Original Article

Molecular Epidemiology of *Trichophyton tonsurans* Strains Isolated in Japan between 2006 and 2010 and Their Susceptibility to Oral Antimycotics

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SUMMARY: *Trichophyton tonsurans* has been isolated among judo practitioners, wrestlers, and sumo wrestlers during an epidemic of tinea corporis and tinea capitis in Japan. A previous study using restriction fragment length polymorphism (RFLP) analysis of the non-transcribed spacer (NTS) region of the ribosomal RNA gene revealed different sources for the causative fungus in epidemics among judo practitioners and among wrestlers. Many different fungal strains have since been isolated from practitioners of these sports. The present study evaluated fungal characteristics of strains newly isolated between July 2006 and December 2010 using this molecular method. PCR-RFLP analysis using *Mva*I and *Ava*I was performed on 263 strains, composed of 186 isolates from judo practitioners, 32 from wrestlers, 30 from sumo wrestlers, 5 from other sports, 7 from family members or friends of the sports practitioner patients, and 3 from sporadic (non-epidemic) cases. Four molecular types, NTS I, II, III, and VII were detected. Of these, NTS I was the most predominant, occurring in 243 of 263 strains (92.4%). All of the 30 strains isolated from sumo wrestlers were classified as NTS I, suggesting that the epidemic among sumo wrestlers originated from an earlier epidemic among judo practitioners. Thirteen strains were classified as NTS II; all were related to wrestling and were isolated mainly from the Chubu and Kansai areas in the central part of Honshu island. NTS III was detected in 6 strains, and one strain classified as NTS VII was isolated from a sporadic case of tinea capitis in a Peruvian immigrant. The minimum inhibitory concentrations (MICs) of terbinafine, itraconazole, fluconazole, and griseofulvin on 10 strains of NTS I and NTS II and 4 strains of NTS III were examined; there were no differences in MIC between these molecular types.

INTRODUCTION

*Trichophyton tonsurans* is known to be the causative agent of a nationwide epidemic of tinea capitis and tinea corporis among judo practitioners and wrestlers in Japan. The epidemic was first identified in a high school wrestling team (1), but an explosive epidemic then occurred among high school and university judo teams (2). An epidemic among young trainees of sumo wrestling, a form of traditional Japanese wrestling, was reported in 2005 (3).

Several recent studies have examined the variable internal repeat (VIR) region of the non-transcribed spacer (NTS) region of ribosomal RNA (rRNA) genes, thus detecting different molecular types among *T. tonsurans* isolates (4–8). Gaedigk et al. (4) detected 5 variants differing in size by polymerase chain reaction (PCR) targeting the VIR region in the NTS region; among these, they identified 7 sub-variants with different single nucleotide polymorphisms (SNPs) identified by restriction fragment length polymorphism (RFLP) analyses using 7 restriction enzymes. In 2006, Sugita et al. (5) sequenced the VIR region in the NTS region from 101 strains isolated from Japanese judo practitioners; based on the genetic homogeneity of the isolates, they concluded that the causative agent was clonal. Our previous study used RFLP analysis of PCR amplicons of the VIR region in the NTS region to examine 232 strains isolated between 2001 and 2006; our results suggested that the epidemics among judo practitioners and wrestlers had different origins (6). The fungal source of the epidemic among sumo wrestlers was probably derived from *T. tonsurans* spread by infected judo practitioners (3).

In order to understand the contemporary epidemic in Japan, we evaluated the molecular characteristics of recently isolated strains, including strains from sumo wrestlers. In vitro susceptibility against oral antifungal agents was also measured in these newly isolated strains to provide insight into clinical treatment.
Table 1. Origins of strains and their molecular types (2006–2010)

<table>
<thead>
<tr>
<th>NTS type</th>
<th>Judo</th>
<th>Wrestling</th>
<th>Sumo</th>
<th>Other sports</th>
<th>Family/Friends</th>
<th>Sporadic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTS I</td>
<td>181</td>
<td>19</td>
<td>30</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>243</td>
</tr>
<tr>
<td>NTS II</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>NTS III</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>NTS VII</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>32</td>
<td>30</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>263</td>
</tr>
</tbody>
</table>

Fig. 1. Geographical origins of examined strains and distribution of NTS I strains (No. of NTS I strains/examined strains) found in Japan (2006–2010).

MATERIALS AND METHODS

Fungal strains (Table 1, Fig. 1): We examined 263 isolates of *T. tonsurans* from 34 of all 47 prefectures in Japan between July 2006 and December 2010. As previously described (6), some of these were cultured from skin lesions of the heads of active martial arts participants, their family members, and close friends using the hairbrush sampling method. This study also included 64 strains isolated from a screening examination among high school judo practitioners at the 2007 All Japan Championships in Saga (9). Individuals' activities were recorded and were classified as follows: judo (*n* = 186), wrestling (*n* = 32), sumo (*n* = 30), other sports (*n* = 5). Other sports comprised 2 martial arts practitioners, 1 kendo player, 1 sports instructor, and 1 gymnast. The analysis also included another 7 strains isolated from friends or family members of tinea patients involved in these activities and 3 strains isolated from patients unrelated to sports, including a Peruvian immigrant (7,10).

The geographical origins of the strains were: Tohoku (northern part of Honshu island: Aomori, Akita, Iwate, Yamagata, Miyagi, and Fukushima; *n* = 26); Kanto-Koshinetsu (central-east Honshu island: Tokyo, Kanagawa, Chiba, Niigata, Ibaraki, Saitama, and Gunma; *n* = 47); Chubu (central Honshu island: Aichi, Shizuoka, Gifu, Fukui, Ishikawa, and Toyama; *n* = 121); Kansai (central-western Honshu island: Kyoto, Osaka, Nara, Shiga, Hyogo, and Wakayama; *n* = 28); and Chugoku, Shikoku, and Kyushu (western Honshu island, Shikoku and Kyushu islands: Hiroshima, Yamaguchi, Ehime, Nagasaki, Fukuoka, Oita, Saga, Kagoshima, and Miyazaki; *n* = 41).

For species-level identification of the strains, we first examined the gross colony morphology and then confirmed that the strains were *T. tonsurans* by PCR-RFLP analysis of the ITS regions of rRNA genes (11).

Preparation of template DNA, PCR conditions, and RFLP analysis: The NTS region was analyzed as previously described (6,7). Briefly, total cellular DNA was extracted from colonies cultured on Sabouraud's dextrose agar plates or plates with or without antibiotics by a rapid preparation method (12) and used as the template for PCR. PCR amplicons were generated using the templates and the primer pair L663 (5'–TTGTAGAATGCTCCAACCAC-3') and R1145 (5'-ACAAGGGCGGGAACTATCCAGC-3'), which targeted the middle part of the NTS region. The thermal cycling conditions were as follows: 4 min at 94°C, followed by 28 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C. The amplicons were digested with *Mva*I and *Ava*I (Toyobo Co., Ltd., Osaka, Japan), separated by 5% polyacrylamide gel electrophoresis, stained with ethidium bromide, and visualized under a UV lamp. The strains were sub-typed based on their banding profiles, as previously reported (6,7).

Susceptibility of the fungal strains against antimycotics in vitro: Ten of 243 NTS I strains, 10 of 13 NTS II strains, and 4 of 6 NTS III strains were selected for further examination based on their geographical origin and the type of sports activity. Susceptibility tests were performed using a slightly modified broth microdilution method proposed by the Japanese Society of Medical Mycology (13). The strains were inoculated onto potato dextrose slants and incubated at room temperature for 2 weeks. Conidia were suspended in saline by vigorously scraping the surface of the colony with a loop; the suspensions were filtered with gauze folded 4 times and adjusted to a final concentration of 2.5 × 10⁴ conidia/mL using a hemocytometer. Serial dilutions of antifungal agents were prepared in 96-well plates using stock solutions...
The MIC range measured for TBF, including a reference strain for the MIC test of *Trichophyton mens-
The ranges of MIC for NTS III strains were similar to FCZ, 0.5–16 μg/mL; and GRF, 0.25–2 μg/mL. The ranges of MIC for NTS II strains were similar to those for NTS I strains: TBF, 1/8–1 ×; ITCZ, 0.001–0.13 μg/mL; FCZ, 0.5–16 μg/mL; and GRF, 0.25–2 μg/mL. The ranges of MIC for NTS III strains were similar to those for NTS I and II strains: TBF, 1/4–1 ×; ITCZ, 0.002–0.03 μg/mL; FCZ, 0.5–32 μg/mL; and GRF, 1–4 μg/mL. Therefore, there were no apparent differences in the MICs between the molecular types. Although the MIC of TBF in NTS II tended to be higher than that in NTS I, the difference was not significant (P > 0.05, Mann-Whitney test). Of the 24 total strains, the MIC values for TBF, ITCZ, FCZ, and GRF were 2 × (n = 24), 0.015 μg/mL (n = 24), 16 μg/mL (n = 24), and 2 μg/mL (n = 24), respectively. GRF had a narrow MIC range, whereas ITCZ and FCZ had wider MIC ranges. The 3 strains with higher MIC of ITCZ (KMU 6241, NTS I: 0.13 μg/mL; KMU 6146, NTS II: 0.015 μg/mL; KMU 6400, NTS II: 0.03 μg/mL) also showed higher MIC of FCZ (KMU 6241: 16 μg/mL; KMU 6146: 32 μg/mL; KMU 6400: 32 μg/mL).

**DISCUSSION**

The results of the present study examining strains isolated between 2006 and 2010 were similar to those of our previous study on strains isolated between 2001 and 2006 (6). The predominant type was NTS I. One hundred eighty-one of the 186 strains isolated from judo practitioners (97.3%) were classified as NTS I; this ratio is almost the same as that in our previous study (160/164 strains, 97.6%) (6). Sugita et al. (5) examined 101 strains isolated from Japanese judo practitioners before 2005 and found only 1 molecular type, “type II,” which corresponds to NTS I in our studies. Thirty-one of these Japanese strains were later found to be identical by a mixed-marker method using 27 sequence variations in 13 gene loci, which discriminated 198 strains into 47 distinct types (8). The molecular profile of their “EvS03” was also detected among strains isolated from USA and Canada (8). Aside from NTS I, the present study identified only a small number of NTS III strains and no NTS II strains among isolates from judo practitioners. Of the 32 strains isolated from wrestlers, 19 (59.4%) were classified as NTS I, and 12 (37.5%) were classified as NTS II in the present study. The incidence of NTS II among wrestlers was 35.4% (17/48 strains) in our previous study (6); the present results indicate that the clone may be preserved among Japanese wrestlers. In addition, all of the NTS II strains were isolated from the Chubu and Kansai regions with the exception of 1 strain isolated from Kanagawa (Kanto-Koshinetu, Fig. 1). The heterogeneous geographic distribution observed in the present study is consistent with previous series, in which 17 of 21 NTS II strains were isolated from these regions (6). The NTS II classification in our study is related to early designations from previous reports; it may be “type II” in Gaedigk’s classification (4), “type IV” in Sugita’s classification (5), and “C” in Abdel-Rahman’s classification (8). The NTS II equivalent type was the most prevalent classification among US and European isolates and the second most prevalent among Mexican isolates (8). Among Korean isolates, the incidence of the molecular type related to NTS II was 45.5% (5/11) among isolates from wrestlers and 27.3% (3/11) among isolates from judo practitioners (Seung and Choi, personal communication).

The present study examined 30 strains isolated from sumo wrestlers who lived in 9 prefectures in 5 regions; Kyushu (n = 9), Kanto-Koshinetu (n = 8), Chubu (n = 7), Tohoku (n = 4), and Kansai (n = 2). The first culture-proven case of T. tonsurans infection among sumo wrestlers was observed in March 2004 in Gifu in the Chubu region (3). All of the subjects were members of a local private sumo studio or high school sumo team, and 1 had trained with a high school judo practitioner with tinea corporis caused by T. tonsurans (3). Subsequently, a recalcitrant epidemic was reported in Nagasaki in Kyushu island between 2006 and 2009 (17,18). During this period, we obtained 30 sumo-related strains from institutes located in the above regions. The results of the present study indicate that all sumo-related strains were NTS I, consistent with the clinical observation that the epidemic among sumo wrestlers originated from judo practitioners (3).

Another important observation from this study is that there was an increase in cases among the friends and families of the epidemic cases. Among them, 1 strain showing NTS II was isolated from a 3-year-old girl in Wakayama in Kansai, who was the daughter of a wrestling coach infected with an NTS II strain. Not only NTS I strains (6) but also NTS II strains may have contributed to epidemic outbreaks in the community. With the exception of this case, all other strains were classified as NTS I in the present study, reflecting the prevalence of NTS I in the present epidemic in Japan.

The biological and pathogenic differences between the molecular types remain an area of interest. The ratio of NTS I to NTS II strains among wrestlers has remained constant over the last 10 years, suggesting that these 2 molecular types do not differ in pathogenesis. In addition, we examined the MICs of oral antifungal agents and found no significant differences in MICs of 4 antifungal agents among the 3 major molecular types, NTS I, NTS II, and NTS III. Further studies are needed to compare the pathogenesis of strains of different molecular types.

The MIC values in the present study were comparable with those of previous studies reported in 2000 (14) and 2001 (15). For example, the MIC values of TBF were 2 × (0.032 μg/mL) in our study, compared with 0.008–0.01 μg/mL in the previous studies (14,15). Here, MIC values of ITCZ were 0.015 μg/mL, as compared with 0.03–0.06 μg/mL in previous studies (14,15); those of FCZ were 16 μg/mL, as compared with 8 μg/mL (14,15), and those of GRF were 2 μg/mL, as compared with 4 μg/mL (14). The present study measured a rather high MIC value of FCZ, although FCZ is not used as first-line therapy for dermatophytes in Japan. The presence of strains showing higher MICs of both ITCZ and FCZ may suggest the existence of azole-resistant tagophytes IFM 5218 (ATCC18748) (13), was considerably higher than in previous studies (14–16). Therefore, we reported the MIC in comparison with that of the tester strain, which was reported to be 0.016 μg/mL (13). The ranges of MIC for the NTS I strains were as follows: TBF, 1/8–1 ×; ITCZ, 0.001–0.13 μg/mL; FCZ, 0.5–16 μg/mL; and GRF, 0.25–2 μg/mL. The ranges of MIC for NTS II strains were similar to those for NTS I strains: TBF, 1/2–4 ×; ITCZ, 0.002–0.03 μg/mL; FCZ, 0.5–32 μg/mL; and GRF, 1–4 μg/mL. Therefore, there were no apparent differences in the MICs between the molecular types.
strains, and the efficacy of FCZ for the treatment of ITCZ-resistant *T. tonsurans* infections may be limited.

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**Conflict of interest** None to declare.

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