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Original Article

Azole-resistant *Aspergillus fumigatus* Containing a 34-bp Tandem Repeat in *cyp51A* Promoter is Isolated from the Environment in Japan

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ABSTRACT

Azole-resistant strains of *Aspergillus fumigatus* containing a tandem repeat in the *cyp51A* promoter and amino acid substitution(s) have been isolated in the environment worldwide; however, this type of resistant strain had never been isolated from the environment in Japan. Our previous study indicated that an azole-resistant *A. fumigatus* strain OKH50 containing a 34-bp tandem repeat in *cyp51A* promoter with L98H substitution in Cyp51A was isolated from a patient in Obihiro of Hokkaido, Japan. In this study, we collected azole-resistant *Aspergillus* spp. by air sampling from the environment in Japan. One *Aspergillus*-like colony was isolated from one of 10 sampling sites surveyed. The strain Env1 was confirmed as *A. fumigatus* by nucleotide sequencing and possessed a 34-bp tandem repeat in the promoter region of *cyp51A* with L98H substitution in Cyp51A. *A. fumigatus* Env1 has the identical short tandem repeat pattern with the OKH50 strain, indicating that these strains are closely related with each other. Additionally, the short tandem repeat pattern is closely related to Danish and Iranian environmental isolates, suggesting that azole-resistant strains have crossed transnational boundaries and are now present in Japan, and therefore, further analysis throughout Japan is required to determine the distribution of this type of azole-resistant *A. fumigatus*.

Key words : *Aspergillus fumigatus*, azole, *cyp51A*, TR34/L98H

Introduction

Aspergillus fumigatus is a ubiquitous fungus found in the environment and the leading causative agent of aspergillosis in humans and animals. Aspergillosis is one of the most common fungal infections in the world[1]. Kume and colleagues have shown that in autopsy cases, the rate of aspergillosis was the highest among fungal infections in Japan since 1994[2]. The mortality rate has been estimated to be 35–95%[1].

Azole antifungals are used not only to treat aspergillosis and other fungal infections but are also used as agricultural fungicides. Because of the extensive use of azole antifungals, azole-resistant *A. fumigatus* strains have been reported globally[3]. The mechanisms responsible for the acquisition of azole resistance are roughly classified into the selections in a patient and in the environment[4]. Tashiro and colleagues have reported the correlation between the duration of azole treatment and the susceptibility of *A. fumigatus* isolates[5], indicating that long-term exposure of patients to azoles increases the likelihood of resistance acquisition. Alternatively,

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recent studies and evidence suggest that the use of azole fungicides has driven the acquisition of azole resistance [6–10].

Most environmental isolates with azole resistance possess a tandem repeat in the *cyp51A* promoter region with amino acid substitution (s) TR_{34}/L98H or TR_{46}/Y121F/T289A [3]. The resistant strains have been isolated worldwide, e.g. in some Eurasian countries, US, and Australia [11]. As shown previously, TR_{34}/L98H-type resistant strain OKH50 was isolated from a patient in Obihiro of Hokkaido, Japan [12], and a TR_{46}/Y121F/T289A-type resistant strain has been reported as a clinical isolate in Tokyo, Japan [13]. However, these types of resistant strains had never been isolated from the environment in Japan. In our previous study, we sought to isolate these types of resistant strains from farm soil samples in Japan, but no resistant strain of *A. fumigatus* was found [14]. In this study, we attempted to isolate azole resistant *A. fumigatus* from the environment in Japan by air sampling.

Materials and methods

Air sampling was performed using M Air T Air Sampler (Merck Millipore, Billerica, MT, USA). Sabouraud dextrose agar (SDA) containing 4 μg/ml itraconazole (ITCZ) or 1 μg/ml voriconazole (VRCZ), and 100 μg/ml chloramphenicol was used for the air sampling. We chose 10 public parks in Obihiro City of Hokkaido as spots for air sampling. Air sampling was performed on Jul. 21st at 1 spot, Aug. 18th at 1 spot, Oct. 12th at 3 spots, Oct. 13th at 3 spots, and Oct. 14th at 2 spots in 2016. Fungi were collected on 2 plates from each spot from 1,000 l air over a 7-min period, and then incubated at 35℃ for up to 7 days. Colonies visually identified as *A. fumigatus* were transferred from these plates onto potato dextrose plates and were used for further analyses.

To determine minimal inhibitory concentrations (MIC) of ITCZ, VRCZ, and Amphotericin B, and minimal effective concentrations (MEC) of micafungin, we used the broth microdilution method based on CLSI M38-A2 with a slight modification. Conidia suspended in 0.05% Tween 20 were diluted in 100μl of RPMI 1,640 medium (Sigma-Aldrich Co., St. Louis, MO) buffered with MOPS (NACALAI TESQUE, Inc., Kyoto, Japan) and pH-adjusted to 7.0 (MOPS-RPMI) at 1×10^4 cells/ml and then were inoculated to each well of 96-well microplates containing medical antifungals at various concentrations (Dry Plate Eiken; Eiken Chemical Co., Ltd., Tokyo, Japan). After 48-h cultivation at 35℃, growth inhibition was determined by visual observation.

Genomic DNA from *A. fumigatus* was prepared as follows. Mycelia cultured in potato dextrose broth were collected and lyophilized. After homogenization with Biomasher IV (Nippi, Inc., Tokyo, Japan), genomic DNA extraction was performed using the procedure described previously [15]. DNA was used for PCR amplification and sequencing of internal transcribed spacer and D1/D2 regions, and partial nucleotide sequences of β-tubulin, *rodA*, and calmodulin genes. For short tandem repeat (STR) analysis, we determined the number of STRs and analyzed them as described previously [13, 16].

Results and discussion

Env1 is the strain isolated from air-sampled plate on Aug. 18th. Macroscopic and microscopic examinations indicated characteristics of *A. fumigatus* (Fig. 1). To confirm whether the strain was *A. fumigatus sensu stricto*, we determined partial nucleotide sequences of β-tubulin, calmodulin, and *rodA* genes. As a result, Env1 strain was accurately identified as *A. fumigatus*.

Both MICs of ITCZ and VRCZ against Env1 strain were > 8 μg/ml (Table 1). These results suggest that Env1 is an azole-resistant *A. fumigatus* isolated from the environment in Japan. Next, we determined the nucleotide sequence of *cyp51A* and the promoter region. The promoter region of *cyp51A* gene contained a 34-bp tandem repeat, and L98H substitution was found in Cyp51A (TR_{34}/L98H). *A. fumigatus* TR_{34}/L98H strain has widespread occurrence in the world; however, this type of strain has never been isolated from the environment in Japan. Recently, we reported that *A. fumigatus* OKH50, a TR_{34}/L98H strain, was isolated from a clinical specimen in Obihiro, Japan [12]. *A. fumigatus* Env1 showed identical STR pattern to *A. fumigatus* OKH50, which is clustered with Danish and Iranian environmental isolates by previous STR analysis (Table 2)[12]. As shown in Fig. 2, no difference in colony morphology between *A. fumigatus* Env1 and OKH50 was observed. The patient with OKH50 had not traveled overseas, suggesting that azole-resistant *A. fumigatus* was brought from overseas to the environment in

Fig. 1. Macroscopic (a) and microscopic (b and c) images of *A. fumigatus* Env1 strain. The colony (a) and conidial heads (b and c) were observed after culturing at 25℃ for 6 days.

Table 1. MIC or MEC (µg/ml) of antifungals against *A. fumigatus* Env1 and OKH50

Strain	Itraconazole	Voriconazole	Amphotericin B	Micafungin[a]
A. fumigatus Env1	> 8	> 8	0.5	≤ 0.015
A. fumigatus OKH50[b]	> 8	4 or 8	0.5	≤ 0.015

[a] MEC are shown for micafungin.
[b] The MIC data are shown in the manuscript by Toyotome et al [12].

Table 2. STR patterns of TR_{34}/L98H *A. fumigatus* strains clustered with Env1 and OKH50 strains

Strain	2A	2B	2C	3A	3B	3C	4A	4B	4C	Country	Source	Reference
Env1	14	21	8	28	9	6	8	10	18	Japan	Environmental	This study
OKH50	14	21	8	28	9	6	8	10	18	Japan	Clinical	12
T18_R	10	20	8	32	9	7	8	9	19	Denmark	Environmental	17
Hamid 02	14	20	8	32	9	6	8	10	20	Iran	Environmental	18
2005-456307L	14	23	8	30	9	6	8	10	20	Netherlands	Clinical	3
2005-456473	14	20	8	40	9	11	8	10	20	Netherlands	Clinical	3
04-202165	14	21	8	31	9	6	8	10	20	Australia	Clinical	19
R5-07-4_R	14	20	8	40	9	11	8	10	20	Denmark	Clinical	17

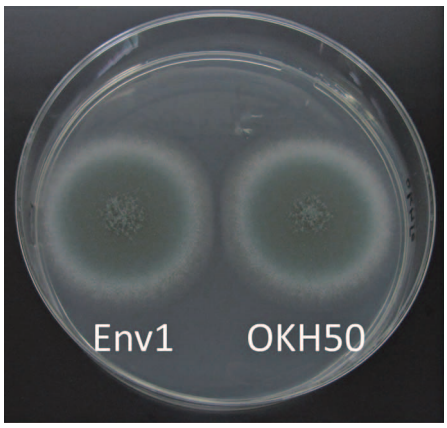

Fig. 2. Comparison of colony morphology between *A. fumigatus* Env1 (Left) and OKH50 (Right) cultured on potato dextrose agar at 35℃ for 2 days.

Japan, and the patient had acquired Env1/OKH50 strain locally.

Lastly, in this study, we examined air sampling on agar plates with ITCZ or VRCZ. In our previous study, we sought to isolate resistant *A. fumigatus* strains from soil samples from farms that used azole fungicides [14]. These studies indicate that the isolation of resistant strains from air samples, as well as from soil samples, is important for this type of survey.

In summary, one azole-resistant isolate was found from the environment in Obihiro, Japan. We found a 34-bp tandem repeat and L98H substitution in the *cyp51A* promoter of *A. fumigatus* Env1. Additionally, the strain showed identical STR

pattern to azole-resistant OKH50 strain previously isolated in Obihiro, Japan. The strain is the first isolate with the tandem repeat from the environment in Japan. Azole-resistant *A. fumigatus* in the environment has not been surveyed in other areas of Japan. A broader analysis is needed to understand the current status in Japan.

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Conflict of Interest

Self-declared COI content: none.

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