First isolation of the terbinafine- and itraconazole-resistant Trichophyton indotineae in China

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Research Article

Keywords: Trichophyton indotineae, terbinafine resistance, azole resistance
Abstract

Background

*Trichophyton indotineae*, a new species of dermatophytes, has become a significant concern in treating dermatophytosis due to the high level of terbinafine resistance reported in India and even worldwide.

Objectives

We aimed to report the first case of the terbinafine- and itraconazole-resistant *T. indotineae* in China, by identifying the phylogenetic classification of the isolate strain, and detecting the drug resistance, gene mutation and expression.

Patients/Methods:

The skin scales of patient were cultured on SDA and authenticated by DNA sequencing and MALDI-TOF MS. Antifungal susceptibility to itraconazole, terbinafine et al was tested following the M38-A2 CLSI protocol to examine MIC. The isolate was screened for mutations in the squalene epoxidase (*SQLE*) gene by Sanger sequencing and detected the expression of CYP51A and CYP51B by qRT-PCR.

Results

We isolated the first multi-resistant ITS genotype VIII sibling of the *T. mentagrophytes* complex (*T. indotineae*) in China. The strain harbored high terbinafine MICs (>32 µg/mL) and had a mutation in the squalene epoxidase gene with amino acid substitution (Phe$^{397}$Leu, mutation 1191C $>$ A). In addition, overexpression of CYP51A and CYP51B was observed. With multiple relapses, the patient finally achieved clinical cure by itraconazole pulse therapy and topical clotrimazole cream for 5 weeks.

Conclusions

We reported the first indigenous case of *T. indotineae* in China, indicating the intensification of drug resistance in dermatophytes. Besides, we found longtime itraconazole pulse therapy (0.2g p. o. BID) may provide a practical reference for clinicians in treating refractory dermatophytes.

Introduction

Dermatophytes is the most common fungal disorders, affecting about 20–25% of the world's population and the prevalence is increasing [1, 2]. *Trichophyton mentagrophytes* have replaced *T. rubrum* infections in frequency, especially in India [1]. In recent years, an alarming increase in the frequency of a difficult-to-
treat dermatophyte lineage of the *Trichophyton mentagrophytes* complex, described as *T. indotiniae* [3], has been witnessed worldwide [4–6]. During the last few years, *T. indotiniae* has caused infections in India [7], Belgium [8], Germany [9], Japan [10], Denmark [11], Iran [12], Greece [13], Switzerland [14], France [15] and Vietnam [16], connected with increased travel and migration between these countries. While in China there was no reported cases of resistant *T. indotiniae* till now.

This emerging novel species resistant to terbinafine were isolated from infected humans in the whole world associated with a high frequency of mutation in the squalene epoxidase (*SQLE*) gene [7]. A part of these strains also showazole resistance[4, 17] leading to the mutation of cytochrome P450 superfamily Cyp51 (*CYP51A* and *CYP51B*) [18] or the overexpression of the multidrug transporters[19]. Although the terbinafine- and itraconazole-resistant *T. indotiniae* is rarely reported, it indicates a huge challenge for treating the double resistance of *T.indotiniae*, resulting in infections with considerable morbidity and economic burden to the healthcare system.

Here, we reported the first indigenous case of *T. indotiniae* with both terbinafine and itraconazole resistance in China. The patient had no history of travel to abroad and contact the overseas good. Moreover, we investigated *SQLE* mutations, expression of *CYP51A /CYP51B* and phylogenetic relationships of *T. indotiniae* strains isolated in China, compared with reference strains from multiple countries. There is an ongoing epidemic of resistant *T. indotiniae* in China, concerning about the large population size and density.

**Materials And Methods**

**Clinical specimens and fungal isolates**

The isolates originated from a patient with tinea corporis and tinea cruris (Fig. 1). Skin scrapings were processed for direct microscopic examination with 10% potassium hydroxide (KOH). Strains were cultured on Sabouraud's Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) containing 300µg/mL chloramphenicol and 500µg/mL cycloheximide at 28°C for 7 days (Fig. 2a~d). Total DNA and RNA were extracted from the strains cultured on the SDA at 28°C for 7 days.

**Identification And Phylogenetic Analysis**

The genomic DNA of *T. indotiniae* strain was extracted from the growing mycelia using EZNA fungal DNA kit (Vazyme, Nanjing, China) according to manufacturer's instructions. Species identification was done using the rDNA internal transcribed spacer (ITS) with primers ITS-1 (TCCGTAGGTGAACCTTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) [20]. PCR conditions for rDNA internal transcribed spacer (ITS) were as follows: 95 °C for 3 min, followed by 35 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and then an extension cycle of 72 °C for 10 min. PCR products were visualized on 2% agarose gel and were sequenced using Sanger (General biol, China). Sequences were aligned using Codon Code Aligner software (Codon Code Corp., Centerville, MA, USA) and compared to data in GenBank. Genotyping
of the isolates was performed by MUSCLE alignment of the ITS sequences constructing by MEGA 11 program (version 11.0.13), using 1000 bootstrap replications with *T. benhamiae* as outgroup.

**MALDI-TOF MS**

MALDI-TOF MS data include *T. mentagrophytes* (*n* = 18), *T. interdigitale* (*n* = 8), *T. indotineae* (*n* = 17), was performed by the formic acid extraction method according to the manufacturer’s instruction with minor modifications[21]. Dermatophytes isolates were cultured on SDA for 7 days at 28°C. After growth, 5mg of cultured hyphae and spores were collected in a 1.5 mL centrifuge tube containing 300µL deionized water. The sample was carefully grinded, and 900µL of absolute ethanol was added to the pellet. After mixing and vortexing, the sample was centrifuged at 12,000×g for 2 min, and the supernatant was discarded. After drying the residue at room temperature for 5 minutes, 80µL of 70% formic acid was added, and vortexed for 20 seconds. Subsequently, standing of the liquid at room temperature for 10 minutes, 80µL of acetonitrile was added, and vortexed for 20 seconds. Afterward, the sample was centrifuged at 12,000 × g for 2 min, and 1µL of the supernatant was transferred on a target plate (Zybio, Chong Qing, China) after drying naturally in a bio-safety cabinet, 1µL of matrix solution was added on the above dried supernatant, and dried again at room temperature. MALDI-tree was built up by software inside of AUTO MS1000 with hierarchical cluster analysis.

**Antifungal Susceptibility Testing**

The broth microdilution method was used following the M38-A2 CLSI protocol to examine and assess MIC (Minimum Inhibitory Concentration) in the strain. Drug stocks were prepared in dimethyl sulfoxide (DMSO); the range of antifungals was as follows: 0.008 ~ 8 mg/L for amorolofine (AMF) and ciclopirox-olanine (CLO); 0.016 ~ 16 mg/L for itraconazole (ITR), miconazole (MCZ) and ketoconazole (KCZ); 0.031 ~ 32 mg/L for terbinafine (TBF), naftifine (NAF) and griseofulvin (GRI); and 0.062 ~ 64 mg/L for fluconazole (FCZ). Then the prepared suspensions (100 µL) of each strain containing 1 ~ 3*10^3 ml/CFU spore were added to the wells. The plates were incubated at 35°C and visually assessed for fungal growth after 96 h.

**SQLE sequencing and expression of CYP51A / CYP51B**

DNA fragments encoding *T. indotineae*, i.e., SQLE (squalene epoxidase), *Cyp51A* (TinCYP51A) and *Cyp51B* (TinCYP51B) were amplified by PCR with a standard protocol. The primers used are listed in Table 2 [6, 22]. PCR was carried out in 50µL reaction mixture volumes, and conditions included an initial denaturation step for 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 59°C, and 100 s at 72°C. PCR products were purified using gel electrophoresis (QIAquick) and sequenced using the primers listed in Table 2. Total RNA was extracted using the universal plant total RNA isolation kit (Vazyme, Nanjing, China). First-strand cDNA was synthesized using a RT mix kit with gDNA clean for qPCR Ver.2 (Accurate Biology, China). The qRT-PCR analysis was performed using SYBR Green Pro Taq HS qPCR kit (Accurate Biology, China) on a Step One real-time PCR system (BIO-RAD, America) under standard
conditions, according to the manufacturer’s recommendations. Gene and amino acid sequences of *SQLE* and those of *Cyp51A* and *Cyp51B* of *T. indotineae* were compared with references deposited in GenBank (accession numbers MT700509.1, OK539857.1 and OK500342.1, respectively).

**Statistics**

Figures were created by using GraphPad Prism version 8.0.0 (GraphPad Software), and ANOVA tests were performed using SPSS version 26 (IBM); differences were considered statistically significant at P values of < 0.05.

**Results**

**Patient**

A 47-year-old man, from Yancheng City Jiangsu Province China, without underlying disease was admitted to Hospital for Skin Diseases, Chinese Academy of Medical Science, in July 2022. This patient, without history travel abroad or contact with imported goods, had been suffering from a recurring centrifugal annular erythema surrounded by scaly plaques on his thighs, hips and neck for more than 3 months. He had been treated by terbinane hydrochloride cream, itraconazole capsules, and triamcinolone acetonide-econazole cream, and clobetasol propionate-ketoconazole cream irregularly, in local hospital or clinics. In our hospital, microscopic examination and cultures of skin scales were performed to confirm the dermatophytosis by *T. mentagrophytes* complex (identified as *T. indotineae* by subsequent molecular analysis); the clinical diagnosis was tinea corporis and t. cruris. Initially, terbinafine tincture and naftifine hydrochloride & ketoconazole cream (twice a day) were prescribed for 2 weeks, but the result was not satisfactory. Then, oral terbinafine (250 mg/day) and ketoconazole cream (twice a day) were applied; the lesions largely disappeared while leaving scattered pruritus erythema and hyperpigmentation. About 10 days later, the patient returned with complaints of recurrence. Oral terbinafine (250 mg/day) and terbinafine cream (twice a day) were applied for 2 weeks. This treatment was effective, but the infection relapsed after discontinuation. Consequently, we upgraded the treatment plan as an itraconazole pulse therapy (0.2 g/twice a day) for 5 weeks; this resulted in no relapse of skin lesions after terminating the medicine after 2 months following up (Fig. 3).

**Fungal Identification And Phylogenetic Analysis**

The strain was initially suspected to be *T. indotineae* due to its resistance to terbinafine treatment. Verification was achieved by DNA sequencing and MALDI-TOF MS. Phylogenetic comparison based using ITS sequences showed that the *T. indotineae* isolate from China was identical to *T. mentagrophytes* internal transcribed spacer type VIII including the *T. indotineae* type strain (CBS 146623). The ITS regions of our isolate had 100% similarity with isolates from India (GenBank accession number MW346137.1), Japan (LC508024.1), Iran (OM366332.1), Germany (MT330250.1), Iraq (MT367568.1), Denmark...
Antifungal Susceptibility Testing

Consistent with our speculation, the strain *T. indotineae* had very high minimal inhibitory concentration (MIC) values of all the tested drugs (Table 1). The MICs of terbinaine were >32µg/mL, naftifine 16 µg/mL, itraconazole 1µg/mL, posaconazole 0.5µg/mL, ketoconazole 2µg/mL, miconazole 2µg/mL, amorolfine 0.5µg/mL, ciclopiroxolamine 1µg/mL, griseofulvin 4µg/mL and fluconazole >64µg/mL, respectively. In contrast, all reference strains had low MICs for terbinaine (0.001 µg/mL), posaconazole (0.06 ~ 0.25µg/mL), and itraconazole (0.06 ~ 0.25µg/mL). We confirmed terbinaine resistance by the EUCAST method (https://eucast.org) with MICs of 2 ~ > 8µg/mL. Our analysis of antifungal susceptibility of the Chinese *T. indotineae* strain indicated high resistance to terbinaine, and suspected drug resistance to itraconazole and ketoconazole.

Sequencing The Sqle Gene And Expression Of Cyp51a And Cyp51b

The *SQLE* gene was amplified using previously reported primer pairs [22]. This revealed that the isolates contained one of a known amino acid substitution (Phe<sup>397</sup>Leu, mutation 1191C > A) (Fig. 6a). Overexpression of CYP51A and CYP51B in azole-resistant *T. indotineae* was also identified, but not in azole-susceptible isolates, as gene amplification of CYP51A and CYP51B may play an important role in reducing sensitivity of *T. indotineae* strains to azoles (Fig. 6b,c). We did not find the previously reported point mutations in CYP51A and CYP51B encoding sterol 14α-demethylase and involving in resistance.

Discussion

In this study, this report has three valuable characteristics. First, we described the indigenous isolation of a TBF-resistant *T. indotineae* strain from a Chinese patient with tinea corporis and t. cruris; second, this strain (designated CAMSTi-001) exhibited double resistance with TBF MIC of > 32 µg/mL and ITZ MIC of 1.0 µg/mL; third, regardless of the refractory and relapse for multiple times, the patient finally clinical cured by oral itraconazole pulse therapy and topical clotrimazole cream for 5 weeks.

Since 2019, the number of *T. indotineae* cases have been reported in India and increased to the whole world. Currently, 76% of the known sequences have been identified in India, 12.8% in the Middle East, 9.6% in Europe, and 1.1% in other countries [23]. Reported ITS genotypes were downloaded from the NCBI database to build a phylogenic tree. The ITS sequence revealed that our isolate was *T. indotineae*, with 100% similarity with isolates from Asian and European countries. China as a large country with various geographical terrains, the world's largest population, boarding India to the southwest, imbalance of economic developing world et al. There may exist drug resistance in China for long time at a background
similar to India of breeding drug-resistance *T.indotinea*, which should arouse our attention to this new emerging pathogen. It is remarkable that our patient did not travel anywhere and denied contact with animals. It means that China automated induce the TBF and ITZ resistant *T. indotinea*. Given the large population of China, this problem may be just the tip of the iceberg and potential emergence may be expected. The outbreak of *T. indotinea* in India is probably mainly due to the unreasonable use of combination creams containing high-potency steroids together with antimycotic and antibiotic agents [24]. In our patient, he once used clobetasol propionate-ketoconazole cream irregularly, which may have promoted resistance. What is worrying is that such abuse is very common in China, as such creams are easily available for any patient to obtain over-the-counter at local pharmacies *T. indotineae* is more prone than other species of the *T. mentagrophytes* complex to show resistance to terbinafine resulting in the necessity of rapid identification. Tang et al [21] showed that, other than sequencing, only MALDI-TOF MS can distinguish the species at sufficient (96.97%) confidence from closely related species. In our case, we first used MALDI-TOF MS to identify the isolate and subsequently compared rDNA ITS sequences as taxonomic standard. MALDI-TOF MS is a convenient method and proved sufficient for reliable identification. The *T. indotineae* specific PCR [25] was positive with our isolate.

In strains retrospectively identified as belonging to the *T. mentagrophytes* species complex, a significant spread of TBF resistance ranging between 0.2%~ 81% is observed in previous statistics [6]. In accordance with our previous report [6], high MICs (16 µg/mL) of TBF were associated with mutations 1189T > C, 1191C > A, or 1191C > G, which led to amino acid substitutions Phe397Leu or Leu393Phe. Sometimes the mutation was combined with the amino acid substitution Ala448Thr. Strains with moderate to low MICs for TBF (0.125 ~ 0.5 µg/mL) showed the amino acid substitutions Phe415Val, Leu393Ser, His440Tyr or Ala448Thr. These mutations are most often found at Leu393 and Phe397 in SQLE. The isolate in the present study revealed a single mutation of the SQLE gene (Phe397Leu, mutation 1191C > A). While the resistance of *T. indotinea* to TBF remains common, it seems to be exceptional with ITC-resistance of *T. indotineae*. Ebert detected the number of 279 *T.indotinea* strains and found that the MIC of ITC was reported to be around 0.016 mg/mL in 90% of the tested strains, while 10% of the strains showed reduced susceptibility to ITC, with a MIC of 0.5 mg/L. It was emphasized that isolates resistant to both ITC and VRC were more prevalent in TRB-sensitive isolates (42%) than in TRB-resistant isolates (18%) [4]. Thus, it was not very common that the terbinafine-resistant isolate also exhibited high MICs for itraconazole, miconazole, ketoconazole and fluconazole in our case. Several mechanisms of azole resistance in human-pathogenic fungi leading to resistance have been described, including point mutations in genes encoding drug targets, overexpression of those targets, and overexpression of multidrug transporters of the ABC family or major facilitator superfamily transporters (MFS) genes involved in drug efflux [22]. Sequencing the CYP51A and CYP51B genes revealed that no mutations of this gene encoding sterol 14α-demethylase and involving in resistance, which is not consistent with the four different types of *Erg11B* mutants detected by Anke Burmester [18]. However, overexpression of CYP51A and CYP51B in azole-resistant *T. indotinea* was also identified, in accordance with the report by Yamada et al [26].
First line systemic treatment for \textit{T. indotineae} with terbinafine is not recommended due to the high number of \textit{SQLE} mutant strains. Fluconazole is also to be avoided in the systemic treatment due to the high intrinsic MIC values of \textit{T. indotineae} strains. In terbinafine-resistant cases, itraconazole as a systemic alternative has been recommended in several studies. However, a significant proportion of \textit{T. indotineae} strains also shows resistance against itraconazole. Thus, treatment for \textit{T. indotineae} will be a challenge in the future due to its dual resistance. Use of relatively new agents, such as voriconazole and luliconazole, as well as combination therapy, could be beneficial for recalcitrant \textit{T. indotineae} infections \cite{27}. In our case, we take itraconazole pulse therapy (0.2 g p.o. BID) and topical clotrimazole cream (twice a day) for 5 weeks with no recurrence of skin lesions until now. It will provide a new measure for the clinician in treating refractory and relapsing dermatophytes.

In summary, the terbinafine- and itraconazole-resistant \textit{Trichophyton indotineae} has been reported in China, which may be rapidly dissemination after stopping lockdown policy of COVID-19 in the future, with increased the risk of human-to human transmission. The longtime of itraconazole pulse therapy (0.2 g p.o. BID) and topical clotrimazole cream (twice a day) as an option for treating refractory and relapsing dermatophytes.

**Declarations**

**Acknowledgements**

This research has been supported by the China National Key R&D Program during the 14th Five-year Plan Period (Grant No.2022YFC2504804), Double-Innovation Doctor Program of Jiangsu province (Grant number JSSCBS20221924), National Natural Science Foundation of China (Grant number 81972949), CAMS Innovation Fund for Medical Sciences (2021-2IM-I-039), National Science and Technology Infrastructure of China (National Pathogen Resource Center NPRC-32), the basic scientific research fund projects of Chinese Academy of Medical Sciences (2021-PT310-006).

**Ethics statement**

The studies involving human participants were reviewed and approved by 2022-KY-037. The patient has written the informed consent in this study.

**Author contributions**

This patient was admitted, treated and followed up by G.L. The study was conceived by G.L and W.L. X.K. and G.L. analyzed and interpreted of the data; H.Z. and M.H. involved in experiment preparation, fungus identification, antifungal susceptibility testing. X.K., G.L. and G.S drafted the manuscript, G.S and C.T. revised it critically for important intellectual content. W.L., X.S. and X.L. provided funds and experiment support. We thank S.X. and Zybio Company (Chong Qing, China) for performing the MALDI-tree.

**Conflict of interest**
The authors declare no conflicts of interest.

References


253;https://doi.org/10.1016/b978-0-12-800271-1.00004-4


Tables

Table 1. MIC (mg/L) of Trichophyton species against nine drugs.

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<th>NAF</th>
<th>ITC</th>
<th>MCZ</th>
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NAF, naftine; ITC, itraconazole; MCZ, miconazole; KTZ, ketoconazole; TBF, terbinafine; AMF, Amorolfine; FCZ, fluconazole; GRI, griseofulvin; CiclopiroxOlanine(CLO);

Table 2. PCR primers used in this study
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**Figures**
The itchy annular erythema and scaly surrounding plaques on patients hips showing tinea corporis and t. cruris.

Figure 1
**Figure 2**

Figure 3

The patient's treatment progress from onset to cure.

Figure 4

The phylogenetic analysis of the *T. mentagrophytes* complex based on the sequencing of the ITS regions of the rDNA. The calculations are based on the maximum likelihood method and the General Time Reversible model. The red and yellow dots represent the strain from China and the type strain (CBS 146623), respectively.
Figure 5

MALDI-TOF tree built by software inside of AUTO MS1000 with hierarchical cluster analysis.
a. Sequencing the SQLE gene revealing amino acid substitution (Phe$^{397}$Leu, mutation 1191C>A). b, c. Expressions of CYP51A and CYP51B in azole-resistant *T. indotineae* being higher than in azole-susceptible isolates ($P \leq 0.05$).

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**Figure 6**

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