Resistance Mechanism in a Terbinafine-Resistant Strain of *Microsporum canis*

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Introduction

Feline dermatophytosis is generally treated with azoles and terbinafine (TRF), compounds that demonstrate efficacy against both human and animal dermatophytoses. TRF is a member of the allylamine class of antifungal agents, which are known to target the squalene epoxidase (SQLE) enzyme in dermatophytes.

We first isolated a TRF-resistant *Microsporum canis* strain from a feline dermatophytosis in China. This strain (designated 47C) exhibited a TRF minimum inhibitory concentration (MIC) of >32 μg/mL but remained susceptible to itraconazole (ITZ) (MIC 0.023 μg/mL) (Table 1). To clarify the resistance mechanism in the 47C strain, the expression of the pleiotropic drug resistance (*PDR1*), multidrug resistance (*MDR1*), *MDR2,* and *MDR4* genes were investigated by real-time quantitative PCR (RT-qPCR) analysis and sequenced SQLE encoding gene.

Material and Methods

Strains examined

Strains are summarized in Table 1. *M. canis* strains 12C, 13C, and 14C, which are known to be susceptible to both TRF and ITZ, were used as comparators (Table 1).

RT-qPCR analysis of the ABC transporter family genes

Strains 12C, 13C, 14C, and 47C were cultured for 4 days at 28 °C in Saboraud's dextrose broth (SDB; 1% peptone and 2% dextrose). TRF exposure was carried out by the previously reported method of Martins *et al.*, who used a concentration of 0.14 μg/mL TRF to evaluate the expression of genes in 4 species of *Trichophyton* [1]. All strains (12C, 13C, 14C, and 47C) were cultured for 4 days at 28 °C in SDB with and without 0.14 μg/mL TRF. Extraction for total RNAs from fungal cells and the reverse transcription procedures were reported [2].

To analyze the expression levels of the *PDR1*, *MDR1, MDR2,* and *MDR4* genes, which encode members of the ABC transporter family, we used real-time quantitative PCR (RT-qPCR) were reported [2].

Sequence for SQLE encoding gene of TRF-resistent strain (47C)

To sequence the SQLE cDNA from isolates, primers were prepared based on the conserved sequence of the *Arthroderma otae* CBS 113480 squalene epoxidase, mRNA (GenBank accession number, XM_002849385).

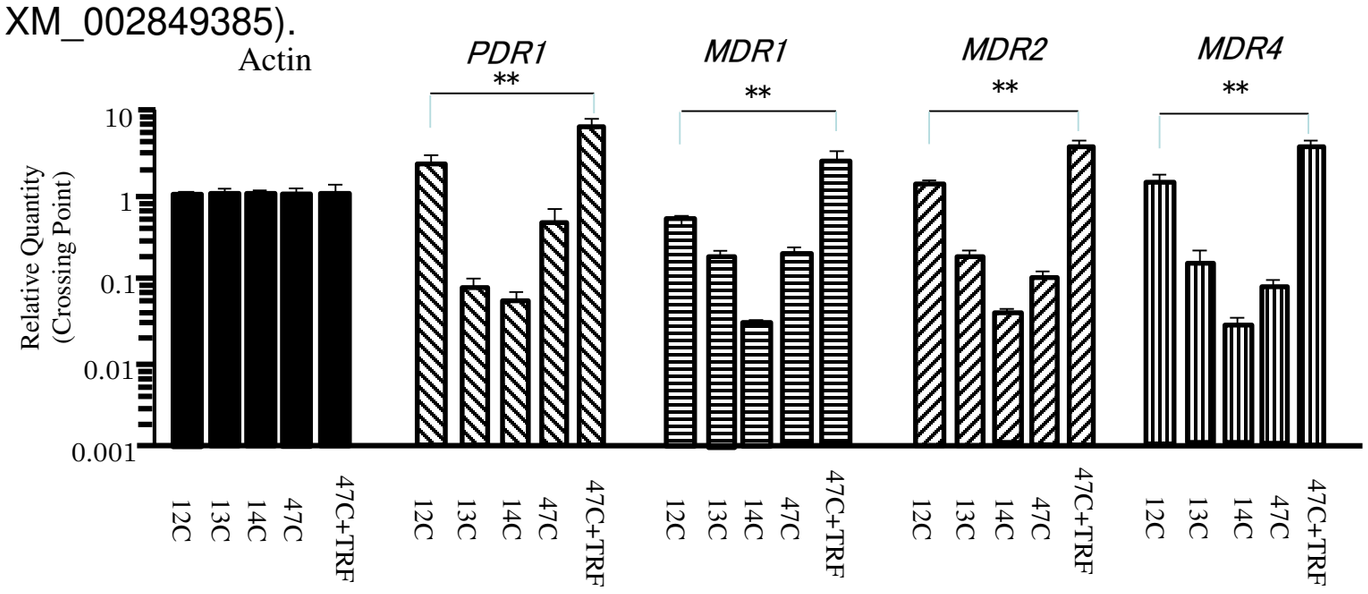

Fig. 1. Transcript levels of the actin-encoding gene and of *PDR1*, *MDR1*, *MDR2*, and *MDR4* in TRF-resistant (47C) and -susceptible (12C, 13C, and 14C) strains of *M. canis.* All strains were cultured in SDB and (for 47C only) in SDB containing 0.14 mg/mL TRF (47C + TRF) for 4 days at 28 °C. Relative Quantity (y axis) indicates that the expression levels of all genes were normalized to that of the actin-encoding gene in the respective strain. Asterisks indicate statistically significant differences by a two-tailed, non-paired *t*-test; ***P<0.01.*

Results

Expression levels of PDR1, MDR1, MDR2, and MDR4

No significant differences were found in basal levels of *actin* expression among any of the strains cultured in SDB in the absence of TRF or compared to strain 47C cultured in SDB the presence of 0.14 μg/mL of TRF (Fig. 1).

The transcript levels of *PDR1*, *MDR1, MDR2,* and *MDR4* in the TRF-resistant strain (47C) cultured in SDB were not significantly higher than those of the respective genes in the TRF-susceptible strains (12C, 13C, and 14C) (Fig. 1). However, the transcript levels of *PDR1*, *MDR1, MDR2,* and *MDR4* in the TRF-resistant strain cultured in SDB containing 0.14 μg/mL of TRF were 2 to 4 times higher than those of the respective genes in the three TRF-susceptible strains (Fig.1).

SQLE sequence of the TRF-resistant isolate

The SQLE gene from the 47C strain encoded a protein with I100M and F395L substitutions (Fig. 2). The sequence determined in this study has been deposited in GenBank (*Microsporum canis* 47C SQEL mRNA for squalene epoxidase, complete cds; GenBank accession no. LC348388).

Discussion

To the best of our knowledge, this work represents the first report that TRF-resistant strains of *M. canis* exhibited the overexpression of ABC transporter proteins and encodes missense mutations in the SQLE. We speculate that the overexpression of genes in the TRF-resistant strain is not sufficient to prevent the antifungal effects of ITZ. To investigate the relationship between these amino acid substitutions and TRF tolerance, point mutations leading to several amino acid substitutions found in the SQLE of the resistance isolate will be introduced into the endogenous *SQLE* gene of the *M. canis* using genetic manipulation tools.

Table 1 MICs (mg/mL) of anti-fungal drugs in the tested strains

Strain	TRF[a]	ITZ[b]	Origin
47C	>32	0.023	feline dermatophytosis in China
12C	0.125	0.064	feline dermatophytosis in Malaysia
13C	0.125	0.023	feline dermatophytosis in Malaysia
14C	0.5	0.25	Pet shop's clipper in Malaysia

[a]MICs were assayed by CLSI protocol M27-A3.
[b] MICs were assayed by E-test.

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M.canis SQLE   SVILLEKSWKEPDRIVGELLQPGGVRALEELGLRDCLEGIDAVRTYGYDVIYFGTGVKIP 120
47C SQLE       ************************************M********************

M.canis SQLE   SNTKLVLKQLSKFHWQRKSLTSVINILAQALYSIFAAGDPNLKVLQRGCFRILDCLQGYT 420
47C SQLE       *********************************L************************
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Fig. 2. Alignment of predicted *M. canis* SQLE amino acid sequence (*Arthroderma otae* CBS 113480 squalene epoxidase, mRNA; Gen Bank Accsession number:XM_002849385) with their counterpart from TRF-resistent strain (47C). Amino acids identical to those of 47C SQLE are shown by asterisk.

References

1. Martins MP, Franceschini AC, Jacob TR, Rossi A, Martinez-Rossi NM. Compensatory expression of multidrug-resistance genes encoding ABC transporters in dermatophytes. J Med Microbiol. 2016;65:605-10.
2. Kano R, Hsiao YH, Han HS, Chen C, Hasegawa A, Kamata H. Resistance Mechanism in a Terbinafine-Resistant Strain of *Microsporum canis*. Mycopathologia. 2018 Jan 16. doi: 10.1007/s11046-018-0242-0.