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An *MDR1* promoter allele with higher promoter activity is common in clinically isolated strains of *Candida albicans*

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Abstract

In the opportunistic fungal pathogen *Candida albicans*, up-regulation of *MDR1*, encoding an efflux transporter, leads to increased resistance to the antifungal drug fluconazole. Antifungal resistance has been linked to several types of genetic change in *C. albicans*, including changes in genome structure, genetic alteration of the drug target, and overexpression of transporters. High-level over-expression of *MDR1* is commonly mediated by mutation in a *trans*-acting factor, Mrr1p. This report describes a second mechanism that contributes to up-regulation of *MDR1* expression. By analyzing the sequence of the *MDR1* promoter region in fluconazole-resistant and fluconazole-susceptible strains, we identified sequence polymorphisms that defined two linkage groups, corresponding to the two alleles in the diploid genome. One of the alleles conferred higher *MDR1* expression compared with the other allele. Strains in which both alleles were of the higher activity type were common in collections of clinically isolated strains while strains carrying only the less active allele were rare. As increased expression of *MDR1* confers higher resistance to drugs, strains with the more active *MDR1* promoter allele may grow or survive longer when exposed to drugs or other selective pressures, providing greater opportunity for mutations that confer high-level drug resistance to arise. Through this mechanism, higher activity alleles of the *MDR1* promoter could promote the development of drug resistance.

Keywords

Candida albicans; Drug resistance; *MDR1*; Fluconazole; Polymorphism; Promoter

Introduction

The diploid yeast *Candida albicans* is a commensal in the human gastrointestinal tract and genitourinary tract. While the organism does not usually cause serious disease in immune-competent hosts, immunodeficient hosts are susceptible to infections ranging from superficial mucosal infections to invasive life-threatening diseases (Odds 1987; Nucci and Anaissie 2001). Fluconazole, an azole that inhibits biosynthesis of the major sterol component in the fungal membrane (Kowalsky and Dixon 1991), is commonly used for treatment of candidiasis (Pienaar et al. 2010; Chalmers and Bal 2011). However, in recent

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years, fungal resistance to fluconazole has emerged as a problem (White et al. 2002; Denning and Hope 2010). Antifungal resistance is often conferred by mutation of the gene encoding the target of fluconazole, lanosterol 14-[alpha]-demethylase, or by up-regulation of efflux transporters such as Mdr1p, Cdr1p, or Cdr2p (recently reviewed in Akins 2005; Sanglard et al. 2009; Morschhauser 2010). A number of molecular mechanisms that allow high-level expression of transporters have been described including genetic rearrangements or mutation of *trans*-acting factors (Sanglard et al. 2009; Morschhauser 2010). The studies described in this report focus on the contributions of the *MDR1* promoter region to the expression of *MDR1* in fluconazole-susceptible and fluconazole-resistant strains of *C. albicans*.

Mdr1p is a major facilitator superfamily transporter and transports drugs and other compounds across the fungal plasma membrane (Fling et al. 1991; Pinjon et al. 2005). *CDR1* and *CDR2* encode drug-transporting ABC transporters (Sanglard et al. 1997; Krishnamurthy et al. 1998) that are overexpressed in some azole-resistant isolates (Akins 2005; Sanglard et al. 2009; Morschhauser 2010). Mutations that result in overexpression of the transporters due to alteration of transcriptional regulators such as Tac1p (for *CDR*; Coste et al. 2004, 2006) or Mrr1p (for *MDR1*; Morschhauser et al. 2007; Dunkel et al. 2008; Schubert et al. 2011) promote drug resistance. Deletion of the *MDR1* gene in drug-resistant, Mdr1p over-expressing strains compromises drug resistance (Wirsching et al. 2000b).

Alterations in chromosome structure represent another important mechanism that contributes to the evolution of drug resistance. Aneuploidies such as the presence of an isochromosome are common in fluconazole-resistant strains (Selmecki et al. 2008, 2009). In a well-characterized example, an isochromosome composed of a centromere flanked by two copies of the left arm of chromosome 5 carries the gene encoding lanosterol 14-[alpha]-demethylase (*ERG11*) and *TAC1* (Selmecki et al. 2008). The presence of the isochromosome increases the copy number of these important genes.

Fluconazole-susceptible, WT *C. albicans* isolates show very low levels of *MDR1* gene expression when grown in laboratory conditions; however, expression can be increased by treatment of cells with some toxic agents (Gupta et al. 1998; Harry et al. 2005; Rognon et al. 2006). On the other hand, many *C. albicans* fluconazole-resistant isolates constitutively express *MDR1* at high levels (Franz et al. 1998; Lopez-Ribot et al. 1998; Lyons and White 2000; Wirsching et al. 2000a; Hiller et al. 2006; Riggle and Kumamoto 2006; Morschhauser et al. 2007). *MDR1* expression is controlled by a transcription factor of the zinc cluster family, termed Mrr1p (multi-drug-resistance regulator) (Morschhauser et al. 2007). Other transcription factors such as Cap1p and Mcm1p have also been implicated in the control of *MDR1* expression (Schubert et al. 2011). Cap1p and Mcm1p both bind to sites within the *MDR1* promoter (Harry et al. 2005; Riggle and Kumamoto 2006; Rognon et al. 2006).

C. albicans is a diploid organism that reproduces mainly by clonal propagation. However, it is capable of true mating and karyogamy between strains of opposite mating type (Hull et al. 2000; Magee and Magee 2000; Lockhart et al. 2003). Clinically isolated strains of *C. albicans* exhibit a significant degree of natural heterozygosity (Whelan et al. 1980; Whelan and Magee 1981). In some cases, one allele is functional, while the other carries a recessive mutation (Whelan et al. 1980; Whelan and Magee 1981; Defever et al. 1982; Whelan and Soll 1982; Gomez-Raja et al. 2008). In the *MDR1* gene, polymorphisms within the promoter region and in the open reading frame (ORF) have been found (Gupta et al. 1998; Wirsching et al. 2000a). Mutations in the ORF of the *MDR1* gene were associated with enhanced induction by benomyl, methotrexate, and several other unrelated drugs (Gupta et al. 1998). However, high-level *MDR1* gene over-expression in fluconazole-resistant strains is

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associated with *trans*-activating mutations rather than *cis*-acting mutations (Wirsching et al. 2000a; Riggle and Kumamoto 2006).

In this communication, we studied polymorphisms in the *MDR1* promoter region that are present in numerous *C. albicans* clinical isolates. The results showed that some of the polymorphisms defined two linkage groups. Alleles from one of the linkage groups showed higher *MDR1* expression compared with alleles from the other group. Strains carrying only the allele with higher promoter activity were significantly more common in collections of clinically isolated strains in comparison with strains carrying only the less active allele. These findings and the results of previous studies showing homozygosis of point mutations in *ERG11* (White 1997), *MRR1* (Dunkel et al. 2008), and *TAC1* (Coste et al. 2006) show that homozygosis is a common mechanism for genetic change in *C. albicans* organisms that are associated with humans.

Materials and methods

Strains

Genotypes of all strains and fluconazole MICs (Minimal Inhibitory Concentration) are listed in Table S1 and Table 1. The clinical strains used in this study were collected from blood and oral mucosa specimens submitted to the clinical microbiology laboratories of Tufts Medical Center, or Beth Israel Deaconess Medical Center, Boston, MA, USA (courtesy of Susan Hadley, MD) or University of Alabama Medical Center, Birmingham, AL, USA (courtesy of John Baddley, MD). The strains were subcultured at the Clinical Microbiology Laboratory, Tufts-New England Medical Center onto Sabouraud's Dextrose agar plates and stored in vials of sterile skimmed milk at −70°C. Five clinically isolated strains were kind gifts of Dr. Michael Pfaller, M.D. and Richard Hollis (Molecular Epidemiology & Fungus Lab Pathology Department University of Iowa Hospitals & Clinics) and were isolated before fluconazole was in widespread clinical use.

Growth media

Yeast extract/peptone/dextrose (YPD) medium, synthetic defined (SD) medium, and complete medium (CM) were as described previously (Rose et al. 1990). RPMI medium 1,640/20 mM MOPS (pH 7.0) was used for MIC determination (Barchiesi et al. 1994). For culture of Ura^- strains, uridine was added to 60 μg ml^{-1}. Fluconazole was added to 8 μg ml^{-1} final concentration.

MIC determination

Susceptibility to fluconazole was analyzed using the standard CLSI (formerly NCCLS) microdilution protocol M27-A (Barchiesi et al. 1994). Briefly, cells at 1×10^3 to 5×10^3 cells/mL were incubated in increasing concentrations of fluconazole (0.0635–128 μg ml^{-1}) in a 96-well plate. The plate was incubated for 46–50 h at 35°C without agitation. The MIC was determined by a lack of growth in the well at certain fluconazole concentrations. For some strains, fluconazole resistance was also confirmed by the semi-solid agar antifungal susceptibility (SAAS) screening method (Provine and Hadley 2000).

DNA analysis

PCR, restriction digestion and gel electrophoresis were performed by standard methods as described previously (Riggle and Kumamoto 2006; Bruzual et al. 2007). Automated DNA sequencing was performed by Michael Berne and coworkers at the Tufts University Core Facility.

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Cloning and sequence analysis of the MDR1 promoter region

The *MDR1* promoter region of each strain was amplified by PCR from genomic DNA prepared as previously described (Riggle and Kumamoto 2006). The product of each PCR reaction was cloned into the pCR2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogene). For each strain, 5–10 independent plasmid clones containing the PCR-amplified *MDR1* upstream region were sequenced to obtain the sequence of both *MDR1* alleles and to exclude PCR artifacts.

Screening alleles by AseI digestion and Southern blotting

Upstream of the *MDR1* ORF there is an *Ase*I restriction site. In addition, in one allele of the *MDR1* promoter, there is another *Ase*I restriction site which includes one of the polymorphic residues. The extra *Ase*I restriction site present in one allele makes it possible to identify the two alleles by Southern blotting or by digestion of PCR products.

Methods for chromosomal DNA isolation and Southern blot hybridization were performed by standard methods as described previously (Riggle and Kumamoto 2006). Genomic DNA was digested with *Ase*I (New England Biolabs). DNA probes were labeled with the Prime-It II Random Primer Labeling Kit from Stratagene and [α-^{32}P]-dATP (New England Nuclear). PCR products from the 1100-bp *MDR*1 gene promoter region (Riggle and Kumamoto 2006) were used for probing Southern blots.

Measurement of gene expression by quantitative real-time reverse transcriptase PCR (qRT-PCR)

The 1,100-bp promoter regions from several clinical isolates were cloned upstream of yEGFP in the vector pLIB1 (Riggle and Kumamoto 2006) and transformed into the Ura$^-$ *C. albicans* strain CAPR514 (Riggle and Kumamoto 2006), a fluconazole-resistant, *MDR1* over-expressing strain. After growth of the cells in medium containing fluconazole (8 μg ml^{-1}), total RNA was extracted from these strains by mechanical disruption using glass beads and a Mini BeadBeater (BioSpecs Products, Inc., Bartlesville, OK, USA) and the RNeasy Mini Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturers' protocols. Strains from Strain group 1 were grown as above and extracted in the same way.

qRT-PCR was performed by standard methods as described previously (White et al. 2007). Briefly, 10 μg of total RNA was converted to cDNA by incubation with Superscript II Reverse Transcriptase (Invitrogen) using an oligo dT primer. After incubation for 1 h at 42°C, RNA was hydrolyzed and the reaction was stopped by addition of NaOH and EDTA to 0.16 N NaOH, 0.08 M EDTA, final concentrations. Following neutralization, cDNA was purified using Qiaquick columns (Qiagen) as described by the manufacturer, except that sodium acetate (pH 5.2) was added to the PB buffer to ensure an acidic pH. cDNA was quantitated by absorbance. Purified cDNA was stored frozen.

The following primer pairs were used to detect the indicated genes: *YEGFP* primers F: CTCCAATTGGTGAT GGTCCAGTCT and R: ACC ATGGGTAATACCAGCAG CAGT; for *MDR*1 primers F: TCTCGGTGGATTCTTTGC TAAT and R: AATGGACCAAAACTAGGACCAC and for ACT1 primers F: TATCATGGGTTGGTATGGG and R: TGTGGTGAACAATGGATG.

qRT-PCR was performed using SYBR green Mastermix and a Stratagene instrument, according to manufacturer's protocols. All reactions were performed in triplicate. Melting curve analysis and/or agarose gel electrophoresis was performed following the reverse transcriptase PCR amplification to verify the presence of a single product. When RNA

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preparations not treated with reverse transcriptase were used as template, the primers failed to amplify products.

Results

Sequence analysis of the *MDR1* promoter region

Polymorphisms in the *MDR1* gene in clinically isolated strains have been previously detected (Gupta et al. 1998; Wirsching et al. 2000a). Since the promoter sequence may have been influenced by the use of fluconazole in the clinic, we first undertook an investigation of the sequence of the *MDR1* promoter region in strains collected prior to 1990 when fluconazole was approved for patient treatment. Five fluconazole-naïve clinically isolated strains were kindly provided by Dr. Michael Pfaller, M.D. and Richard Hollis (Molecular Epidemiology & Fungus Lab, Pathology Department, University of Iowa Hospitals & Clinics). These strains, referred to as strain group 1 (Table 1), were highly susceptible to fluconazole (MIC of fluconazole, <0.25 $\mu g\ ml^{-1}$) (Table 1).

The regulatory regions of the *MDR1* gene, 1,100 bp upstream from the start codon, from these five strains were cloned as described in “Materials and methods”. This region is important for *MDR1* gene overexpression, in fluconazole-resistant strains (Hiller et al. 2006; Riggle and Kumamoto 2006; Rognon et al. 2006). Multiple clones from each strain were sequenced.

Heterozygosities were detected between the two *MDR1* alleles in strains 978–28, 979–15, and 979–33 (Figure S1). Strain 978–28 showed 20 differences within the 1,100-bp region between the two alleles. Strains 979–15 and 979–33 had 13 and 10 polymorphisms, respectively. The polymorphisms included single-base deletions and different single-base exchanges. For the remaining two strains (978–14 and 979–05), no differences were detected between any of the clones sequenced, suggesting that either the strains contained two identical alleles or that one of the two alleles was not amplified or cloned.

Interestingly, at positions −137, −152, −154, −306, and −343 [numbered relative to the start of the *MDR1* open reading frame using the sequence from the reference strain SC5314 (Jones et al. 2004)], particular bases frequently occurred together (Fig. 1). Thus, the sequence in this region of the promoter defined two linkage groups, corresponding to two alleles of the *MDR1* promoter. These polymorphisms were located near a functionally important sequence termed the MDRE (CGGTAAAATCCTAATTG GGAAAAATACCGAGAATGACACA, located at −261 to −295), which contains a binding site for Mcm1p and contributes to *MDR1* expression in over-expressing strains (Riggle and Kumamoto 2006; Rognon et al. 2006). These polymorphisms thus could directly or indirectly affect promoter activity or they may be linked to other changes that influence promoter activity. Therefore, the occurrence and activity of these 2 *MDR1* promoter alleles in *C. albicans* strains was analyzed.

One set of polymorphisms was termed the A allele based on the polymorphism at position −306. This allele occurred in strains 978–14, 979–15, 979–33, and 978–28 (one of two alleles; Table 1). The other set of polymorphisms was termed the G allele based on the same polymorphism, and included the alleles from strain 979-05 and the second allele from 978-28. In addition to the polymorphisms noted in Fig. 1, a variety of other polymorphisms (base substitutions, additions or deletions) occurred throughout the promoter region in these strains, as shown in Figure S1. Wirsching et al. previously described several of these additional polymorphisms (Wirsching et al. 2000a). In addition, some of the polymorphisms were found in the two alleles present in the sequenced reference strain SC5314 (Jones et al. 2004), or its derivative, CAI-4 (data not shown).

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The polymorphism at position −306 caused the formation of a restriction site polymorphism. When the residue A was present (as in the A allele) an *AseI* restriction site was created, whereas when G was present, the sequence was not a site for *AseI*. Therefore, the G allele yielded a large *AseI* fragment of 1492 bp on a Southern blot while the A allele yielded a 1,351-bp *AseI* fragment. This polymorphism allowed us to analyze the alleles by Southern blotting. Consistent with the results of sequencing, this analysis showed that strain 978-28 was heterozygous in this region of the promoter, strains 978-14, 978-15, and 979-33 carried only A alleles and strain 979-05 carried only G alleles (Fig. 1b).

Strains commonly used in laboratories represent another group of strains that were collected before the extensive use of fluconazole. We analyzed the alleles in a collection of five different fluconazole-susceptible strains (SC5314 (Gillum et al. 1984), WO-1 (Slutsky et al. 1985), SGY-243 (Kelly et al. 1987), ATCC10261 (Odds and Hierholzer 1973) and 981 (Goshorn and Scherer 1989) (Goshorn and Scherer 1989) (referred to as strain group 2, Table 1) and strains derived from them (Table S1). Although these laboratory strains have been cultured under non-physiological laboratory conditions and in some cases, manipulated genetically through transformation, all strains tested were heterozygous for the polymorphism at position −306. Fluconazole-resistant strains selected from SC5314-derived strains by laboratory growth in fluconazole (Riggle and Kumamoto 2006) were also heterozygotes. Thus, strains collected before fluconazole was widely used for patient treatment (strain groups 1 and 2) included 6 heterozygotes, 3 A/A strains, and 1 G/G strain; the frequency of A/A strains was 3/10.

A/A strains are more common than G/G strains in recently collected *C. albicans* clinically isolated strains

To determine whether there was a difference in *MDR1* promoter genotypes among more recent isolates, the *MDR1* promoter was studied in a collection of strains isolated from patients in two different cities. Twenty-seven clinically isolated, fluconazole-susceptible strains (strain group 3, Table 1) and 22 highly resistant strains (fluconazole MIC >128 mg/L; strain group 4, Table 1) from Tufts Medical Center (formerly New England Medical Center; Boston, MA, USA) or Beth Israel Deaconess Medical Center (Boston, MA, USA) were kindly provided by Dr. Susan Hadley. An additional 11 fluconazole-resistant strains (strain group 5, Table 1) were isolated at the University of Alabama Medical Center (Birmingham, AL, USA) and were kindly provided by Dr. John Baddley and Dr. Susan Hadley.

Genomic DNA was isolated from each strain and characterized by Southern blotting as described in Materials and methods. The 1,100-bp PCR amplified *MDR1* promoter region from laboratory strain CAI4 was used as probe. Results showed that among the fluconazole-susceptible strains from group 3, 11 were heterozygous in this region of the *MDR1* promoter, yielding both a 1.5 and 1.3 Kb band (Fig. 2a). Thirteen isolates were A/A strains and three isolates were G/G strains (Table 1).

The isolates that were highly resistant to fluconazole were screened by the same methods (Fig. 2b). From the 22 isolates from Boston (strain group 4), 8 were heterozygous, 11 were A/A strains, and 3 were G/G strains. The 11 fluconazole-resistant clinically isolated strains from the University of Alabama Medical Center (strain group 5) contained 10 isolates that were A/A strains and one heterozygous strain (Fig. 2c). In this group of isolates, we did not identify any G/G strains.

Thus, among recently collected clinical isolates (strain groups 3, 4 and 5), the frequency of A/A strains was 34 out of 60 (Fig. 3). Among the strains collected recently in Boston, MA, fluconazole-susceptible (strain group 3) and fluconazole-resistant strains (strain group 4) gave similar frequencies of heterozygotes and A/A strains. The proportion of A/A strains

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among the recent isolates (34/60) (strain groups 3, 4 and 5) was not statistically significantly different from the proportion seen in older isolates (3/10) (strain groups 1 and 2; $p < 0.18$, Fisher's exact test). However, there was a difference between strains that have been propagated under laboratory conditions (5 out of 5 heterozygotes; strain group 2) and the clinically isolated strains (37 A/A strains out of 64 isolates; strain groups 1, 3, 4 and 5) ($p < 0.02$, Fisher's exact test). Although strains used in the laboratory were originally isolated from clinical samples, at least some of these strains were collected many years ago when environmental exposures to compounds and clinical practices were different. In addition, laboratory strains are commonly stored under conditions intended to minimize genetic changes (e.g. frozen), unlike strains in nature which have the opportunity to evolve. These facts may explain why all of these strains were A/G heterozygotes.

Among all of the homozygous strains, there were significantly more A/A strains (37 out of 44 total) than G/G strains (7 out of 44 total) ($p < 0.0001$, sign test). This observation suggests that, in some settings, the A/A genotype provides a selective advantage relative to the G/G genotype.

Functional differences in *MDR1* gene expression from A alleles and G alleles

To determine whether A alleles and G alleles differed in expression of the *MDR*1 gene, we analyzed *MDR1* expression in the fluconazole-susceptible strains isolated before fluconazole was widely used. Strains 978-14, 979-15, and 979-33 (A/A strains), and 979-05 (G/G strain) were analyzed. *MDR1* expression in fluconazole-susceptible strains is very low, and we detected basal *MDR1* gene expression using quantitative real-time reverse transcriptase PCR (qRT-PCR). For the analysis, RNA was prepared from *C. albicans* cells cultured in the presence of fluconazole (8 $\mu g\ ml^{-1}$). cDNA prepared from the RNA was used as the template for qRT-PCR amplification, as described in Materials and Methods. Results were normalized using *C. albicans ACT1* (encoding actin) and are shown relative to expression in a reference strain, the laboratory strain CAI-4.

As shown in Fig. 4, strain 978-14 (A/A strain) showed more than tenfold higher levels of *MDR1* expression compared with strain 979-05 (G/G strain). The other two strains, 979-15 and 979-33 (A/A strains), showed more than fivefold higher levels of *MDR1* expression in comparison with 979-05 (G/G strain). These results showed that strains carrying the A allele expressed higher levels of *MDR1* transcript than the strain carrying the G allele. Expression in a drug-resistant A/G strain, CAPR514A, that overproduces Mdr1p is shown for comparison.

To eliminate other differences in the strain background of various clinical strains, the promoter regions of the alleles carried in several strain group 4 strains including G alleles from CI 127, 172, 189, and 196 and A alleles from CI 124, 166, and 167, were cloned upstream of the promoterless *yEGFP* gene in the vector pLIB1 (Riggle and Kumamoto 2006). The sequences of several of these *MDR1* promoter alleles were also determined and showed that the alleles belonged to either the A or G allele group (Fig. 1 and Fig. S1). The P_{MDR1}–*yEGFP* fusions were integrated onto the chromosome of the drug-resistant, *MDR1*-over-expressing laboratory strain CAPR514 (Riggle and Kumamoto 2006). Under these conditions, GFP fluorescence in the strains carrying either the A or G allele was high (data not shown), presumably due to the *trans*-activating *MRR1* mutation (Morschhauser et al. 2007; Dunkel et al. 2008; Schubert et al. 2008). In order to quantify expression from the *MDR1* promoters, RNA was isolated from cells grown in fluconazole (8 $\mu g\ ml^{-1}$) and *yEGFP* expression was measured by quantitative real-time RT PCR (Fig. 5). *ACT1* was used to normalize expression. Results showed that strains carrying A alleles expressed higher levels of the reporter gene yEGFP than strains carrying G alleles ($p < 0.001$, *t* test). The average expression of A alleles was fivefold higher than the average expression of G alleles.

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Thus, in an isogenic strain background, A alleles were more active in promoting expression from the *MDR1* promoter than G alleles.

Discussion

With the approval of fluconazole by the FDA in 1990, the drug became the most popular antifungal to treat candidiasis. The extensive use of fluconazole was mainly due to its effectiveness and low toxicity (Graybill 1989; Larsen 1990). Recently, widespread use of fluconazole in candidiasis patients has led to a higher frequency of treatment failures due to drug resistance (Ruhnke et al. 1994; Chakrabarti et al. 2009). Because of this observation, there has been strong interest in understanding how drug resistance in *C. albicans* evolves. Our results show that recently isolated clinical strains commonly carry a more active allele of the *MDR1* promoter, often on both homologues. Because two copies of the higher activity *MDR1* promoter allele would result in higher basal expression of *MDR1* due to increased gene dosage, we propose as a model that higher basal expression of Mdr1p allows strains to grow or survive longer in the presence of fluconazole or another compound. As a result, spontaneous, advantageous mutations have a greater opportunity to arise in a strain carrying higher activity *MDR1* promoter alleles. Over evolutionary time, the more active *MDR1* promoter alleles thus contribute to the evolution of drug resistance.

Fluconazole resistance in clinical isolates of *C. albicans* has been associated with a combination of several distinct mechanisms (White 1997; Franz et al. 1998; Lopez-Ribot et al. 1999; Morschhauser 2002, 2010; Anderson 2005; Sanglard et al. 2009). The order of the events that gives rise to a drug-resistant phenotype does not seem to be fixed. Rather, *C. albicans* cells can generate a partially resistant phenotype through different mechanisms, and the partially resistant mutant cells then overgrow the more susceptible cells in the population in the presence of drug (White 1997; Franz et al. 1998; Anderson 2005).

The results described in this communication add to the growing literature demonstrating the importance of alterations in genome structure during the evolution of drug resistance. Selmecki et al. showed that growth in the presence of an antifungal drug led to selection for a specific isochromosome that increased the copy number of *EFG11* and *TAC1* (Selmecki et al. 2009). Other chromosomal rearrangements were also noted during the evolutionary process. However, A/A strains were not isolated from A/G strains during laboratory selection of fluconazole-resistant strains or following passage through a mouse (data not shown). The length of a laboratory selection experiment may be insufficient for selection of A/A strains or the relevant selective pressure may not be replicated in a laboratory experiment.

The observation that several of the fluconazole-naive strains collected prior to the widespread use of fluconazole were A/A strains shows that the selective pressure leading to the high frequency of A/A strains was not related to fluconazole use. Older antifungals such as ketoconazole or itraconazole are unlikely sources of selection pressure because increased expression of *MDR1* does not increase the MIC for these drugs (White 1997; Franz et al. 1998; Lopez-Ribot et al. 1999; Wirsching et al. 2000b; Harry et al. 2005; Cheng et al. 2007). There may be natural compounds produced by a host or therapeutic compounds other than azoles that require efflux by Mdr1p for detoxification. Exposure of *C. albicans* to such compounds during host colonization may have provided the selective pressure that resulted in the frequent occurrence of A/A strains. In addition, some compounds, such as the antibacterial drug rifampicin, induce *C. albicans MDR1* expression (Vogel et al. 2008). Exposure to such a drug could amplify the effect of the A allele because higher *MDR1* expression could give an A/A strain a greater advantage over a G/G strain. Since antibacterial drugs have been in use far longer than fluconazole, the use of such compounds

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may have increased the effect of the selection pressure that favored A/A strains as opposed to G/G strains.

Our results show that in fluconazole-susceptible and fluconazole-resistant strain backgrounds, the A alleles had increased promoter activity relative to the G alleles. In fluconazole-susceptible strains, *MDR1* expression was much lower than the expression in a strain carrying a *trans*-activating *MRR1* mutation such as strain CAPR514. Nevertheless, differential expression between A and G alleles was observed under these basal expression conditions. In an *MRR1* mutant strain, expression from the *MDR1* promoter was much higher. When clones carrying an A allele or a G allele were introduced into this mutant background, a difference in the level of expression was also observed. Therefore, the A allele leads to higher *MDR1* expression in strains carrying either a WT or mutant allele of *MRR1*. Increased *MDR1* expression, however, is not sufficient to produce a measurable increase in fluconazole MIC in an A/A strain relative to a G/G strain.

Differences in the promoter activity of two homologs have been observed previously in *C. albicans*. Staib and coworkers (Staib et al. 2002) showed that an increase or a reduction in the copy number of pentameric repeats (R1 and/or R2) in the promoter of the *SAP2* gene affected expression from the promoter within infected organs but not during laboratory culture. The *SAP2-2* promoter allele, containing five copies of R1 and five copies of R2, was induced more easily than the *SAP2-1* promoter, which contains four copies of R1 and six copies of R2. In the *CHS7* promoter, polymorphisms are also associated with differences in promoter activity (Sanz et al. 2007).

The five residues that segregated together and defined the A and G alleles (−137, −152, −154, −306, and −343) lie on either side of an element in the promoter termed the MDRE, composed of bases −261 to −295. This region is important for expression from the *MDR1* promoter in an *MRR1* mutant strain (Riggle and Kumamoto 2006). Therefore, it is possible that the particular bases that make up either the A or G alleles play a direct role in determining the activity of the promoter. Alternatively, these bases may be linked to other bases that play a direct functional role. As there are many polymorphisms in the *MDR1* promoter region, the precise residues that confer the differences in activity of the A and G alleles are currently unknown. Given the intricate regulation of *MDR1* expression with several *trans*-acting factors that bind to the *MDR1* promoter and many regions that are important for expression (Harry et al. 2005; Hiller et al. 2006; Riggle and Kumamoto 2006; Rognon et al. 2006; Morschhauser et al. 2007; Mogavero et al. 2011), it is likely that the differences in activity will involve multiple, co-segregating residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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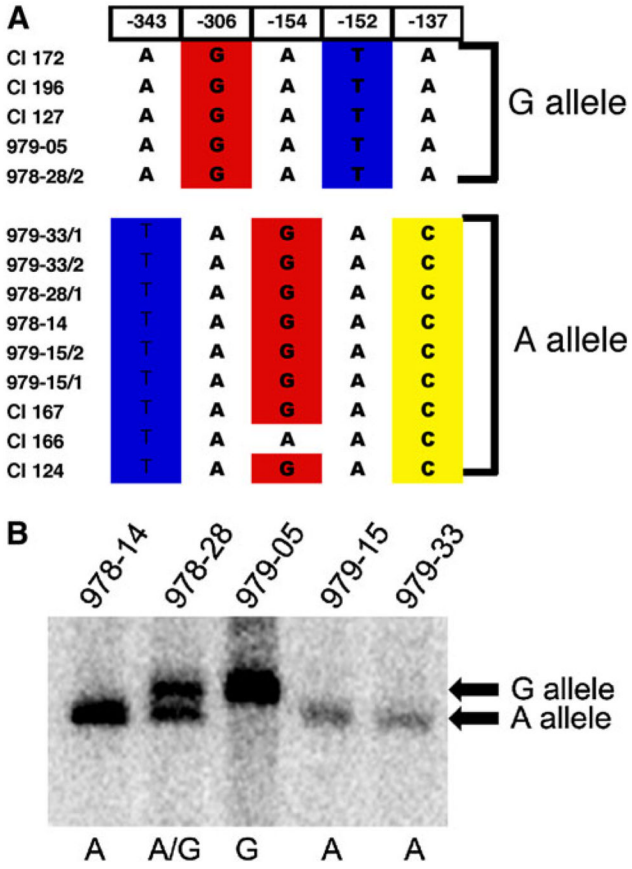

Fig. 1.

Sequences of alleles of the *MDR1* promoter region in several strains show polymorphisms. *Panel* **a** Strain names are given at *left*. For heterozygotes, the two alleles are denoted 1 and 2. The position of the residue relative to the start of the *MDR1* open reading frame using the sequence from SC5314 is shown above. The sequence at each position in the allele indicated is shown. The nucleotides at these five positions were used to define two alleles of this *MDR1* promoter region, termed the A allele and the G allele. *Panel* **b** Twenty micrograms of genomic DNA extracted from the indicated strains was digested with *AseI* and probed in a Southern blot. The mobilities of the bands produced from the G allele and A allele are indicated at *right*. The *letters* shown below indicate the alleles present in each strain

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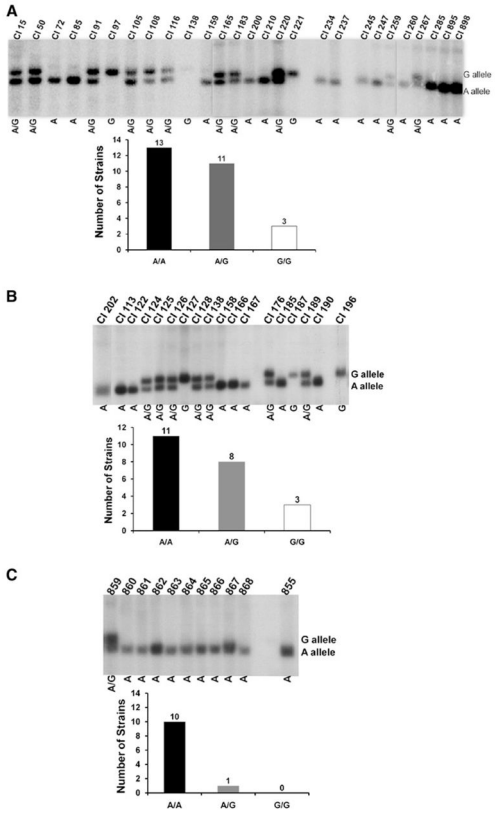

Fig. 2.

Southern blot analysis of genomic DNA from clinically isolated strains of *C. albicans. Panel* **a** The gel at the top shows 20 μg of genomic DNA extracted from the indicated fluconazole-susceptible strains (strain group 3), digested with *AseI* and probed in a Southern blot. The mobilities of the bands produced from the G allele and A allele are indicated at *right*. The *letters* shown below indicate the alleles present in each strain. The *graph* shows the numbers of strains of each genotype. *Panel* **b** The same analysis for fluconazole-resistant strains isolated in Boston, MA, USA (strain group 4). *Panel* **c** The same analysis for fluconazole-resistant strains isolated at Alabama Medical Center (strain group 5)

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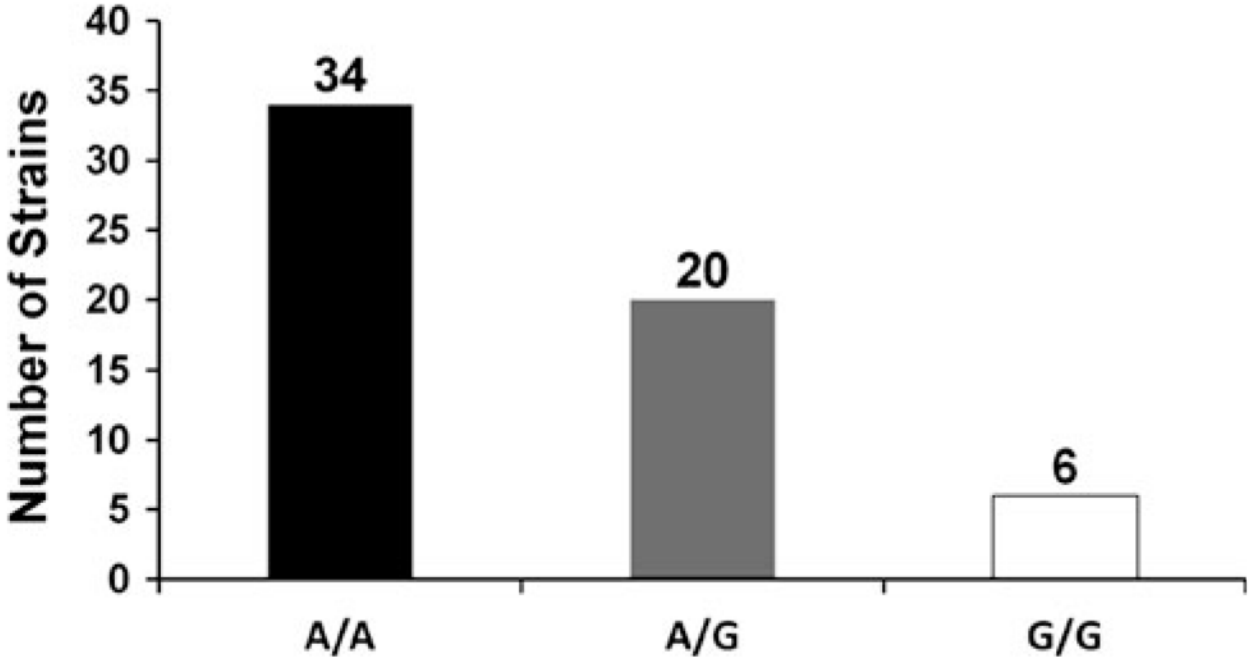

Fig. 3.

Summary of all recently isolated clinical strains studied. The graph includes fluconazole-susceptible and -resistant strains isolated in Boston, MA, USA or at Alabama Medical Center (strain group 3, 4 and 5)

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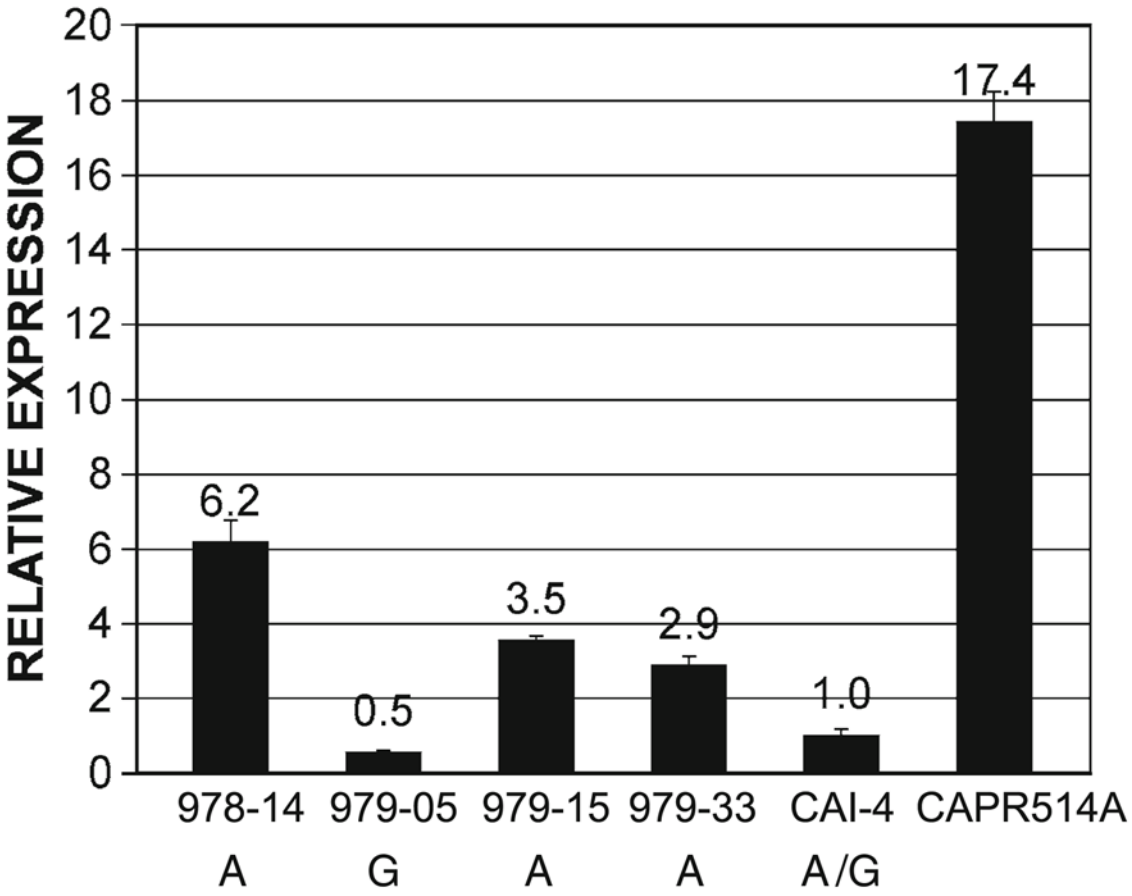

Fig. 4.

Higher levels of *MDR1* transcript in A allele homozygotes. Expression of the *MDR1* gene in strains indicated at *bottom* was analyzed by qRT-PCR as described in "Materials and methods". Results were normalized using actin expression and are expressed relative to expression in the laboratory reference strain CAI-4, grown in the presence of fluconazole. The average of results from three experiments, each run in triplicate, is shown, with the standard deviation. Strains 978-14, 979-05, 979-15, 979-33, and CAI-4 are fluconazole susceptible (MIC < 0.25 μg ml^{-1}). Alleles present in these strains are shown. Strain CAPR514A is a fluconazole-resistant, *MDR1*-overexpressing A/G strain (MIC >64 μg ml^{-1}), shown for comparison

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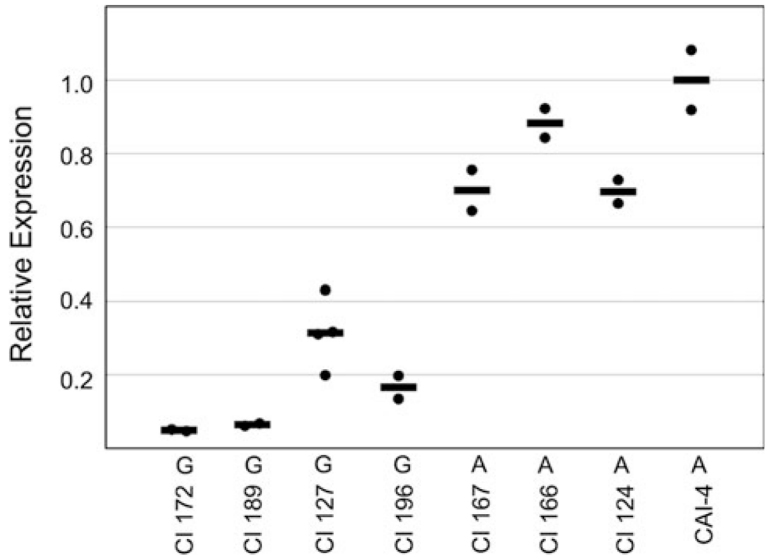

Fig. 5.

Expression of yEGFP from A or G alleles of the *MDR1* promoter. CAPR514-derived cells carrying fusions of the yEGFP reporter gene under control of *MDR1* promoter A or G alleles from the indicated strains were grown in the presence of fluconazole and RNA was isolated from the cells. Expression of the *yEGFP* gene was analyzed by qRT-PCR as described in "Materials and methods". Results were normalized using actin expression and are expressed relative to expression of yEGFP from an A allele from reference strain CAI-4. Each *filled circle* shows the average of a measurement performed in triplicate. *Black bar* indicates the mean of the two or four measurements

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Table 1

Summary of strains

Name	Alleles	O/E gene[a]	MIC (mg/L)	Number/total
Strain Group 1 Clinical isolates, pre-1990, Fluconazole susceptible				
978-14, 979-15, 979-33	A/A	NA	<0.25	3/5
978-28	A/G	NA	<0.25	1/5
979-05	G/G	NA	<0.25	1/5
Strain Group 2 Strains used in laboratories, Fluconazole susceptible				
SC5314, WO-1, SGY-243, 981, ATCC10261	A/G	NA	<0.25	5/5
Strain Group 3 Clinical isolates, Boston, MA, Fluconazole susceptible				
CI 072, CI 085, CI 159, CI 200, CI 210, CI 234, CI 237, CI 245, CI 247, CI 260, CI 285, CI 895, CI 898	A/A	NA	<0.25	13/27
CI 015, CI 050, CI 091, CI 105, CI 108, CI 116, CI 165, CI 183, CI 220, CI, 259, CI 267	A/G	NA	<0.25	11/27
CI 097, CI 138, CI 221	G/G	NA	<0.25	3/27
Strain Group 4 Clinical isolates, Boston, MA, Fluconazole resistant				
CI 158	A/A	*MDR1*	>128	11/22
CI 122, CI 190	A/A	*CDR*	>128	
CI 113, CI 166, CI 167, CI 168, CI 177, CI 185, CI 199, CI 202	A/A	unknown	>128	8/22
CI 124	A/G	*CDR*	>128	
CI 125, CI 126, CI 128, CI 138, CI 172, CI 176, CI 189	A/G	unknown	>128	
CI 127, CI 187, CI 196	G/G	unknown	>128	3/22
Strain group 5 clinical isolates, Birmingham, AL, Fluconazole resistant				
AL 864, AL 868	A/A	*CDR*	>128	10/11
SH 855, AL 860, AL 861, AL 862, AL 863, AL 865, AL 866, AL 867	A/A	unknown	>128	
AL 859	A/G	unknown	>128	1/11

NA not applicable

[a]Overexpressed gene