Short Report

ATP-binding Cassette (ABC) Transporter Proteins in Highly Terbinafine-resistant Strains of *Trichophyton indotineae* (Former Species Name: *Trichophyton interdigitale*)

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**ABSTRACT**

To clarify the terbinafine (TRF) resistance mechanism in highly TRF-resistant [minimum inhibitory concentration (MIC) > 32 µg/mL] strains of *Trichophyton indotineae* (NUBS19006 and NUBS19007), we investigated the expression of squalene epoxidase (*SQLE*), pleiotropic drug resistance 1 (*PDR1*), multidrug resistance 2 (*MDR2*), and *MDR4* genes by real-time quantitative PCR analysis, given the known interaction of the corresponding proteins with antifungals and the efflux blocker tacrolimus (FK506). *SQLE*, *PDR1*, *MDR2*, and *MDR4* transcript levels in TRF-resistant strains cultured in SDB were not significantly higher than those of the respective genes in TRF-susceptible strains (1 and 10). By contrast, *PDR1*, *MDR2*, and *MDR4* transcript levels in TRF-resistant and TRF-susceptible strains cultured in SDB containing 10 µg/mL TRF were 5-100 times higher than those of the respective genes in strains grown in the absence of TRF. However, no differences in *PDR1*, *MDR2*, and *MDR4* transcript levels were found between TRF-resistant (NUBS19006 and NUBS19007) and TRF-susceptible strains cultured in SDB containing 10 µg/mL TRF. The interaction between TRF and FK506 on antifungal activity was not detected in TRF-resistant strains. These results indicate that ATP-dependent efflux pumps do not confer TRF-resistance mechanisms in TRF-resistant strains.

**Key words**: ATP-binding cassette (ABC) transporter proteins, resistance, squalene epoxidase, terbinafine, *Trichophyton indotineae*

*Trichophyton interdigitale* and *Trichophyton rubrum* are anthropophilic species that are frequently isolated from tinea unguium and tinea pedis worldwide. Itraconazole (ITZ) and terbinafine (TRF) have been used in the treatment of infections by these causative pathogens for more than 20 years.

Recently, *T. interdigitale* and *T. rubrum* isolates were reported to exhibit TRF resistance. These resistant strains have missense mutations (Leu393Ser/Phe or Phe397Leu) in the squalene epoxidase-encoding gene, *SQLE*. *Trichophyton indotineae*, a species newly designated in 2020 independent of *T. interdigitale*, comprises highly TRF-resistant dermatophytoses that are epidemic in North India. *T. indotineae* harbors a missense mutation (Phe397Leu) in *SQLE*. This high level of TRF resistance (minimum inhibitory concentration (MIC) > 32 µg/mL) in Indian dermatophyte isolates seems to be driving an ongoing outbreak of dermatophytoses in countries other than India.

*T. rubrum* isolated from human dermatophytosis was also reported to exhibit TRF resistance. Antifungal resistance of dermatophytes frequently results from overexpression of genes encoding ATP-binding cassette (ABC) transporter proteins, including pleiotropic drug resistance 1 (*PDR1*), multidrug resistance 2 (*MDR2*), and *MDR4* genes.

We speculated that highly TRF-resistant *T. interdigitale* strains have other antifungal resistance mechanisms. The overexpression mechanism results in squalene epoxidase accumulation and activation of squalene epoxidase efflux pumps, whose activities in turn prevent TRF accumulation in dermatophyte cells.

Tacrolimus (FK506) was previously shown to reverse multidrug resistance in multiple types of eukaryotic cells, an effect that is mediated via blockade of ATP-dependent efflux pumps, including human P-glycoprotein, *Candida albicans* Cdr1p/Cdr2p, and *Candida krusei* Abc1p. Synergistic effects...
of FK506 in combination with azoles have been detected in all tested azole-resistant *Candida* strains, verifying that efflux pumps contribute to antifungal drug resistance in this genus. We speculated that FK506 could enhance fungicidal activity for highly TRF-resistant *T. interdigitale* by blocking overexpression of genes encoding ATP-dependent efflux pumps.

In the present study, we investigated the expression of *SQLE*, *PDR1*, *MDR2*, and *MDR4* in *T. indotineae*. Moreover, we examined the interaction of TRF and the efflux blocker FK506 on the expression of these genes in TRF-resistant strains.

In 2019, two highly TRF-resistant (MIC > 32 µg/mL) *T. indotineae* clinical isolates (NUBS19006 and NUBS19007) were obtained from two human cases of tinea corporis in Shinjuku, Tokyo and Urayasu, Chiba in Japan. The patients had traveled regularly and frequently between Japan and Nepal/India and were treated with TRF in Japanese hospitals; however, no response was seen. After antifungal susceptibility testing, the patients were cured with itraconazole (ITZ) or fosravuconazole (F-RVZ) administration (Table 1). Genetic analyses showed that these strains contained a missense mutation (Phe397Leu) in *SQLE*.

Two TRF-susceptible (MIC < 0.03 µg/mL) *T. interdigitale* clinical isolates (strains 1 and 10) were also evaluated in this study (Table 1). All isolates were identified based on morphological characteristics and analysis of internal transcribed spacer region sequences.

To analyze expression levels of the *SQLE* and ABC transporter family (*PDR1*, *MDR2*, and *MDR4*) genes, we used real-time quantitative PCR (RT-qPCR) as follows. Strains were cultured for 4 days at 28°C in Sabouraud dextrose broth (SDB; 1% peptone and 2% dextrose). TRF exposure was carried out using the previously reported method of Martins et al., who added TRF to evaluate the expression of genes in four *Trichophyton* species. All strains were cultured for 1 h at 28°C in SDB with and without 10 µg/mL TRF.

Samples of fungal cells cultured in SDB were collected by centrifugation at 1600 × *g* for 5 min, and the resulting pellet was flash-frozen in liquid nitrogen and ground to homogeneity. Total RNA was extracted from approximately 200 mg of each sample using the RNaseasy total RNA kit (QIAGEN, Tokyo, Japan). Reverse transcription of poly(A) tail RNA was performed using the QuantiTect Reverse Transcriptase kit (QIAGEN). Aliquots (100 ng) of the resulting cDNA samples were then amplified by RT-qPCR (Thermal Cycler Dice; Takara, Kyoto, Japan) and analyzed as described previously.

Primer pairs used to amplify fragments (sizes as indicated) of gene ORFs were as follows: *SQLE* (137 bp), forward primer *SQLE-RT1S* (5′-TTCCCCACCGATGGCCACAAG-3′) and reverse primer *SQLE-RT1R* (5′-CATCTCCCTGTGTCGCTGGAT-3′), which corresponded to nucleotides 367-386 and 523-504 (respectively) of *T. indotineae* NUBS19006 *SQLE* gene (complete cds: GenBank accession no. LC510258); *PDR1* (130 bp), forward primer 5′-CCTAATGGCCCTCTGAGT-3′ and reverse primer 5′-AAATGCCAGCCTGTTCTGT-3′; *MDR2* (83 bp), forward primer 5′-TGACTCTGAATCCGAAAAGG-3′ and reverse primer 5′-GTCGGTGAGCAACAGGAAATA-3′; *MDR4* (113 bp), forward primer 5′-GGAAATTGAGCTTCGAGACG-3′ and reverse primer 5′-TTCCAACGATAGCAGTGTGTC-3′; and the actin-encoding gene (89 bp), forward primer 5′-CCATCTCATCCCGTGTGTTG-3′.

Experimental design and data analysis (∆∆*Ct* method) were carried out by relative quantitation using the Thermal Cycler Dice Real Time System Software Ver. 5.1C (Takara). Basal expression levels were estimated based on normalization to the level of the actin-encoding transcript in RNA from cultures, with the assumption that equivalent total RNA input and observed equal PCR efficiency would provide comparable *Ct* (threshold cycle) values. Gene expression levels were normalized to that of the actin-encoding gene, and values were compared in strains cultured in SDB with and without 10 µg/mL TRF. The RT-qPCR experiments were performed in duplicate.

No significant differences were found in basal actin and *SQLE* expression levels among strains cultured in SDB in the absence of TRF or compared with strains cultured in SDB in the presence of 10 µg/mL TRF (Fig. 1).

*PDR1*, *MDR2*, and *MDR4* transcript levels in TRF-resistant strains NUBS19006 and NUBS19007 and TRF-susceptible strains 1 and 10 cultured in SDB were not significantly different (Fig. 1). By contrast, *PDR1*, *MDR2*, and *MDR4* transcript levels in TRF-resistant and TRF-susceptible strains cultured in SDB containing 10 µg/mL TRF were 5-100 times higher than those of strains cultured in SDB in the absence of TRF (Fig. 1). Furthermore, *PDR1*, *MDR2*, and *MDR4* expression levels were similar between TRF-resistant and TRF-susceptible strains cultured in SDB containing 10 µg/mL TRF.

TRF-resistant strains NUBS19006 and NUBS19007 were still able to grow in SDB supplemented with 10 µg/mL TRF, whereas TRF-susceptible strains 1 and 10 were not.

Drug interaction in susceptibility tests was examined in accordance with the CLSI protocol M38-A2 microdilution technique and evaluated using the microdilution checkerboard method. Experiments were performed in duplicate. The interaction between TRF and FK506 (Sigma-Aldrich, St. Louis, MO, USA) was evaluated in TRF-resistant NUBS19006 and NHBS19007 strains after 7 days of incubation at 28°C in RPMI 1640 medium. TRF and FK506 concentrations ranged from 0.3 to 320 µg/mL.

The fractional inhibitory concentration (FIC) was calculated for each agent by dividing the MIC of each drug in the
combination by the MIC of the drug alone. FIC values were then summed to determine the fractional inhibitory concentration index (FICI) that resulted from the drug combination using the following equation: $FICI = \frac{FICA + FICB}{C_A \text{Comb}/MIC_A \text{Alone} + C_B \text{Comb}/MIC_B \text{Alone}}$, where $MIC_A \text{Alone}$ and $MIC_B \text{Alone}$ are the MICs of drugs A and B, respectively, when acting alone, and $C_A \text{Comb}$ and $C_B \text{Comb}$ are the concentrations of drugs A and B, respectively, when combined.

FICI values were categorized as follows: synergy, FICI value ≤ 0.5; no interaction, FICI value = 0.5-4; and antagonism, FICI value > 4.

Antifungal activities of TRF and FK506, alone and in combination, were tested in TRF-resistant strains cultured in SDB after 5 days of incubation at 28°C. MICs for 90% inhibition of each drug alone or in combination were determined (Table 2). TRF-resistant strain NUBS19006 exhibited TRF MIC of 160 µg/mL. The addition of FK506 attenuated TRF MIC to 0.625 µg/mL, and the TRF FICI value was 1.0039 (Table 2). TRF-resistant strain NUBS19007 exhibited TRF MIC of 40 µg/mL. Addition of FK506 attenuated the TRF MIC to 2.5 µg/mL, and the TRF FICI...
NUBS19007 strains did not correlate with resistant strains NUBS19006 levels. However, µg/mL TRF were 5-100 times higher than those in all susceptible strains 1 and 10 cultured in SDB containing 10 µg/mL TRF. Furthermore, these results were consistent with previous reports that suggest different expression levels among all strains cultured in SDB in the presence of 10 µg/mL TRF. Therefore, these results are consistent with the idea that TRF resistance in NUBS19006 is related to the TRF-resistance mechanism. Thus, it is necessary to investigate whether MDR3 of terbinafine (TRF) -resistant isolates. It is revealed that TRF resistance in NUBS19006 in the presence of 10 µg/mL TRF. Therefore, these results are consistent with the idea that TRF resistance in NUBS19006 and NUBS19007 strains did not correlate with SLOE transcript levels.

PDR1, MDR2, and MDR4 expression levels in TRF-resistant strains NUBS19006 and NUBS19007 and TRF-susceptible strains 1 and 10 cultured in SDB containing 10 µg/mL TRF were 5-100 times higher than those in all strains cultured in the absence of TRF. However, PDR1, MDR2, and MDR4 transcript levels were not significantly different between TRF-resistant and TRF-susceptible strains cultured in SDB containing 10 µg/mL TRF. These results suggested that TRF induces overexpression of genes encoding members of the ABC transporter family. However, overexpression of these genes was not sufficient to prevent the antifungal effects of TRF.

Moreover, interaction between TRF and FK506 on antifungal activity of TRF was not detected in TRF-resistant strains. These results indicate that ATP-dependent efflux pumps do not confer TRF-resistance mechanisms in TRF-resistant strains.

In a recent study, Monod et al. reported TruM3, a newly identified transporter of the ABC family in T. rubrum that can confer azole resistance if overexpressed. However, they did not investigate terbinafine (TRF) -resistant isolates. It is necessary to investigate whether MDR3 of T. indotinae is related to the TRF-resistance mechanism.

**Conflicts of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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**Table 2. Combined drug effects against TRF-resistant strains evaluated by the FICI model**

| Strain   | MIC of drug mg/L | MIC of drug mg/L | FICI | INT  |
|----------|------------------|------------------|------|------|
|          | TRF alone | FK506alone | TRF and FK506 | FICI | INT  |
| NUBS19006 | 160 | 0.625 | 0.625 | 1.0039 | NIT  |
| NUBS19007 | 40 | 2.5 | 2.5 | 1.0625 | NIT  |

FICI values are shown as median of three independent experiments, INT: interpretation, NIT: no interaction.

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