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Clinical *Trichophyton rubrum* Strain Exhibiting Primary Resistance to Terbinafine

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The in vitro antifungal susceptibilities of six clinical *Trichophyton rubrum* isolates obtained sequentially from a single onychomycosis patient who failed oral terbinafine therapy (250 mg/day for 24 weeks) were determined by broth microdilution and macrodilution methodologies. Strain relatedness was examined by random amplified polymorphic DNA (RAPD) analyses. Data obtained from both broth micro- and macrodilution assays were in agreement and revealed that the six clinical isolates had greatly reduced susceptibilities to terbinafine. The MICs of terbinafine for these strains were >4 μg/ml, whereas they were <0.0002 μg/ml for the susceptible reference strains. Consistent with these findings, the minimum fungicidal concentrations (MFCs) of terbinafine for all six strains were >128 μg/ml, whereas they were 0.0002 μg/ml for the reference strain. The MIC of terbinafine for the baseline strain (cultured at the initial screening visit and before therapy was started) was already 4,000-fold higher than normal, suggesting that this is a case of primary resistance to terbinafine. The results obtained by the broth macrodilution procedure revealed that the terbinafine MICs and MFCs for sequential isolates apparently increased during the course of therapy. RAPD analyses did not reveal any differences between the isolates. The terbinafine-resistant isolates exhibited normal susceptibilities to clinically available antimycotics including itraconazole, fluconazole, and griseofulvin. However, these isolates were fully cross resistant to several other known squalene epoxidase inhibitors, including naftifine, butenafine, tolnaftate, and tolciclate, suggesting a target-specific mechanism of resistance. This is the first confirmed report of terbinafine resistance in dermatophytes.

In recent years, the incidence of infections caused by dermatophytes and other fungi has increased considerably (2, 13), especially among pediatric and geriatric populations. *Trichophyton rubrum*, among other dermatophytes, is a major causative agent for superficial dermatomycoses like onychomycosis and tinea pedis (19, 26) and is known to account for as many as 69.5% of all dermatophyte infections (4, 5, 18). Common therapeutic strategies based on the use of terbinafine and griseofulvin are generally considered effective (6, 7, 11). Terbinafine is very widely used, both orally and topically, in the therapy of dermatophyte infections, with no reports of resistance against this allylamine antifungal (16, 24).

Infections due to *T. rubrum* are often associated with frequent relapses following cessation of antifungal therapy, but the relapses have so far not been related to resistance to the antifungals (15). Although acquired resistance to terbinafine has not been reported for any pathogen, routine antifungal susceptibility testing is not carried out in the case of dermatophyte infections. Thus, it is likely that resistance occurs but is not detected. This is likely to be true in the case of onychomycosis, which involves prolonged therapy, relatively low drug concentrations, and a significant proportion of clinical failures. In a previous study, we evaluated a group of patients from a multicenter study designed to assess the efficacy of terbinafine in patients with known cases of onychomycosis. Nail samples from this patient group were colonized with *T. rubrum* throughout the terbinafine therapy. Antifungal susceptibility testing and random amplified polymorphic DNA (RAPD) analyses revealed that the failure of patients to clear *T. rubrum* is not related to the development of resistance to the drug and that failure of terbinafine therapy may be dependent on host-related factors (2).

However, in one patient belonging to the same cohort, all isolates including baseline isolates were found to have greatly reduced susceptibilities to terbinafine in vitro. Here we describe the characterization of this first known series of *T. rubrum* strains with intrinsic resistance to terbinafine, as determined by two susceptibility testing methods. We also demonstrate that the susceptibilities of these strains to other antifungals are unaffected. In contrast, the terbinafine-resistant isolates exhibited marked cross-reactivities to inhibitors of squalene epoxidase, indicating that a common mechanism of resistance to squalene epoxidase inhibitors could be operative in *T. rubrum* strains.

MATERIALS AND METHODS

Fungal cultures. *T. rubrum* isolates were cultured from a selected group of 30 patients who failed oral terbinafine therapy (2). The 30 patients were from among 1,432 subjects with onychomycosis of the toenail who were enrolled in a multicenter open-label study assessing the efficacy and safety of a terbinafine treatment regimen of 250 mg/day for 12 weeks. The patients had received no systemic or topical antifungal treatment within the 3 months before the beginning of this study. After 12 weeks of treatment, patients showing less than 25% nail improvement were continued on the treatment for an additional 6 weeks. Patients were reassessed at week 18; if less than 25% improvement was seen, an additional 6 weeks of treatment was given. The maximum treatment time was 24 weeks. Follow-up was done at approximately 30, 36, 48, and 72 weeks. No other systemic or topical antifungal agents were permitted during the terbinafine treat-

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ment. The group of 30 patients selected for this investigation was chosen on the basis of the following criteria: (i) toenail scrapings were culture positive for *T. rubrum* at the initial medical visit, (ii) nail scrapings remained culture positive for *T. rubrum* at one or more subsequent visits during the study, and (iii) nail scrapings were culture positive for *T. rubrum* at the end of the study. A total of 104 sequential isolates were obtained from these patients. All isolates were confirmed to be *T. rubrum* at the Center for Medical Mycology, University Hospitals of Cleveland, Cleveland, Ohio. The identification protocol for *T. rubrum* required equal growth on Trichophyton T1 and T4 agars (Becton Dickinson), negative urea fixation and hair perforation tests, and microscopic visualization of elliptical conidia along the hyphae. Isolates were subcultured onto potato dextrose agar in triplicate and stored at −80°C until antifungal susceptibility testing was performed.

Antifungal drugs. The following antifungal agents were used in this study: terbinafine, naftifine, and butenafine (all from Novartis, East Hanover, N.J.), fluconazole (Pfizer Inc., New York, N.Y.), tolnaftate (H. Lundbeck Co., Copenhagen, Denmark), tolciclate (Montedison Co., Milan, Italy), itraconazole (Janssen Research Foundation, Beerse, Belgium), and griseofulvin (Sigma Chemical Co., St. Louis, Mo.).

Antifungal susceptibility testing. (i) Microdilution method. The broth microdilution assay for antifungal susceptibility testing of dermatophytes was previously developed as a modification of the National Committee for Clinical Laboratory Standards (NCCLS) M27-A method (17, 22). RPMI 1640 medium (American Biorganics Inc., Niagara Falls, N.Y.) with L-glutamine but without sodium bicarbonate and buffered at pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid, monosodium salt, was the medium used for broth microdilution susceptibility testing. Serial twofold dilutions were prepared by the NCCLS M27-A method (21). The final concentrations of fluconazole and griseofulvin used were 0.13 to 64.0 μg/ml, while those of itraconazole and terbinafine were 0.06 to 32.0 μg/ml. A standardized inoculum was prepared by counting the microconidia microscopically. Cultures were grown on oatmeal cereal agar slants for 4 days at 35°C to produce conidia. Sterile normal saline (85%) was added to the agar slant, and the cultures were gently swabbed with a cotton-tipped applicator to dislodge the conidia from the hyphal mat. The suspension was transferred to a sterile centrifuge tube, and the volume was adjusted to 5 ml with sterile normal saline. The resulting suspension was counted on a hemacytometer and was diluted in RPMI 1640 medium to the desired concentration. Microdilution plates were set up in accordance with the NCCLS M27-A reference method; the exception was the inoculum preparation, which was set up as described above. Column 1 was filled with 200 μl of medium to serve as a sterility control. Columns 2 through 11 were filled with 100 μl of the inoculum and 100 μl of the serially diluted antifungal agent. Column 12 was filled with 200 μl of the inoculum and served as a growth control. The microdilution plates were incubated at 35°C and were read visually after 4 days of incubation. The MIC was defined as the concentration at which the growth of the organism was inhibited 80% compared with the growth in the control well. All isolates were run in duplicate, and the results were read visually.

(ii) Macrodilution method. The antifungal susceptibilities of the isolates were also determined by a broth macrodilution assay according to the NCCLS M27-A protocol (21). Inocula for the assays were prepared from stocks frozen at −80°C by dilution in RPMI 1640 medium (Cellgro Mediatech, Inc., Herndon, Va.) to give a final viable cell count of 2.5×10^3 to 3×10^3 CFU/ml. Each assay was performed with a duplicate series of drug dilutions. The tubes were incubated for 7 days at 30°C. The MIC was defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth. *T. rubrum* strains ATCC 18759 (American Type Culture Collection, Manassas, Va.) and NFI 1895 (a clinical isolate) served as reference control strains in all susceptibility assays. All experiments were repeated at least once.

MFC determination. For determination of the minimum fungicidal concentration (MFC), 100-μl aliquots were removed from the assay tubes or wells showing no visible growth at the end of incubation and streaked onto Sabouraud dextrose agar plates. The plates were incubated at 30°C for 7 days. The MFC was defined as the lowest drug concentration at which no visible fungal growth or colonies developed.

Fungal DNA isolation. Genomic DNA was isolated from each isolate and the reference strains by a previously described method (2). Briefly, filtered mycelia (1 g) were suspended in 1.25 ml of 1% (wt/vol) cetyltrimethylammonium bromide (Sigma Chemical Co.) in a sterile 15-ml tube, and the mixture was incubated for 1 h at 65°C. An equal volume of phenol-chloroform-isoamyl alcohol (24:24:1; vol/vol/vol) was then added to this mixture. The suspension was vortexed with sterile glass beads (diameter, 0.5 mm) for 20 min with an automated multitube vortexer (VWR Scientific Products, Philadelphia, Pa.) and centrifuged at 2,400 × *g* for 10 min. The supernatant was extracted with an equal volume of phenol-chloroform (24:1; vol/vol). The final supernatant was precipitated with 70 μl of 10 M ammonium acetate and 3 ml of ethanol, and the mixture was centrifuged at 12,500 rpm for 5 min. The genomic DNA pellet was washed with 70% ethanol, air dried, resuspended in Tris-EDTA (containing RNase), and diluted to 20 ng/μl.

TABLE 1. MICs and MFCs of terbinafine for *T. rubrum* isolates

Visit no.[a]	Isolate	Macrodilution method		Microdilution method	
		MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
0	NFI 5146[b]	4	>128	4	128
2	NFI 5147	4	>128	4	128
3	NFI 5148	8	>128	4	128
4	NFI 5149	32	>128	4	128
7	NFI 5150	>128	>128	4	128
8	NFI 5151	>128	>128	4	128
	NFI 1895[c]	<0.0002	0.0002	<0.0002	0.0002
	ATCC 18759[c]	<0.0002	0.0002	<0.0002	0.0002

[a] Visit 0 was for screening and culture; therapy lasting 24 weeks started at visit 1, and subsequent visits were at 6-week intervals.
[b] Baseline isolate.
[c] Reference strains.

RAPD analysis. RAPD analysis was performed as described previously (2) in a total reaction volume of 100 μl containing 5 μl of genomic DNA (100 ng), 2.5 μl of deoxynucleoside triphosphates (10 mM each deoxynucleoside triphosphate), 8 U of *Taq* DNA polymerase (Gibco BRL), 10 μl of 10× PCR buffer, and 10 pmol of random primer OPK-17 (Biosynthesis Inc., Lewisville, Tex.) per μl. This primer was selected on the basis of the results of previous studies (2, 27). The reaction was performed in a GeneAmp PCR System 9600 (Perkin-Elmer) under the following conditions: 1 cycle of denaturation at 94°C for 1 min; 45 cycles of denaturation at 94°C for 20 s, annealing at 35°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

RESULTS

Detection of *T. rubrum* strains exhibiting reduced susceptibilities to terbinafine. Six sequential *T. rubrum* isolates (isolates NFI 5146 through NFI 5151) were cultured from a patient enrolled in a clinical trial (see above) for the treatment of onychomycosis. Testing of the terbinafine susceptibilities of these isolates by a microdilution assay revealed that all six isolates exhibited greatly reduced susceptibilities (MIC, 4 μg/ml) compared to those of the reference strains (MICs, <0.0002 μg/ml for both ATCC 18759 and NFI 1895).

A trend of increasing terbinafine MICs for sequential isolates is shown. To confirm that the high terbinafine MICs were independent of the antifungal microdilution susceptibility method used, we reassayed the *T. rubrum* isolates for their terbinafine susceptibilities using a broth macrodilution method by the NCCLS M27-A protocol (21) and compared the data with those obtained by the broth microdilution assay. As shown in Table 1, both the microdilution and the macrodilution assays revealed that the isolates were resistant to terbinafine. Interestingly, unlike the microdilution method, which showed that the MICs at which 80% of isolates are inhibited were 4 μg/ml for the sequential strains, irrespective of the number of patient visits, the macrodilution method showed that the MICs gradually increased with each visit, from an MIC of 4 μg/ml for the isolate from the first patient visit to one of >128 μg/ml for the isolate from the eighth patient visit. The MFCs determined for

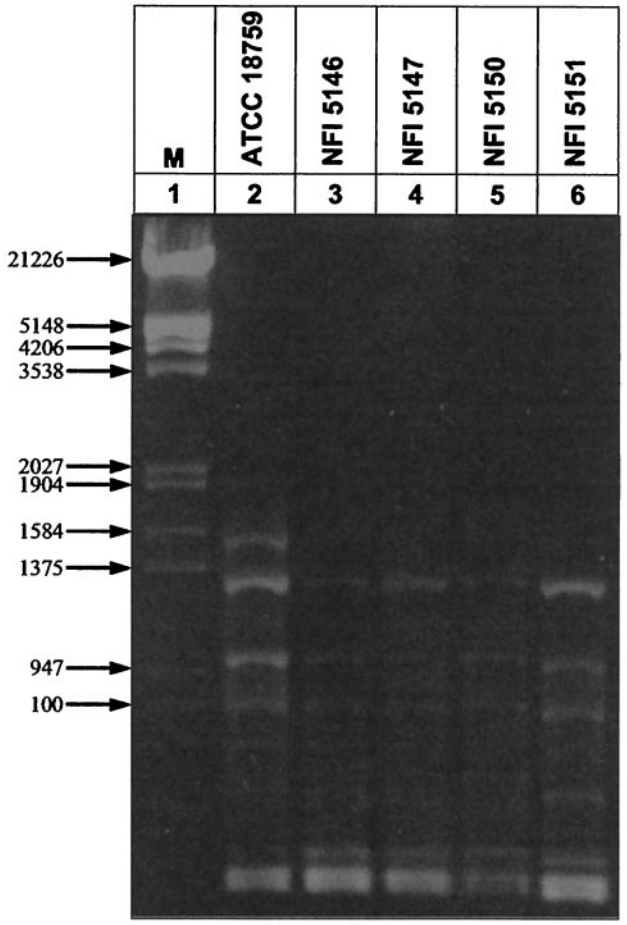

FIG. 1. RAPD analysis of terbinafine-resistant sequential *T. rubrum* isolates. Lane 1 (M), molecular size marker (0.1 to 21.2 kbp); lane 2, ATCC 18759 (reference strain); lane 3, NFI 5146 (baseline strain); lane 4, NFI 5147 (visit 2); lane 5, NFI 5150 (visit 7); lane 6, NFI 5151 (visit 8). The sizes of the markers (in base pairs) are indicated by the arrows on the left.

the cells by both the microdilution and the macrodilution methods were elevated for all sequential isolates compared to those for susceptible reference strain NFI 1895. The MFCs did not change for isolates from sequential visits (Table 1). Thus, the macrodilution assay data confirmed that the isolates had primary resistance and extend the observation to suggest that exposure to the drug resulted in increased resistance.

RAPD analysis revealed that the sequential *T. rubrum* isolates are represented by a single strain. To determine whether the sequential isolates represented a single strain or different strains indicating new infections at each visit, we performed RAPD analysis to detect differences in the amplification patterns among the isolates. Significant differences in banding patterns would suggest that the infections during the course of terbinafine therapy were caused by nonidentical strains. As shown in Fig. 1, *T. rubrum* NFI 5146 (the baseline isolate, obtained from the patient before treatment was initiated) and *T. rubrum* NFI 5147, NFI 5150, and NFI 5151 (obtained from the same patient during visits 2, 7, and 8, respectively) had similar banding patterns. The reference strain (ATCC 18759) had a similar DNA profile, differing from those of the clinical isolates by only one band (Fig. 1, lane 2). This difference is not considered significant (3, 23). Isolate NFI 1895 (the reference strain) and isolates NFI 5148 and NFI 5149 (obtained during visits 3 and 4, respectively) also had banding patterns similar to those of the other sequential isolates (data not shown). Therefore, the isolates obtained at sequential visits represent a single strain of *T. rubrum*.

TABLE 2. MICs of azoles and griseofulvin for sequential *T. rubrum* isolates

Visit no.	Isolate	MIC (μg/ml)[a]					
		Macrodilution method			Microdilution method		
		ITRA	FLU	GRISEO	ITRA	FLU	GRISEO
0	NFI 5146[b]	0.03	2	4	<0.06	0.50	0.25
2	NFI 5147	0.03	0.5	1	<0.06	0.25	0.125
3	NFI 5148	0.03	1	2	<0.06	0.50	0.25
4	NFI 5149	0.03	1	2	<0.06	0.50	0.25
7	NFI 5150	0.03	4	2	<0.06	0.50	0.50
8	NFI 5151	0.03	1	2	<0.06	0.50	0.50

[a] ITRA, itraconazole; FLU, fluconazole; GRISEO, griseofulvin.
[b] Baseline isolate.

Terbinafine-resistant *T. rubrum* isolates do not develop cross-resistance to azoles and griseofulvin. In an attempt to gain further insight into the extent and spectrum of drug resistance in the sequential *T. rubrum* isolates, their susceptibilities to clinically used oral antifungals including itraconazole and griseofulvin were determined by both the microdilution and the macrodilution methods. As shown in Table 2, all isolates tested exhibited normal susceptibilities to the two azoles and griseofulvin (Table 2). Moreover, the MICs of all three antifungal agents for the isolates obtained during sequential visits remained the same. Thus, our results indicate that the *T. rubrum* strains isolated are specifically resistant to terbinafine.

Terbinafine-resistant *T. rubrum* isolates are fully cross resistant to other squalene epoxidase inhibitors. The target of terbinafine in fungal cells is the enzyme squalene epoxidase, which is involved in the conversion of squalene to squalene epoxide, a precursor in the biosynthesis of ergosterol (10, 24). To determine whether the terbinafine-resistant strains isolated are cross resistant to other squalene epoxidase inhibitors, the susceptibilities of one isolate obtained at visit 2 (NFI 5147) and a second isolate collected at visit 7 (NFI 5150) to known squalene epoxidase inhibitors (naftifine [an allylamine], butenafine [a benzylamine], and tolnaftate and tolciclate [thiocarbamates]) were determined (10). The susceptibilities of the two reference strains, strains ATCC 18759 and NFI 1895, to the same inhibitors were tested simultaneously. As indicated in Table 3, the MICs of the squalene epoxidase inhibitors tested were high for the two terbinafine-resistant isolates, whereas the reference strains were highly susceptible to these inhibitors (Table 3). These studies show that the terbinafine-resistant isolates are fully cross resistant to other squalene epoxidase inhibitors.

DISCUSSION

Close examination of clinical data revealed that the patient whose isolates were evaluated in the present study was a 31-year-old male who was fully compliant with the treatment

TABLE 3. MICs and MFCs of squalene epoxidase inhibitors for three *T. rubrum* isolates

Drug and isolate[a]	Macrodilution method		Microdilution method	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Naftifine				
NFI 5147	2	>128	4	>128
NFI 5150	4	>128	4	>128
NFI 1895	0.004	0.016	0.008	0.03
Butenafine				
NFI 5147	>128	>128	64	>128
NFI 5150	>128	>128	>128	>128
NFI 1895	0.0005	0.016	0.0002	0.008
Tolnaftate				
NFI 5147	8	>128	0.25	>128
NFI 5150	>128	>128	>128	>128
NFI 1895	0.0002	0.004	0.0002	0.004
Tolciclate				
NFI 5147	>128	>128	16	>128
NFI 5150	>128	>128	>128	>128
NFI 1895	0.0005	0.06	0.0002	0.03

[a] NFI 5147 was isolated from the patient during visit 2, and NFI 5150 was isolated from the same patient during visit 7. NFI 1895 is a reference strain of *T. rubrum*.

regimen, had no significant medical condition, was not taking any other medications during terbinafine therapy, and had received no prior antifungal therapy. The terbinafine MICs for the six *T. rubrum* clinical isolates investigated in this study were confirmed to be approximately 3 orders of magnitude higher than normal. The fact that the MIC for the baseline isolate (NFI 5146) was approximately 4,000-fold higher than normal suggests that resistance did not develop as a result of continued therapy. Therefore, this is most likely an example of primary resistance to terbinafine, confirmed by using two antifungal susceptibility testing methods (microdilution and macrodilution techniques). Interestingly, the results obtained by the macrodilution assay showed that the MICs increased during the course of treatment, implying that resistance was being exacerbated by prolonged exposure to the drug. In contrast, there were no differences in the susceptibilities of the six isolates when the microdilution procedure was used. Although the significance of this discrepancy is not clear and could be attributed to differences between the two methodologies, the results from the broth macrodilution assay support the possibility that long-term exposure of an already resistant pathogen to terbinafine leads to secondary changes manifesting as increased resistance.

Our findings show that the terbinafine-resistant isolates identified in this study do not develop resistance to azoles and griseofulvin. These classes of antifungals have different modes of action. Azoles (fluconazole and itraconazole) inhibit fungal growth by blocking the cytochrome P450-type 14α-demethylase enzyme involved in ergosterol biosynthesis (12, 14, 25), while griseofulvin affects cell division and hyphal formation by disrupting spindle and cytoplasmic microtubule function (1, 8, 9). Terbinafine specifically inhibits squalene epoxidase, blocking the synthesis of squalene epoxide from squalene and resulting in the accumulation of toxic levels of squalene and decreased levels of ergosterol production (20, 24). High intracellular squalene concentrations are believed to interfere with fungal membrane function and cell wall synthesis (24). Squalene epoxidase is not an enzyme of the cytochrome P450 type; therefore, potential inhibition of this class of enzymes can be avoided (24). Thus, the observation that no cross-resistance to azoles and griseofulvin was observed in the terbinafine-resistant isolates is not surprising.

Our data showed that although the terbinafine-resistant isolates were fully susceptible to azoles and griseofulvin, they were resistant to a number of squalene epoxidase inhibitors (naftifine, butenafine, tolnaftate, and tolciclate). This observation indicates that these isolates have a common mechanism of resistance involving squalene epoxidase. The finding that the terbinafine-resistant isolates were fully cross resistant to several classes of squalene epoxidase inhibitors but displayed normal sensitivities to azoles and other antifungals suggests that resistance may be the result of a mutation in the gene encoding squalene epoxidase in *T. rubrum*. Further biochemical and molecular biological studies will be required to fully elucidate this point. The low frequency of detection of dermatophytes exhibiting terbinafine resistance suggests that this is not a common cause of therapeutic failure. However, the findings of the present study demonstrate that terbinafine-resistant dermatophyte strains can exist and may, on occasion, be the reason for the lack of clinical response to the drug.

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