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Distinct Patterns of Gene Expression Associated with Development of Fluconazole Resistance in Serial *Candida albicans* Isolates from Human Immunodeficiency Virus-Infected Patients with Oropharyngeal Candidiasis

JOSE L. LOPEZ-RIBOT,[1]* ROBERT K. McATEE,[1] LINDA N. LEE,[1] WILLIAM R. KIRKPATRICK,[1]
THEODORE C. WHITE,[2] DOMINIQUE SANGLARD,[3] AND THOMAS F. PATTERSON[1]

Department of Medicine, Division of Infectious Diseases, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7881[1]; Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, and Seattle Biomedical Research Institute, Seattle, Washington, 98109[2]; and Institut de Microbiologie, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland[3]

Resistance to fluconazole is becoming an increasing problem in the management of oropharyngeal candidiasis in human immunodeficiency virus-infected patients. Strains obtained from five patients developed decreased fluconazole susceptibility over time. DNA strain typing confirmed the high degree of relatedness among isolates from one patient and the variability among isolates from different patients. Expression of genes involved in development of fluconazole resistance was monitored in each isolate using probes specific for *ERG11* (lanosterol 14α-demethylase), *MDR1* (a major facilitator), and *CDR* (ATP-binding cassette or ABC transporter) genes. Increased expression of *CDR* genes was detected in the series of isolates from two patients. Isolates from one of the two patients also demonstrated increased *ERG11* expression, whereas isolates from the other patient did not. Increased levels of *MDR1* mRNA correlated with increased resistance in sequential isolates from another patient. Initial overexpression of *MDR1* with subsequent overexpression of *CDR* genes and a final isolate again overexpressing *MDR1* were detected in serial isolates from another patient. In another patient, overexpression of these genes was not detected despite an eightfold increase in fluconazole MIC. In this patient, sequence data of the *ERG11* gene revealed no point mutations associated with decreased susceptibility. Five different patterns of gene expression were observed in isolates recovered from five patients who developed resistance. Therefore, these experiments demonstrate that a variety of mechanisms or combinations of mechanisms are associated with the development of fluconazole drug resistance. Additional studies are needed to estimate the frequency and clinical impact of these mechanisms of resistance.

Resistance to fluconazole and other azole antifungal drugs has become an important clinical problem in the management of human immunodeficiency virus (HIV)-infected patients with recurrent oropharyngeal candidiasis (OPC) (17, 20). Recent advances in our understanding of the molecular mechanisms leading to azole resistance in *Candida albicans*, the main etiologic agent of OPC, suggest the multifactorial nature of resistance. Alterations in the target enzyme (lanosterol 14α-demethylase), including point mutations (10, 11, 22, 28, 32) and overexpression (31), lead to decreased susceptibilities to azole drugs. Increased efflux of drug, mediated by multidrug pumps belonging to two different families, the major facilitators and the ATP-binding cassette (ABC) transporters, also confers resistance to azole antifungal agents (1, 23–25, 29, 31). The genes coding for several ABC transporters in *C. albicans* have been identified, including several *CDR* genes (2, 16, 24, 25, 30). These ABC transporters, which have been associated with drug resistance in a variety of eukaryotic cells, include a membrane pore composed of transmembrane segments and two ATP-binding cassettes on the cytosolic side of the membrane, which provide the energy source for the pump (3, 7).

The *MDR1* gene (also called *BEN*[r]) is the only gene coding for a major facilitator that has been identified in *C. albicans* so far (5), and its overexpression leads to fluconazole resistance exclusively among azole drugs (23, 25, 31). The major facilitators contain a transmembrane pore but use proton motive force as their energy source (12).

In the present study, we have investigated the expression of *C. albicans ERG11* (encoding lanosterol 14α-demethylase, formerly designated *ERG16* [8, 9]), *MDR1*, and *CDR* genes in sequential clinical isolates obtained from HIV-infected patients with OPC in order to assess the distribution and frequency of various mechanisms responsible for the development of antifungal drug resistance and their clinical impact.

MATERIALS AND METHODS

Clinical samples and isolates. *C. albicans* isolates were obtained by direct swab or by oral saline rinses from five HIV-infected patients with recurrent OPC enrolled in a longitudinal study to assess significance of fluconazole resistance (Table 1). Patients were treated initially with fluconazole at 100 mg/day. Doses were increased up to 800 mg/day in an effort to achieve therapeutic response after development of clinical resistance. In all five patients, sequential isolates showed decreased susceptibility to fluconazole, and in all five patients, therapeutic response was achieved by increasing the dose of fluconazole to a range of 200 to 800 mg/day (Table 1). In this study, clinical resistance was defined as the clinical requirement for increasing fluconazole doses for response. Resistance refers to mycological, in vitro resistance, which was the detection of increased MICs. The identity of these clinical isolates as *C. albicans* was confirmed by standard biochemical and microbiological procedures, including carbohydrate assimilation patterns (API 20C; Analytab Products, BioMerieux, France), germ tube formation in serum-containing medium, and color of colonies in chromo-

* Corresponding author. Mailing address: Department of Medicine, Division of Infectious Diseases, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-7881. Phone: (210) 567-1981. Fax: (210) 567-3303. E-mail: ribot@uthscsa.edu.

TABLE 1. Serial isolates of *C. albicans* from patients with OPC[a]

Patient	Isolate	OPC episode	Day	FLU dose (mg/day)	Cumulative FLU dose (g)	Previous azole use	MIC (µg/ml)			DNA type
							FLU	ITRA	AMB	
7	412	1	1	100	8.5	ITRA, KETO	0.5	0.03	0.5	A
	1907	3	210	200	11.2		8	0.5	0.25	A
	2307	6	280	400	18.1		>128	0.5	0.25	A
9	437	1	1	100	3.0		1	0.015	0.25	B
	1002	3	61	100	5.7		0.25	0.015	0.25	B
	1442	4	119	100	7.5		0.125	0.015	0.25	B
	2271	5	273	100	8.4		16	0.03	0.25	B
	2823	8	411	800	44.4		16	0.06	0.25	B
14	580	1	1	100	31.7	CLO	4	0.03	0.25	C
	649	1	7	200	32.6		16	0.25	0.25	C
	2438	3	296	200	90.1		32	0.25	0.25	C
40	1490	1	1	100	4.6		0.5	0.125	0.25	D
	1587	2	15	100	5.5		8	0.125	0.25	D
	1622	2	15	100	5.5		0.5	0.015	0.25	D
	1780	4	58	100	8.2		16	0.5	0.25	D
	2225	6	149	200	11.8		4	0.125	0.25	D
	2512	8	179	800	20.8		32	0.03	0.25	D
43	1649	1	1	100	0	CLO	0.25	0.06	0.25	E
	1831	2	44	100	0.9		8	0.03	0.25	E
	2183	3	100	100	1.8		>128	0.5	0.25	E

[a] Drug abbreviations: FLU, fluconazole; ITRA, itraconazole; KETO, ketoconazole; CLO, clotrimazole; AMB, amphotericin B.

genic medium (CHROMagar Candida, CHROMagar, Paris, France). Isolates were stored at room temperature as suspensions in sterile water and subcultured onto plates containing Sabouraud dextrose agar 48 h prior to propagation in YEPD medium (2% yeast extract, 1% peptone, 2% glucose).

Strain identification. Strain identity was established by karyotyping, restriction fragment length polymorphism (RFLP), and DNA fingerprinting using the moderately repetitive probe Ca3 (a gift from D. Soll, University of Iowa) as previously described (18, 26). Briefly, chromosomes from the different isolates were prepared in agarose plugs and separated by pulsed-field gel electrophoresis (Bio-Rad, Hercules, Calif.). RFLP patterns were generated by digestion of genomic DNA with *Sfi*I (Boehringer-Mannheim, Indianapolis, Ind.). After documentation, the materials present in the RFLP gels were transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, N.H.) and hybridized with a Ca3 probe radioactively labeled by random priming (Random Primers DNA Labeling System; Gibco-BRL, Gaithersburg, Md.). The membranes were then washed and exposed to autoradiography film (Du Pont, Wilmington, Del.).

Drug susceptibility testing and MIC determinations. Initial fluconazole susceptibility at the time of primary isolation was determined by an agar dilution method as described previously by our group (14, 15). Antifungal susceptibilities to fluconazole, itraconazole, and amphotericin B were determined by the National Committee for Clinical Laboratory Standards standard procedures using macrobroth techniques and reading the endpoints at 48 h (13).

Northern (RNA) blot analysis. Total RNA from the different isolates grown to mid-logarithmic phase in YEPD medium was obtained by using the RNAeasy mini kit (Qiagen Inc., Santa Clarita, Calif.) following the manufacturer's instructions. Equal amounts (approximately 5 µg) of RNA as determined by A_{260} measurements were separated by electrophoresis (21) and subsequently transferred to nylon membranes (Nytran; Schleicher & Schuell) using the Turboblotter apparatus (Schleicher & Schuell). Probes for *ERG11*, *MDR1*, and *CDR* genes were purified from plasmids containing inserts of the respective genes as described before (31). The resulting *CDR* probe is based on the whole sequence of *CDR1* and has been shown to cross-hybridize with other members of this gene family (24, 25, 31). Probes specific for *CDR1* and *CDR2* genes were prepared as described by Sanglard and coworkers (24) by PCR amplification from plasmids containing these sequences (pDS243 and pDS246 for *CDR1* and *CDR2*, respectively), with the following primers: primers 5′-GAG ATC TAC CCT TTA AGA TA (forward) and 5′-TCT GAA TCG GGA TTC AAT TG (reverse) for *CDR1* and primers 5′-GGTATATAAACTGGACAACA (forward) and 5′-CGGAAT CTGGGTCTAATTGT (reverse) for *CDR2*. The identity between the two resulting probes at the level of nucleotide sequence was below 50%. All probes were labeled by random priming (Random Primers DNA Labeling System; Gibco-BRL), and hybridizations were performed with Rapid-hyb buffer (Amersham Life Science Inc., Arlington Heights, Ill.) following the manufacturer's instructions. After hybridization, blots were washed and exposed to autoradiography film (Du Pont). Nylon membranes were probed sequentially with the

different probes following stripping the previously bound probe with 95°C-heated 1× SSC buffer (1× SSC is 0.15 M sodium chloride plus 15 mM sodium citrate [pH 7.0]) with 0.5% sodium dodecyl sulfate twice for 15 min. For densitometric analysis, autoradiograms were scanned with the Adobe Photoshop program (Adobe Systems Inc., Mountain View, Calif.), and signals were quantitated with Dendron (Solltech Inc., Oakdale, Iowa). Relative values were adjusted for differences in sample loading based on quantification of 18S rRNA levels. For preparation of figures, digital images were processed by using the Adobe Photoshop program.

PCR amplification and sequencing. The *ERG11* genes encoding lanosterol 14α-demethylase from several isolates were amplified by PCR. Genomic DNA from the different isolates was extracted with YeaStar Genomic DNA (Zymo Research, Orange, Calif.) and used as a template for amplification of *ERG11* genes. PCR was carried out with high-fidelity *Pwo* DNA polymerase (Boehringer Mannheim) using the following primers: 5′-GTT GAA ACT GTC ATT GAT GG (forward) and 5′-TCA GAA CAC TGA ATC GAA AG (reverse). Amplicons were purified with Wizard PCR Preps (Promega, Madison, Wis.), and their nucleotide sequences for both strands were determined by primer elongation with an ABI automated DNA sequencer (Applied Biosystems, Foster City, Calif.).

RESULTS

Clinical response. Patients were treated initially with fluconazole at 100 mg/day and with increased doses of up to 800 mg/day if necessary for clinical resolution after development of refractory disease. In all five patients, clinical resistance to initial doses occurred, requiring increased doses of fluconazole (Table 1). However, therapeutic responses were seen in all patients with increased fluconazole doses. Table 1 shows the sequential isolates from each patient, the elapsed period between times of isolation, the dose of fluconazole required, the cumulative dose of fluconazole, prior regimens with other azoles, and the MICs for fluconazole, itraconazole, and amphotericin B as determined by a macrodilution method by National Committee for Clinical Laboratory Standards techniques. The initial isolate of the series for each patient was fluconazole susceptible (MIC of ≤8 µg/ml) (13, 19). Cross-resistance between itraconazole and fluconazole was detected in isolates from patients 7, 14, and 43 with an 8- to 16-fold
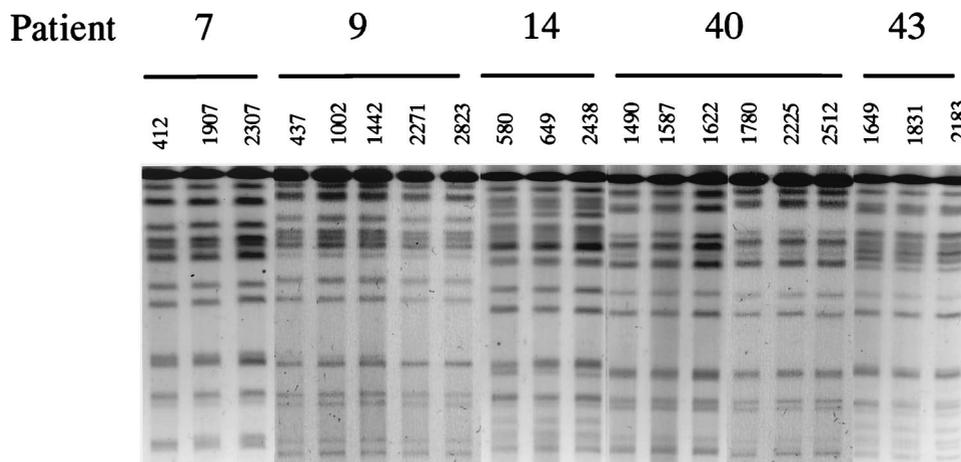
FIG. 1. RFLP patterns generated by digestion with *Sfi*I of genomic DNA from the different clinical *C. albicans* isolates recovered from successive episodes of OPC. The image is a composite of image scans from two different gels run under the same electrophoretic conditions.

increase in itraconazole resistance in the final isolates. Only one isolate from patient 40 showed decreased susceptibility to itraconazole, whereas all other isolates had the same or increased susceptibility to itraconazole relative to that of the first isolate obtained. On the other hand, all isolates from patient 9 remained susceptible to itraconazole (MIC of ≤0.125 μg/ml) despite increasing fluconazole MICs. All isolates remained susceptible to amphotericin B.

The identities of strains from a given patient were confirmed by karyotyping, RFLP, and fingerprinting with the *C. albicans* Ca3 probe. In each of these methods, sequential isolates from each patient were demonstrated to be highly related and distinct from isolates recovered from other patients by all typing methods employed (Table 1). RFLP patterns generated by digestion with *Sfi*I of genomic DNA from the different isolates followed by separation by pulsed-field electrophoresis is shown in Fig. 1.

Expression of *ERG11*, *MDR1*, and *CDR* genes in clinical isolates. Total RNA extracted from the different isolates growing in YEPD medium in the absence of antifungal drug was analyzed by a Northern blot technique with probes specific for *ERG11*, *MDR1*, and *CDR* genes (Fig. 2).

In patient 7, with sequential isolates showing decreased susceptibility to fluconazole, mRNA levels for *CDR* genes were dramatically increased along the series of isolates. Densitometric analysis showed overexpression of *CDR* genes by a factor of 7 in the second isolate of the series (isolate 1907; fluconazole MIC = 8 μg/ml) and by a factor of 10 in the third highly resistant isolate (isolate 2307; fluconazole MIC > 128 μg/ml). The third isolate also demonstrated moderate overexpression of *ERG11*. *MDR1* levels were negligible for all three isolates.

In contrast, study of five sequential isolates from patient 9, also requiring increasing fluconazole MICs, revealed the overexpression of the *MDR1* gene and no increase in *CDR* gene expression. Levels of *MDR1* mRNA were undetectable in the first three susceptible isolates of the series, but *MDR1* was markedly overexpressed in the fourth and fifth less susceptible isolates. Levels of expression for *ERG11* remained constant throughout the series. Expression of *CDR* genes was detected at very low levels for all isolates in this series, without significant changes as resistance developed.

In patient 14, although antifungal susceptibility testing revealed the decreasing susceptibility of isolates, the Northern blot technique failed to detect overexpression for any of the
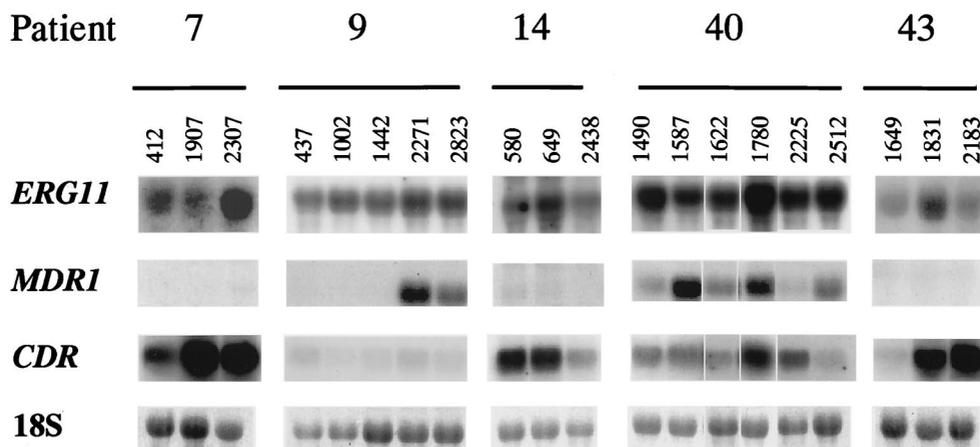

FIG. 2. Northern blots of total RNA from clinical *C. albicans* isolates probed with *ERG11*, *MDR1*, and *CDR* probes. Hybridizations were performed as described in Materials and Methods. The bottom series show the amounts of 18S rRNA used to standardize signal levels according to lane loading parameters.
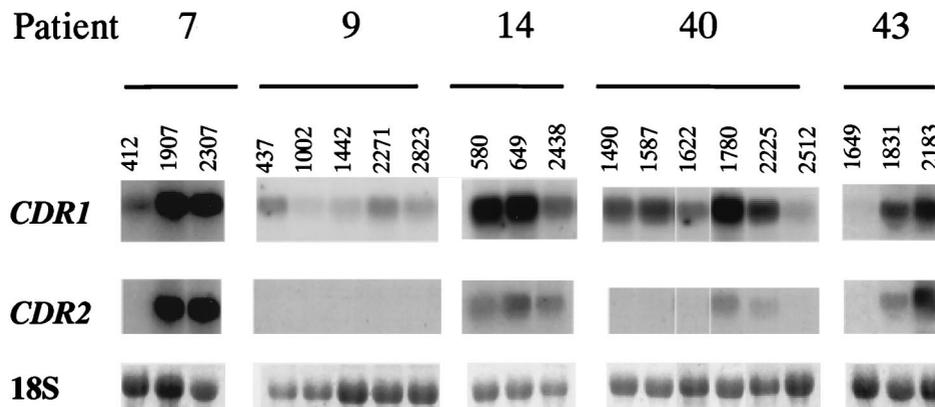
FIG. 3. Northern blot analysis of total RNA from clinical *C. albicans* isolates using probes specific for *CDR1* and *CDR2* (see Materials and Methods). The bottom series show the amounts of 18S rRNA used as a control for lane loading in order to normalize signal levels.

genes in the study. However, high constitutive levels of expression of *CDR* genes were noted for all isolates of the series, although decreased *CDR* message was detected in the third, most resistant isolate. Because the third isolate recovered from patient 14 showed decreasing susceptibility to fluconazole that did not correlate with overexpression of any of the genes studied (whereas the first and second isolates demonstrated elevated levels of *CDR* message), the *ERG11* gene coding for lanosterol 14α-demethylase was sequenced to detect point mutations which may have resulted in decreased fluconazole susceptibility. *ERG11* genes from these isolates were obtained by PCR. Single fragments of the expected length (1.6 kb) were obtained in each case. Compared to a published *ERG11* sequence (9), a total of seven silent (not resulting in amino acid substitutions) nucleotide changes were found in isolate 580. *ERG11* sequences from isolates 649 and 2438 were identical and showed a total of 10 base differences compared to the published sequence. For these two isolates, the only nucleotide change that had an effect in the amino acid sequence was the single amino acid substitution V437I. However, we have detected this same substitution in *ERG11* genes from susceptible *C. albicans* isolates (data not shown). In addition, Sanglard and colleagues (22) have shown by functional expression of these genes in *Saccharomyces cerevisiae* that this mutation does not confer resistance to fluconazole. Therefore, the mechanism(s) of increased drug resistance for the third isolate (isolate 2438) is unknown.

Six sequential isolates from patient 40 were included in the study. Northern blot analysis revealed that levels of *ERG11* mRNA remained constant for all isolates examined, including the first and third isolates that were sensitive to fluconazole (isolates 1490 and 1622; MIC = 0.5 μg/ml). Strong expression of *MDR1* in the second (isolate 1587; fluconazole MIC = 8 μg/ml) and fourth (1780; MIC = 16 μg/ml) isolates with less expression in the first (1490), third (1622), and sixth (2512) isolates mirrored MIC results with the exception of the sixth isolate, which required the highest fluconazole MIC (32 μg/ml) but did not exhibit the strongest *MDR1* expression. In isolate 1780 (fluconazole MIC = 16 μg/ml), increased message for *MDR1* was accompanied by overexpression of *CDR* genes (by a factor of 2.5). A twofold increase in *CDR* message with no overexpression of *MDR1* was detected in the fifth isolate (2225; fluconazole MIC = 4 μg/ml).

Isolates from patient 43 showed overexpression of *CDR* genes (by a factor of approximately 10 for both the second and third isolates in the series relative to the first isolate). In

the case of the second isolate (isolate 1831; fluconazole MIC = 8 μg/ml), but not the third, more resistant isolate (2183; fluconazole MIC > 128 μg/ml) of the series, this change was accompanied by a moderate increase in *ERG11* message. For all isolates from patient 43, *MDR1* expression was below the detection limit.

Differentiation between levels of expression of *CDR1* and *CDR2*. In order to discriminate between *CDR1* and *CDR2* gene expression, specific probes for *CDR1* and *CDR2* were used in Northern blots of total RNA extracted from sequential isolates from the different patients (Fig. 3). These experiments revealed that these two genes were simultaneously overexpressed in all cases in which *CDR* overexpression was observed. *CDR1* was expressed constitutively but at low levels in azole-susceptible strains and overexpressed as azole resistance developed (isolates 1907 and 2307 from patient 7, isolates 1780 and 2225 from patient 40, and isolates 1831 and 2183 from patient 43). Levels of *CDR2* mRNA were below detection limits in the same azole-susceptible isolates (with the exception of isolate 580, from patient 14, which required a fluconazole MIC of 4 μg/ml), and overexpressed in more-resistant isolates from patients 7 (isolates 1907 and 2307), 40 (isolates 1780 and 2225), and 43 (isolates 1831 and 2183). The relative *CDR2* mRNA levels were equal to those of *CDR1* in isolate 1907 (MIC = 8 μg/ml) and exceeded those of *CDR1* in highly resistant isolates 2307 and 2183 (MICs >128 μg/ml) by factors of 1.5 and 1.2, respectively. Conversely, *CDR1* mRNA levels exceeded those of *CDR2* in all other, less resistant, isolates.

DISCUSSION

Increasing reports of clinical and mycological fluconazole resistance in *C. albicans* have lead to the examination of the molecular mechanisms responsible for development of decreased fluconazole susceptibility. Under selective pressure from treatment with fluconazole, yeast cells can generate resistance through a variety of mechanisms (27, 33). Factors contributing to resistance include alterations in the target enzyme, including overexpression and point mutations, and increased efflux of drug mediated by ABC transporters and major facilitators (33). These studies have been performed in vitro, and their clinical significance is still unknown (1, 4, 10, 30). A limited number of studies support the role of these mechanisms in the development of *C. albicans* resistance in a small number of clinical isolates (11, 22, 23, 25, 31, 32). The aim of this study was to assess the frequency in which specific, known

mechanisms of resistance occurred in series of isolates with decreasing fluconazole susceptibility recovered from OPC patients followed longitudinally.

In all five patients studied, isolation of strains with decreased fluconazole susceptibility was associated with clinical resistance requiring increased fluconazole doses for clinical response. However, it should be noted that clinical response to fluconazole was achieved in all five patients with increased doses of drug. Similar dose-dependent responses have been reported with OPC and decreased fluconazole susceptibility (17, 19).

Increased mRNA levels for *ERG11*, *MDR1*, and *CDR* genes are associated with resistance (1, 24, 25, 31). In the present study, we have evaluated expression of these genes in *C. albicans* isolates from HIV-infected patients with OPC. A total of 20 isolates from five different patients were included in this study. Successive isolates from one patient were determined to represent the same strain by three molecular typing methods. Antifungal susceptibility testing of isolates recovered from successive OPC episodes in each patient indicated that progressive decrease in fluconazole susceptibility occurred during the longitudinal study. No one mechanism was predominant for the development of drug resistance in all isolates. The mode of acquisition of drug resistance by a particular mechanism or mechanisms for isolates from the different patients was complex. In a series of isolates from two patients (patients 7 and 43), overexpression of *CDR* genes (both *CDR1* and *CDR2*) was associated with the development of resistance. This increase was obvious even in the second isolate of each series, with fluconazole MICs still in the susceptible range (8 μg/ml), which could possibly indicate a tendency toward development of resistance. In one of these patients (patient 7), increased *CDR* expression was accompanied by increased *ERG11* expression, which was not the case in patient 43. In the case of patient 43, we speculate that other undetermined mechanisms of resistance could have combined with *CDR* overexpression in the third, highly resistant isolate (as compared to the second, still susceptible isolate), since *CDR* mRNA levels were similar for both isolates and could not explain the dramatic increase in fluconazole MIC observed for the third isolate.

Increased mRNA levels for *MDR1* were detected in two isolates with decreased fluconazole susceptibility (MIC = 16 μg/ml) from patient 9 compared to prior susceptible isolates. These isolates, however, remained itraconazole susceptible. An alternation in overexpression of *MDR1* and *CDR* genes was detected in isolates from another patient (patient 40), suggesting the presence of unstable phenotypes in the same strain with progression of infection due to the selective pressure of the antifungal treatment.

No clear correlation between expression of these genes and development of fluconazole resistance was observed in isolates from patient 14, where expression of *CDR* genes (mainly *CDR1*) seemed to decrease, rather than increase, in the final, less susceptible isolate. Sequencing of the *ERG11* genes from isolates from this patient revealed the absence of point mutations that could result in decreased susceptibility to azole drugs; and thus, another, yet uncharacterized, mechanism(s) may be responsible for their decreased susceptibility. Thus, for these five patients, five different patterns of resistance were detected, suggesting that *C. albicans* isolates develop resistance through a variety of molecular mechanisms and that the acquisition of fluconazole resistance is complex.

In at least one case (patient 7), cumulative changes in the expression of genes related to fluconazole resistance appeared to be responsible for decreased susceptibilities to the drug during progression of infection. However, in other cases, a single mechanism seemed to be clearly dominant and intimately associated with development of resistance (e.g., *MDR* overexpression in patient 9). Overall, these experiments confirm previous results suggesting a major role for efflux pumps in the development of resistance to azoles. In addition, others have demonstrated that *ERG11* overexpression alone does not account for high-level azole resistance (1, 25, 31). Since the present study is limited to the study of genes known to be involved in fluconazole resistance, it should be noted that other yet unrecognized mechanisms may be operational in this series of isolates and may contribute to the overall decrease in fluconazole susceptibilities (22).

In *C. albicans*, the *CDR* genes constitute a large multigene family coding for highly related proteins (2, 24, 25, 30). Some, but not all, members of this family are associated with resistance to antifungal drugs. *CDR1* and *CDR2* were the first two members of this family associated with drug resistance in *C. albicans*, and both *CDR1* and *CDR2* have been reported to play a role in fluconazole resistance (24, 25, 31). The probe used in the initial experiments to monitor expression of *CDR* genes was based on the whole sequence of *CDR1* and has been shown to cross-hybridize with other members of this gene family, including *CDR2* (24, 25, 31). Preparation of probes specific for *CDR1* and *CDR2* allowed differentiation of the expression of these two highly related genes belonging to the same multigene family. Increased mRNA levels for both *CDR1* and *CDR2* were detected in those resistant isolates found to overexpress *CDR* genes (compare Fig. 1 and Fig. 2). *CDR1* was constitutively expressed at low levels in susceptible isolates, but *CDR2* mRNA was absent in the same susceptible isolates (i.e., isolate 412 from patient 7 and isolates 1490 and 1622 from patient 40). Moreover, as previously reported (24), levels of expression of *CDR2* were greater than or equal to those of *CDR1* in highly resistant isolates. These results confirmed those of Sanglard and coworkers on the participation of both *CDR1* and *CDR2* in the development of fluconazole resistance in clinical isolates, as well as the different patterns of expression observed for both genes (24, 25).

Consistent with previous observations (23, 25, 31), overexpression of *CDR* genes resulted in decreased susceptibility to other azole drugs (i.e., patients 7 and 43), whereas increased *MDR1* levels conferred resistance to fluconazole only, as demonstrated by the susceptibility of isolates from patient 9. None of the changes observed in these isolates resulted in increased resistance to amphotericin B, which has been associated with changes in the ergosterol biosynthetic pathway (33).

Finally, fluconazole resistance may affect virulence, as has been demonstrated using some of the isolates described in the present study in a murine model of systemic candidiasis (6). However, the correlation between increased resistance and decreased virulence was not always observed and may be dependent on the underlying mechanism of resistance, thus indicating a more complex relationship between antifungal susceptibility and clinical outcome.

Overall, the present study indicates a high degree of complexity in the molecular mechanisms responsible for fluconazole resistance, with five different patterns of resistance observed in five different series of isolates with decreasing susceptibilities to this antifungal drug. Additional studies are needed to fully understand how specific mechanisms of antifungal drug resistance are induced and to determine the impact of clinical management on the development of resistance to antifungal agents.

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