Heterozygous G448R/G mutation was found in one sensitive (SH23) and one resistant (SH22) isolate. Their contribution to fluconazole resistance is still uncertain and requires further study. Single mutations of D116E, K128T, E266D, V437I or V488I may not contribute to azole resistance because they were found in both azole-resistant and azole-susceptible strains (Marichal et al., 1999; Morio et al., 2010; Perea et al., 2001; Sanglard et al., 1998; Ying et al., 2013). The novel V332L mutation was detected in nine strains, three of which were azole-resistant (33.33%), two were S-DD (22.22%) and four were azole-susceptible (44.44%); therefore we suggest that V332L mutation probably does not contribute to azole resistance. It is worth mentioning that in four azole-resistant strains (SH37, SH59, SH76 and SH174), no upregulation of pump-related genes or ERG11 gene expression was found. In addition, their ERG11 sequences did not reveal mutations unique to resistant strains. It is possible that other genes, such as ERG5 and ERG3, contribute to azole resistance (Martel et al., 2010a, 2010b), which deserves further investigation.

In summary, our genetic and phenotypic analyses revealed the highly diversified nature of C. albicans infection in infectious disease patients. Host niches and genotype mass are associated with a high biofilm-formation phenotype. Our work indicates that azole resistance in C. albicans frequently results from multiple molecular mechanisms. The mutations in key residues (A114S, Y132H and Y257H) in the ERG11 gene may primarily account for resistance to azole drugs. The development of antifungal drugs that target these mutations may improve the overall efficacy of severe candidiasis treatments.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (grants 81171623, 81261120387, 81322025 and 81371875), Outstanding Young Talent Plan of Shanghai (XYQ2011039), and Shanghai Shuguang Talent Project (12SG03).

REFERENCES


