Discovery of Terbinafine Low Susceptibility 
*Trichophyton rubrum* strain in Japan

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This is the first confirmed report of terbinafine low susceptibility *Trichophyton rubrum*, BGUTR13, in Japan collected from the whole sole of the elderly over 65s with cotton swab sampling method at the special nursing care-home in 2016. We revealed BGUTR13 showed low susceptibility (MIC, >128 µg/mL) against terbinafine. But, BGUTR13 exhibited normal susceptibility to itraconazole, did not showed cross-resistance. Also, the squalene epoxidase gene of terbinafine low susceptibility strain BGUTR13 which is the target of terbinafine contained newly confirmed one mismatch. We suggested the possibility that the resistance mechanism of terbinafine low susceptibility strains is due to the loss of sensitivity of squalene epoxidase inhibitors and does not affect antifungal drugs with other different mechanisms of action.

Key words: Low susceptibility / Terbinafine / *Trichophyton rubrum*.

Dermatophytosis is a common infectious disease of the keratinized tissues in the skin, hair, and nails, which is caused by dermatophytes (Osborne et al., 2005), and can affect a large proportion of the population (Vander et al., 2003). Among the three known genera of dermatophytes, *Epidemophyton, Microsporum*, and *Trichophyton*, especially *Trichophyton*, species such as *Trichophyton mentagrophytes* (*T. mentagrophytes*), *Trichophyton tonsurans*, and *Trichophyton rubrum* (*T. rubrum*), are the most common pathogens, with *T. rubrum* being the most prevalent isolated organism (Osborne et al., 2005). *T. rubrum*, among other dermatophytes, is a major causative agent for superficial dermatomycosis like onychomycosis and tinea pedis (Kemna and Elewski, 1996; Weitzman and Summerbell, 1995).

Terbinafine whose target is squalene epoxidase, part of the ergosterol biosynthesis pathway (Osborne et al., 2006), is an allylamine antifungal that is frequently used for the dermatophytosis treatment. Terbinafine is highly effective in treating fungal infections. But, despite extensive use of the drug, the reports of *T. rubrum* resistant to terbinafine are rare (Osborne et al., 2006; Hossain and Ghannoum, 2001). There are no reports of terbinafine-resistant *T. rubrum* in Japan yet. On the other hand, *T. rubrum* which showed remarkably low susceptibility to terbinafine has been slightly confirmed in other countries (Osborne et al., 2006; Mukherjee et al., 2003).

Generally, it is said that the prevalence of Japanese in both tinea pedis and onychomycosis is very high compared to other countries, and environmental and cultural complex factors such as high temperature and high humidity climate (Ide et al., 1999) and public bathing (Watanabe et al., 2000) may influence. Therefore, many epidemiological surveys have been conducted in Japan (Watanabe et al., 2010; Kasai, 2000; Kasai, 2001; Nishimoto, 2006; Sei, 2012; Sei, 2015). It is reported that the morbidity rate of tinea pedis and tinea unguium in dermatology outpatients...
was 40%, of first dermatology outpatients was more than 6%.

Also, direct infection of *Trichophyton* species from patients suffering from tinea pedis is rare (Katoh et al., 1996), the main route of infection is to invade into the stratum corneum after dermatophytes scattered in the environment from the carriers adhere to uninfected persons (Morishita et al., 2003). Therefore, many epidemiological surveys are also conducted on the scattering rate (possession rate) which is an important factor in infection (Fujihiro, 1993; Nishimoto et al., 1991). These studies suggested the scattering rate was very high, more than 65.0%, from patients suffering from tinea pedis. Our epidemiological survey targeted 159 people in 2016 showed that the scattering rate of *Trichophyton* species from the whole sole is high, 23.3% (Suzuki et al., 2017). Further, elders over 65 were very higher, 40.8%. Nevertheless, in the special nursing care-home for the elders, there was not necessarily an appropriate medical treatment given (Suzuki et al., 2017), furthermore there seemed to be some cases in which the wrong administration of antifungal drugs was done. There might be a concern that the appearance of drug-resistant fungi will also be promoted by the misuse of antifungal drugs.

Therefore, we investigated the possibility of the occurrence of drug low susceptibility strain by broth microdilution susceptibility testing, with using *Trichophyton* species collected at the special nursing care-home for the elderly people. And some analysis was conducted on that drug resistant strain that we collected.

Strains tested were from the Bunkyo Gakuin University collection, which were named BGU. *T. rubrum* strain BGUTR15 was the reference strain. *T. rubrum* strain BGUTR13 is a low susceptibility to terbinafine and shows high MIC (terbinafine >128 µg/mL) by broth microdilution susceptibility testing. BGUTR13 and BGUTR15 were collected from the whole sole by cotton swab sampling method (Nishimoto et al., 1991) from elderly people over 65s at the special nursing care-home for the elderly people, which were located in Kanto-area in 2016. These strains were identified *T. rubrum* by macroscopic observation of colony morphology (pigmentation, growth rate, texture) on Sabouraud dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), microscopic examination of conidia, PCR-RFLP (Shehata et al., 2008). Strains were stored at -80°C until experiments was carried out.

The following antifungal agents, which are designated as first-line drug or alternative drug in the guidelines (Hay and Moore 2004), were used in this study: terbinafine (Tokyo Chemical Industry Co., Tokyo, Japan), and itraconazole (Wako Pure Chemical Industries Ltd., Tokyo, Japan).

The measurement of MIC was conducted by broth microdilution susceptibility testing complying with CLSI M38-A2 standard (CLSI, 2008). RPMI 1640 (Sigma-Aldrich Co., St. Louis, N.Y., USA) with L-glutamine but without bicarbonate, buffered to pH 7.0 with 0.165 M 3-N-morpholinopropanesulfonic acid (Wako Pure Chemical Industries Ltd., Tokyo, Japan), was the medium used for broth microdilution susceptibility testing. Two antifungal agents were obtained in powder form and dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries Ltd., Tokyo, Japan), and then they were serially diluted using RPMI 1640 to yield concentrations with double the desired final concentrations of 0.001 to 128 µg/mL for terbinafine and 0.004 to 4 µg/mL for itraconazole. The antifungal agents were prepared in 96-well plates. *T. rubrum* strains were cultured on Sabouraud dextrose agar diluted 10-fold at 35°C for 7 to 15 days. BGUTR13 and BGUTR15 sporulated well after this period. Inoculum suspensions were obtained from each strain by covering the fungal colonies with 5.0 mL of sterile saline with 0.1% tween 80 (Tokyo Chemical Industry Co., Tokyo, Japan) and by gently rubbing the colonies with a sterile swab. Collected conidia were adjusted to the desired density by adding RPMI 1640 to obtain a conidial suspension of 2 x 10^3 to 6 x 10^5 CFU/mL. 100 µL of the prepared cell suspension of each strain was added to each well of 96-well plates containing 100 µL of previously prepared antifungal drugs in RPMI 1640 to bring the drug dilutions and inoculums to the final desired test concentrations. After these steps, the final concentration of DMSO in test wells became 1.0%. Growth and sterility controls were included for each strain tested. Each organism was tested in duplicate. The inoculated plates were incubated at 35°C for 4 days. MICs were determined visually and were defined as the lowest drug concentration that caused 80% inhibition of the growth in comparison to the growth control. *T. interdigitale* ATCC MYA-4439 was used for quality control strain, and we confirmed that concentration of antifungal agents (terbinafine and itraconazole) are within the range of quality control.

Genomic DNA were isolated from *T. rubrum* mycelium, and a genomic fragment covering the whole coding region of the squalene epoxidase gene was amplified with the primers (5-CCTCTAGACCCATCAATAAGTTA CTACAATG and 5-CCTCTAGAGATTTAGATAAGCC TATCTGCCTA) (Osborne et al., 2005). Amplification was carried out in a PCR Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan). After an initial denaturation for 5 min at 94°C, 30 cycles were completed, each consisting of 30s at 94°C, 30s at 58°C, and 30s at 72°C. Subsequently, the amplified squalene epoxidase gene was sequenced with a 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). The
complete gene sequence data for the squalene epoxidase genes of terbinafine low susceptibility strains BGUTR13 have been assigned GenBank accession number LC311882.

*T. rubrum* strain BGUTR13 and BGUTR15 were collected from elderly people. Testing of the terbinafine susceptibility of these strains by broth microdilution susceptibility testing revealed that BGUTR13 reduced susceptibility (MIC, >128 µg/mL) compared to the reference strains BGUTR15 (MIC, 0.002 µg/mL) (Table 1). We revealed that BGUTR13 was low susceptibility to terbinafine.

To gain further insight, the susceptibility to clinically used antifungals, itraconazole, were determined. As shown in Table 1, tested strains exhibited normal susceptibility to itraconazole. Also, terbinafine low susceptibility strain BGUTR13 showed similar susceptibility compared to the reference strains (MIC, 0.016 µg/mL for BGUTR15) (Table 1). These results indicated that the strain BGUTR13 was a low susceptibility to terbinafine and did not develop the cross-resistance to itraconazole.

We sequenced the squalene epoxidase gene from a terbinafine-susceptible strain, BGUTR15, and confirmed its nucleotide sequence to be identical to NFI1895 and NFI5182 reported as a sensitivity strains (Osborne et al., 2005). In contrast, the squalene epoxidase gene sequence from the terbinafine low susceptibility strain BGUTR13 contained one mismatch, $117^{\text{T}} \text{TAA} \rightarrow \text{TTC}$, in comparison with the BGUTR15 gene sequence.

This is the first confirmed report of terbinafine low susceptibility *T. rubrum* in Japan and we found that the gene of squalene epoxidases of the strain BGUTR13 contained one mismatch point.

There are several studies that investigated MICs of *T. rubrum* isolated in Japan, but there is no report of resistant strain that shows very low susceptibility to terbinafine (Ghannoum et al., 2010; Jo et al., 2013; Tabata et al., 2015). We reported the first occurrence of terbinafine low susceptibility *T. rubrum* in Japan on this paper. The appearance of terbinafine low susceptibility *T. rubrum* in Japan may make clinical therapy hard and may influence future medical treatment policy.

On the other hand, MIC of terbinafine low susceptibility strain BGUTR13 to itraconazole of triazole antifungal agent showed approxitmate same values as the reference strain BGUTR15. MIC of BGUTR13 showed values within the MIC range reported in the previous articles (Jo et al., 2013; Tabata et al., 2015). Also, terbinafine-resistant *T. rubrum*, NFI5146, isolated in other country did not show resistance toazole antifungal agent (Mukherjee et al., 2003). But NFI5146 showed cross-resistance to natifine of allylamine antifungal agent and butenafine of benzylamine antifungal agent (Mukherjee et al., 2003). Allylamine and benzylamine antifungal agent specifically inhibit the squalene epoxidase, which is part of the ergosterol synthesis pathway (Favre and Ryder, 1996), blocking the synthesis of squalene epoxide from squalene and resulting in the accumulation of toxic levels of squalene and decreased levels of ergosterol production, rapidly and bactericidially acts (Leyden, 1998). Terbinafine and itraconazole inhibits the growth of fungi by interfering with the synthesis of ergosterol in the cell wall, butazole works at a later step than terbinafine and blocks the formation of ergosterol from lanosterol (Leyden, 1998). Thus, the mechanism of action is different between terbinafine and itraconazole, cross-resistance to itraconazole was not observed in the terbinafine low susceptibility isolates is no wonder. From results of these studies, we suggested the possibility that the resistance mechanism of terbinafine low susceptibility strains is due to the loss of sensitivity of squalene epoxidase inhibitors and dose not affect antifungal drugs with other different mechanisms of action.

Subsequently, in order to confirm the mechanism of terbinafine low susceptibility molecularly, the squalene epoxidase gene of the low susceptibility strain BGUTR13 and the reference strain BGUTR15 were sequenced. We revealed the squalene epoxidase gene sequence of the BGUTR13 contained one mismatch, $117^{\text{T}} \text{TAA} \rightarrow \text{TTC}$, in comparison with the BGUTR15 gene sequence. In terbinafine-resistant strain NFI5146, different mismatch ($117^{\text{T}} \text{TAA} \rightarrow \text{TTT}$) of the same part confirmed, and this mismatch reported to leads the missense mutation (L393F) (Osborne et al., 2005). A mismatch in this region inhibits the binding affinity of terbinafine resulting the reduction of the squalene epoxidase activity, which may lead to the appearance of terbinafine low susceptibility strains. Also, we suggested possibility that mutation of squalene epoxidase gene is the main resistant mechanism.

The report of terbinafine-resistant *T. rubrum* was few (Osborne et al., 2006; Hossain and Ghannour, 2001), and there were no reports of resistant strain in Japan. Results of these studies suggest that the appearance of terbinafine low susceptibility *T. rubrum* is rare, it is unlikely that terbinafine low susceptibility strain becomes a problem in the clinic immediately. But, terbinafine low

**Table 1.** MICs of two antifungals against *T. rubrum* BGUTR13 and the reference strain BGUTR15 determined using the broth microdilution susceptibility testing

| Compound         | Antifungal MIC (µg/mL) |
|------------------|------------------------|
|                  | BGUTR13                | BGUTR15                |
| Terbinafine      | >128                   | 0.002                  |
| Itraconazole     | 0.032                  | 0.016                  |
susceptibility *T. rubrum* may have the influence on future medical treatment policy, and we show that it is necessary to continue to investigate variation of MICs of terbinafine and other antifungal drugs not included in clinical routine work. If treatment efficacy is not confirmed in terbinafine therapy, it may be better to use antifungal drugs with different mechanisms. Also, this study has not elucidated the factors and backgrounds of the occurrence of terbinafine low susceptible strain. Therefore, additional study needs to be done in the future.

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