



Clinical Isolate of a Multi-Antifungal-Resistant *Trichophyton rubrum*

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ABSTRACT The multi-antifungal drug-resistant strain (NUBS21012) of *Trichophyton rubrum* was isolated from a patient with recurrent tinea corporis. The resistant strain encoded Phe at codon 393 instead of Leu (L393F) in the squalene epoxidase (*SQLE*) gene. The expression of genes encoding ATP-binding cassette transporter proteins increased in the strain compared to that of other strains. This result provides evidence that ATP-binding cassette transporter proteins are closely associated with azole resistance.

KEYWORDS deletion mutation, onychomycosis, squalene epoxidase, terbinafine, *Trichophyton rubrum*, azole, dermatophytes, dermatophytoses

Dermatophytoses are common fungal skin infections in humans (1–3). More than 60% to 70% of the infections are caused by dermatophytes, predominantly *Trichophyton rubrum* (50%) (3). Terbinafine (TRF) has been used as a treatment for dermatophytosis; however, TRF-resistant strains have recently been isolated from human patients around the world (4–7). Almost all TRF-resistant strains encode F397L or L393F amino acid substitutions in the squalene epoxidase (*SQLE*) gene (4–7).

In this study, we isolated a TRF-resistant strain of *T. rubrum* from a patient with recurrent tinea corporis. The patient had been treated with TRF and had been switched to ravuconazole (RVZ) for 1 year and 6 months. However, the treatment did not cure the tinea corporis completely, and the systemic skin lesions progressed to fungal granuloma. We show that a susceptible strain can become resistant to azole compounds after overmedication. Switching from TRF to azoles in a case of TRF resistance can generate a strain resistant to TRF and azoles.

Case report. A 60-year-old female with Cushing's syndrome had tinea corporis on the face, back, chest, abdomen, buttocks, crotch, and both thighs. She had dermatophytosis for more than 10 years and experienced chronic and recurrent tinea corporis that was treated with TRF hydrochloride cream. Subsequently, in 2020, she experienced tinea corporis on the back that was treated with oral and topical TRF, but the treatment failed. She was then treated with oral fosravuconazole (F-RVZ) and topical luliconazole (Table 1). The first isolate (strain NUBS21011) was obtained from a tinea corporis lesion on the face on 11 March 2020; a L393F mutation was detected in the *SQLE* gene of this TRF-resistant isolate (Table 1) (8). In this study, an additional strain (NUBS21012) was later isolated from a recurrent tinea corporis lesion on the face on 6 September 2021 (after 1 year and 6 months of oral F-RVZ treatment) (Table 1).

To assess the susceptibility of the isolates to amphotericin B (AMB), griseofulvin (GRF), TRF, itraconazole (ITZ), voriconazole (VRZ), and RVZ, the broth microdilution assay was performed based on the Clinical and Laboratory Standards Institute (CLSI)

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TABLE 1 Strains, MICs of antifungal drugs, and mutation sites in the *SQLE* and *TERG* genes^a

Strain	Origin	Isolation date	Treatment history (administration periods)	MICs ($\mu\text{g/mL}$)						Amino acid substitution in <i>SQLE</i> ^b	Amino acid substitution in <i>TERG</i> ^c
				AMB	GRF	ITZ	VRZ	RVZ	TRF		
NUBS21011	Tinea corporis	11 March 2020	Oral and topical TRF (1 yr)	1	0.25	0.5	0.25	<0.03	32	L393F	Not detected
NUBS21012	Tinea corporis (fungal granuloma)	6 September 2021	Oral fosravuconazole and topical LCZ (1 yr 6 mo)	0.5	8	16	8	16	32	L393F	Y136H
N45	Tinea unguium	8 June 2020	Unknown	NT	NT	<0.03	NT	<0.03	<0.03	NT	NT
N46	Tinea corporis	2 June 2020	Unknown	NT	NT	<0.03	NT	<0.03	<0.03	NT	NT

^aNUBS, Nihon University College of Bioresource Sciences; AMB, amphotericin B; GRF, griseofulvin; TRF, terbinafine; ITZ, itraconazole; VRZ, voriconazole; RVZ, ravuconazole; LCZ, luliconazole; *SQLE*, squalene epoxidase; *TERG*, cytochrome P450 51; NT, not tested.

^bAmino acid sequences encoded by the *SQLE* gene in *T. rubrum* CBS 118892 (GenBank accession no. [XM_003233797](#)).

^cAmino acid sequences were compared to those encoded by the *Trichophyton rubrum* CBS 118892 cytochrome P450 51 (*TERG_05717*) mRNA, complete coding DNA sequence (CDS) (GenBank accession no. [XM_003236932.1](#)).

M38-A2 guidelines with modifications, as previously described (9, 10). The MICs against NUBS21011 and NUBS21012 are listed in Table 1.

To sequence the hot spot mutations in *SQLE* of the isolates, primers were prepared based on the conserved sequence of the *T. rubrum SQLE* (see Table S1 in the supplemental material) (8). PCR amplification and sequencing analysis were conducted as reported previously (8). The *SQLE* gene of the TRF-resistant strains (NUBS21011 and NUBS21012) encoded the L393F mutation (Table 1).

To mRNA sequence the azole target cytochrome P450 51 genes of the clinical isolates (NUBS21011 and NUBS21012), primers were prepared based on the conserved sequence of the *Trichophyton rubrum* CBS 118892 cytochrome P450 51 (GenBank accession no. [XM_003236932.1](#)) (Table S1). The cDNA synthesis process and sequencing analysis were done according to a previous report (8). Sequences of the cytochrome P450 51 genes in NUBS21011 and NUBS21012 were deduced to be 1,584 bp long, and they encoded a protein of 528 amino acids. The homology for the gene of NUBS21011 was 100% identical to that of the *T. rubrum* CBS 118892 cytochrome P450 51. In addition, the Y136H mutation was identified in the gene of NUBS21012 (Table 1).

To analyze the expression levels of the ABC transporter family (*PDR1*, *MDR2*, *MDR3*, and *MDR4*) genes, we performed real-time quantitative PCR (RT-qPCR) as follows. Strains were cultured for 4 days at 28°C in Sabouraud's dextrose broth (SDB; 1% peptone and 2% dextrose). ITZ exposure was carried out according to the previously reported method of Monod et al., who added ITZ to evaluate the expression of genes in four *Trichophyton* species (11). All strains were cultured for 3 h at 28°C in SDB with 0.05 $\mu\text{g/mL}$ ITZ. The cDNA synthesis process, RT-qPCR (Thermal Cycler Dice; TaKaRa), and analysis were done as described previously (12). The primer pairs of *PDR1*, *MDR2*, *MDR3*, and *MDR4* were used according to previous reports (11, 13). The *MDR3* and *MDR4* transcript levels were 10 to 100 times higher in NUBS21012 than in the TRF-resistant strain NUBS21011 and antifungal-susceptible strains N45 and N46 (Fig. 1).

It is speculated that the RVZ treatment induced the additional multidrug resistance in the TRF-resistant strain. Therefore, the long-term administration of antifungal agents may lead to the development of multidrug resistance.

Several single-point mutations in the *SQLE* gene have been identified in TRF-resistant strains of *T. rubrum* isolated from tinea pedis and tinea unguium around the world, including I121M, V237I, L393F, L393S, F397L, F415S, H440Y, and F484Y in *SQLE* (4–7). Previously, we performed an epidemiological study of the prevalence of TRF-resistant *T. rubrum* in Japanese patients (8). All TRF-resistant strains carried the L393F mutation in the *SQLE* gene (8). Therefore, TRF resistance seems to mainly result from single-point mutations in the *SQLE* gene.

Resistance to azoles can be linked to the overexpression of genes encoding ABC transporter proteins, including the *PDR1*, *MDR2*, *MDR3*, and *MDR4* genes in dermatophytes (11, 12, 14). In this study, the *MDR3* and *MDR4* transcript levels were 10 to 100 times higher in NUBS21012 than in the TRF-resistant strain (NUBS21011) and antifungal-susceptible strains (N45 and N46). The transcript levels of *MDR2* and *MDR3* were also increased by the ITZ addition stimulation (11, 13). Our result provides evidence

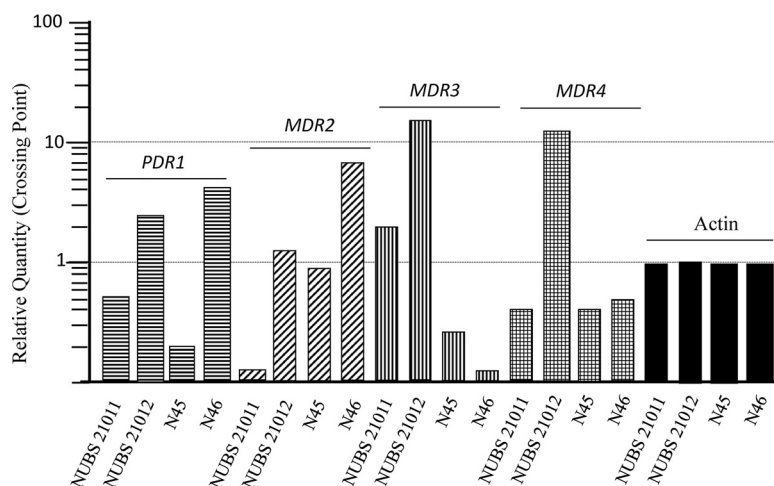


FIG 1 Transcript levels of the actin-encoding gene, *PDR1*, *MDR1*, *MDR2*, *MDR3*, and *MDR4* in TRF-resistant strains (NUBS21011 and NUBS21012) and TRF-susceptible strains (N45 and N46) of *T. rubrum*. All strains were cultured in SDB containing 0.05 $\mu\text{g/mL}$ ITZ for 3 h at 28°C. Expression levels of all genes were normalized to that of the actin-encoding gene and are expressed as a relative quantity.

that ABC transporter proteins of *MDR3* and *MDR4* are closely associated with azole resistance. In addition, the multidrug-resistant strain NUBS21012 was also less susceptible to GRF (MIC, 8 $\mu\text{g/mL}$) than that of NUBS21011 (MIC, 0.25 $\mu\text{g/mL}$) (Table 1). *MDR3* and *MDR4* also might be related to resistance for GRF.

Moreover, Y136H was identified in the P450 51 gene of NUBS21012, but it was not detected in the first isolated strain (NUBS21011). Thus, it has not been reported to demonstrate the gene mutation and azole drug resistance. However, we could not clarify in this study whether this point mutation is related to the drug resistance mechanism.

Data availability. The sequences determined in this study have been deposited into the GenBank database (GenBank accession no. [LC662767](https://www.ncbi.nlm.nih.gov/nuccore/LC662767) and [LC662768](https://www.ncbi.nlm.nih.gov/nuccore/LC662768)).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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