Prevalence, mechanisms, and genetic relatedness of the human pathogenic fungus *Aspergillus fumigatus* exhibiting resistance to medical azoles in the environment of Taiwan

Hsuan-Chen Wang,[1] Jui-Chang Huang,[2] Yong-Hong Lin,[3] Yu-Hsin Chen,[4] Ming-I Hsieh,[1] Pui-Ching Choi,[1] Hsiu-Jung Lo,[1] Wei-Lun Liu,[5] Ching-Shan Hsu,[6] Hsin-I Shih,[7] Chi-Jung Wu,[1,8,*] Yee-Chun Chen[9]

[1]National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Zunan, Taiwan

[2]Tainan District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Tainan, Taiwan

[3]Kaohsiung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Pingtung, Taiwan

[4]Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Changhua, Taiwan

[5]Department of Emergency and Critical Care Medicine, Fu Jen Catholic University Hospital, New Taipei, Taiwan

[6]Department of Environmental Resource Management, Chia Nan University of Pharmacy and Science, Tainan, Taiwan

[7]Department of Emergency Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[8]Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[9]Department of Internal Medicine, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan

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Running title: Azole-resistant *Aspergillus fumigatus* in Taiwan

Originality-Significance Statement: This study has three major merits. First, although Asian countries contribute 24% of the global agricultural fungicide usage, knowledge regarding the molecular epidemiology of azole-resistant *A. fumigatus* in Asia is limited. Azole fungicides have been widely used for decades in Taiwan, which is an East Asian island country. This study represents one of the largest nationwide environmental surveillance studies investigating azole-resistant *A. fumigatus* in Asia and reveals how resistance develops and evolves on a geographically isolated island in the era of globalization. Second, in contrast to the common ancestral lineage of azole-resistant *A. fumigatus* carrying TR_{34}/L98H mutations across Europe, our study found heterogeneity in azole-resistant isolates, revealing both the global spread of TR_{34}/L98H isolates and the occurrence of TR_{34}/L98H/S297T/F495I isolates that belong to local microsatellite genotypes. Furthermore, this is the first study to demonstrate the clonal expansion of TR_{34}/L98H/S297T/F495I isolates belonging to a distinct local microsatellite genotype; these isolates have become widely disseminated and have been recovered from patients. Third, azole-resistant isolates without *cyp51A* mutations were also examined for *cyp51A*-independent resistance mechanisms, which are rarely addressed in environmental surveillance studies. The constitutive overexpression of *AfuMDR3* and *atrF*, two drug efflux transporter genes, in two individual resistant isolates provides additional evidence supporting their roles in azole resistance. Overall, this study emphasizes the need for periodic environmental surveillance of azole resistance in *A. fumigatus* at the molecular level in regions in which azole fungicides are applied to detect epidemiological trends and aid the development of treatment recommendations for human *Aspergillus*-related diseases.

Key words: *Aspergillus fumigatus*, *atrF*, azole fungicide, azole resistance, efflux transporter, MDR3, Taiwan, TR_{34}/L98H

***Author for correspondence:**

Chi-Jung, Wu, MD, PhD

5F., No.367, Shengli Road, North District, Tainan, 70456, Taiwan; National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Taiwan

TEL: 886-6-7000123 ext. 65220;

FAX: 886-6-208-3466;

E-mail: wucj@nhri.org.tw

Summary

Emerging azole resistance in *Aspergillus fumigatus* poses a serious threat to human health. This nationwide surveillance study investigated the prevalence and molecular characteristics of azole-resistant *A. fumigatus* environmental isolates in Taiwan, an island country with increasing use of azole fungicides. Of the 2,760 air and soil samples screened from 2014-2016, 451 *A. fumigatus* isolates were recovered from 266 samples, and 34 isolates from 29 samples displayed resistance to medical azoles (itraconazole, voriconazole, or posaconazole). The resistance prevalence was 10.9% and 7.5% in *A. fumigatus*-positive samples and isolates, respectively. Most (29, 85.3%) azole-resistant isolates harbored TR_{34}/L98H mutations, which were widely distributed, clustered genetically with clinical isolates, and had growth rates that were similar to those of the wild-type isolates. Microsatellite genotyping revealed both the global spread of the TR_{34}/L98H isolates and the occurrence of TR_{34}/L98H/S297T/F495I isolates belonging to local microsatellite genotypes. *AfuMDR3* and *atrF*, two efflux transporter genes, were constitutively upregulated in two individual resistant isolates without *cyp51A* mutations, highlighting their potential roles in azole resistance. These results emphasize the need for periodic environmental surveillance at the molecular level in regions in which azole fungicides are applied, and agricultural fungicide management strategies that generate less selective pressure should be investigated.

Introduction

Invasive aspergillosis is an important human fungal disease causing substantial mortality that affects an ever-increasing population of immunocompromised patients, and most infections are caused by *Aspergillus fumigatus*, a saprophytic mold that produces airborne conidia and is ubiquitously distributed in the environment (Verweij *et al.*, 2015). The recommended therapies for aspergillosis include voriconazole and other mold-active azoles, which target sterol 14α-demethylase encoded by *cyp51A* (Verweij *et al.*, 2015). The prevalence of azole-resistant *A. fumigatus* has significantly increased over the past two decades, threatening the effectiveness of first-line antifungal therapy (Verweij *et al.*, 2016). Azole resistance in *A. fumigatus* may develop during patient therapy with medical azoles or through exposure to azole fungicides in the environment. TR_{34}/L98H and TR_{46}/Y121F/T289A mutations, which combine tandem repeats in the *cyp51A* promoter region and amino acid substitution(s) in its coding sequence, are the two major environmentally-derived resistance mutations that are prevalent among both clinical and environmental azole-resistant isolates (Chowdhary *et al.*, 2017). Additionally, *cyp51A*-independent resistance mechanisms, such as the upregulation of drug efflux transporters, have also been increasingly recognized in clinical and environmental settings (Slaven *et al.*, 2002; Fraczek *et al.*, 2013; Meneau *et al.*, 2016). Considering the global emergence of azole-resistant *A. fumigatus* isolates and the dismal clinical outcomes associated with delayed effective treatment, experts discourage voriconazole monotherapy for the treatment of invasive aspergillosis in regions with a high environmental resistance rate (>10%) (Verweij *et al.*, 2015).

To halt the progression of azole resistance and identify potential targets for the discovery of new antifungals, understanding how resistance develops and evolves in different parts of the world is important. Asian countries contribute 24% of the global agricultural fungicide usage (Stensvold *et al.*, 2012). However, knowledge regarding the prevalence and clonality of

azole-resistant *A. fumigatus* in Asia based on nationwide environmental surveillance is limited, except for reports from China and India (Chowdhary *et al.*, 2012; Chen *et al.*, 2016a). Taiwan is an East Asian island country geographically separated from the main Eurasian countries but with frequent international business and travel. Agriculture is one of the main industries in Taiwan, and azole fungicides have been widely used in Taiwan for at least three decades. Our earlier work identified clinical TR_{34}/L98H isolates that were genetically related or unrelated to overseas isolates from azole-naïve patients, suggesting an environmental niche for such isolates (Wu *et al.*, 2015). Thus, undertaking a wide environmental survey of azole resistance in *A. fumigatus* in Taiwan in the era of globalization is warranted and ecologically interesting. In this study, we aimed to delineate the prevalence and resistance mechanisms of azole-resistant *A. fumigatus* environmental isolates in Taiwan and their genetic relatedness to strains from international collections.

Materials and Methods

A. fumigatus isolates

Air and soil samples from different locations across Taiwan were investigated from January 2014 to June 2016. Air samples were obtained from the indoor areas of 28 hospitals, communities, and farmlands using an air sampler (1,000 L in 10 min) (Air IDEAL®; bioMérieux, Marcy l'Etoile, France), and soil samples were collected from communities and farmlands. Two grams of each soil sample was suspended in 4 mL of 0.85% NaCl and 0.3% Triton X-100, vortexed, and allowed to settle for 30 seconds. The cultivation of *Aspergillus* conidia from the air and soil samples (100 μL of each supernatant) was performed on Sabouraud dextrose agar (SDA) plates supplemented with 50 mg/L chloramphenicol (Sigma-Aldrich, St. Louis, USA), which were incubated at 37°C for one day and then at 50°C for up to 2 days to select for heat-tolerant *A. fumigatus.* A maximum of 3 colonies per plate

were purified. *A. fumigatus* was identified by morphological characteristics and sequence analysis of the internal transcribed spacer region and calmodulin genes (Balajee *et al.*, 2007).

Antifungal susceptibility testing

To detect the azole-resistant isolates, azole-containing agar plates were used. The aforementioned colonies were inoculated onto three SDA plates supplemented with respectively itraconazole (4 mg/L), voriconazole (1 mg/L), and posaconazole (0.5 mg/L) (all Sigma-Aldrich, St. Louis, USA), and incubated at 37°C. Colonies that grew after 2-4 days on any of the azole-containing agar plates were selected for susceptibility testing. The minimum inhibitory concentrations (MICs) of the medical azoles (itraconazole, posaconazole, and voriconazole), amphotericin B (Sigma-Aldrich, St. Louis, USA), and azole fungicides (difenoconazole and tebuconazole [both Chem Service, Pennsylvania, USA]) were determined using the Clinical Laboratory Standard Institute (CLSI) broth microdilution method (M38-A2 document) (CLSI, 2008). The final drug concentrations used for testing were 0.015 to 8 mg/L for amphotericin B, itraconazole, posaconazole, and voriconazole and 0.06 to 32 mg/L for difenconazole and tebuconazole. According to the clinical breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), an isolate with an itraconazole, voriconazole, or posaconazole MIC of >2, >2, or >0.25 mg/L, respectively, was considered "azole-resistant" (EUCAST).

Resistance mechanisms

All azole-resistant isolates and randomly selected azole-susceptible *A. fumigatus* isolates were examined for mutations in *cyp51A* and its promoter region using previously described methods (Snelders *et al.*, 2010). For resistant isolates without typical *cyp51A* mutations known to be associated with azole resistance, *cyp51A*-independent resistance mechanisms were investigated.

hapE was sequenced as described previously (Camps *et al.*, 2012b), *srbA* was sequenced using the laboratory-designed PCR primers and conditions described in Table S1 (Willger *et al.*,

2008), and both sequences were compared to those of *A. fumigatus* Af293 to detect any mutations. Biofilms were prepared for the gene expression analysis of *cyp51A*, *cyp51B*, and drug efflux transporter genes (*AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, *atrF*, *cdr1B*, and *MFS56*). In brief, for each isolate, three 75T flasks were inoculated with 25 ml of spore suspensions (10^5 spores/mL) prepared in RPMI-1640 medium and incubated at 37°C until a layer of adherent mycelia (biofilm) formed (usually 16 hours). The biofilms were then washed thoroughly three times with sterile PBS by repeated pipetting to remove non-adherent cells. After washing, itraconazole was added to the first flask to a final concentration of 4 mg/L, voriconazole to the second flask at a concentration of 1 mg/L, and an equivalent volume of DMSO to the third flask as a control. After 8 hours of treatment, the biofilm material was scraped and removed from each flask and placed into a 2-ml screw-cap vial. Excess medium was removed by centrifugation at 13,000 rpm for 2 min. RNA was extracted from 100 mg of the fungal mycelia using a MagCore Total RNA Cultured Cells Kit (RBC Bioscience, New Taipei City, Taiwan). cDNA was subsequently synthesized with GoScript™ reverse transcriptase (Promega, Wisconsin, USA) according to the manufacturer's instructions. The basal and azole-induced mRNA expression levels were then assessed by quantitative reverse transcription (RT)-PCR using the 2X KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Massachusetts, USA) with previously described primers and modified PCR conditions (95°C for 3 min, followed by 40 cycles of 95°C for 5 sec and 65°C for 30 sec) (Fraczek *et al.*, 2013). For each strain and each gene examined, three biological replicates were performed with three technical replicates. Transcript levels were normalized to a housekeeping gene, β-tubulin, and compared to *A. fumigatus* ATCC MYA-3626, except for expression of the *AfuMDR4* gene, which was compared to ATCC 16903. The results were analyzed using the $2^{-\triangle\triangle Ct}$ method. Five susceptible strains and 2 TR_{34}/L98H isolates were also evaluated for *cyp51A*-independent resistance mechanisms as controls.

Microsatellite genotyping

Genotyping was performed based on a cluster analysis of nine *A. fumigatus* short tandem repeat (STR) markers with interlaboratory standardization, as described previously (de Valk *et al.*, 2005; de Valk *et al.*, 2009). For phylogenetic analysis, we included environmentally resistant and randomly selected susceptible isolates in this study along with 13 local clinical isolates (including 7 TR_{34}/L98H isolates) identified in our collection from 2011-2016 and 23 isolates from other countries (Chowdhary *et al.*, 2012; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Kidd *et al.*, 2015; Steinmann *et al.*, 2015; Chen *et al.*, 2016a; Alvarez-Moreno *et al.*, 2017; Toyotome *et al.*, 2017). The dendrogram was prepared using the Unweighted Pair Group Method with an Arithmetic mean (UPGMA) algorithm using BioNumerics v7.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

Growth assays

To assess the fitness costs of azole resistance, radial growth and sporulation were observed in selected TR_{34}/L98H isolates of different microsatellite genotypes, resistant isolates without *cyp51A* mutations, and susceptible reference and clinical strains. A conidial suspension (10 μL, 10^5 conidia/mL) was inoculated in the center of a SDA plate and incubated at 35°C. The diameter of the growing colony and sporulation were examined every 24 hours for up to 72 hours.

This study was approved by the Institutional Review Board (EC1040502-E) of the National Health Research Institutes, Taiwan.

Results

A total of 2,760 environmental samples, comprising mainly soil samples (2480, 89.9%) from farmlands, were collected, among which 564 (20.4%), 452 (16.4%), 1232 (44.6%), and 512 (18.6%) samples were from northern, central, southern and eastern Taiwan, respectively.

Overall, 451 *A. fumigatus* isolates were recovered from 266 samples, and 34 isolates from 29 samples displayed azole resistance. Resistant isolates were recovered from all four geographic regions of Taiwan, mainly from farmland soils (31, 91.2%) but also from air in urban communities (3, 8.8%). Overall, the prevalence of azole resistance was 10.9% and 7.5% in *A. fumigatus*-positive samples and isolates, respectively, and compared with that in the other regions, the prevalence was the lowest in northern Taiwan (Figure 1; Table S2).

The majority (29, 85.3%) of azole-resistant isolates harbored TR_{34}/L98H mutations with or without S297T/F495I substitutions, which are described as TR_{34}/L98H isolates in this study. All TR_{34}/L98H isolates were resistant to both itraconazole and posaconazole, and 11 (37.9%) isolates (10 with TR_{34}/L98H mutations and one with TR_{34}/L98H/ S297T/F495I mutations) were also resistant to voriconazole and exhibited the pan-azole resistant phenotype. Notably, the TR_{34}/L98H/ S297T/F495I isolates had lower voriconazole MICs than the TR_{34}/L98H isolates without S297T/F495I substitutions, as demonstrated by the lower geographical mean MIC (1.69 *vs.* 3.56 mg/L) and MIC_{90} (2 *vs.* 4 mg/L). A reduced susceptibility to the azole fungicides tebuconazole and difenoconazole was also demonstrated in all TR_{34}/L98H isolates (Table 1).

Five (14.7%) resistant isolates, SD359, SD116A3, TN061-2, KP158A2, and KP183A1, did not harbor the *cyp51A* mutations associated with azole resistance, and all isolates, except for SD116A3, also expressed reduced susceptibility to tebuconazole and difenoconazole (Table 2). SD359 and TN061-2 were pan-azole-resistant, while SD116A3 was resistant to posaconazole and voriconazole, and KP158A2 and KP183A1 were resistant to posaconazole. Of the 45 susceptible isolates, the majority (37, 82.2%) carried wild-type *cyp51A*, and nucleotide polymorphisms were identified in a small number of isolates (8, 17.8%) (Table 1).

Of 5 resistant isolates without *cyp51A* mutations and 7 control strains examined for *cyp51A*-independent mechanisms, sequence analysis did not identify amino acid substitutions

in *hapE*, but analysis of *srbA* revealed the presence of V37D or E957D amino acid substitutions in both the susceptible and resistant isolates and an additional L68F substitution in TN061-2 (Table 2).

Gene expression analyses revealed remarkable upregulation of *AfuMDR3* expression in SD359 and *atrF* expression in SD116A3 (Figure 2). SD359 had higher basal (5.5x), itraconazole-induced (25.4x), and voriconazole-induced (4.5x) *AfuMDR3* expression levels; SD116A3 had higher basal (93.7x), itraconazole-induced (20.9x), and voriconazole-induced (20.0x) *atrF* expression levels. In addition, SD359 had 2 to 5.5-fold increases in its basal expression of A*fuMDR2, AfuMDR3,* and *atrF*. SD116A3 had increased basal expression of *cyp51A*, *cyp51B, AfuMDR1*, *AfuMDR2*, and *AfuMDR4* (2.5-5.0x), *cdr1B* (11.2x), and *MFS56* (14.3x). KP183A1 and the susceptible isolate F02411 had similar patterns of increased basal expression of *cyp51B, AfuMDR1*, *AfuMDR2, AfuMDR4, cdr1B*, *atrF*, and *MFS56*, which were 2.6-8.3x and 3.6-8.8x higher for KP183A1 and F02411, respectively. The transporters in TN061-2 and KP158A2 were only upregulated to a lesser extent (Figure S1).

Microsatellite genotypic analysis revealed heterogeneity and two major clades among the TR_{34}/L98H isolates in Taiwan (Figure 3). The clade in the upper part of the phylogenetic tree mainly includes TR_{34}/L98H/ S297T/F495I isolates from Taiwan and China; the clade in the lower part of the tree mainly contains TR_{34}/L98H isolates without S297T/F495I substitutions from Taiwan and other Eurasian countries, Australia, and Colombia. Twenty-nine TR_{34}/L98H isolates could be grouped into 5 major microsatellite genotypes (A-E) (isolates with >90% similarity based on STR patterns) and others. Using ≥90% as a similarity cut-off, the majority (20, 69%) (genotypes A, C, E, and others) of the TR_{34}/L98H isolates in this study were genetically distinct from overseas TR_{34}/L98H isolates, while a minority (9, 31%) (SD264A1, TD027-1 and genotypes B and D) clustered with TR_{34}/L98H isolates from other Eurasian countries. Notably, a major cluster was identified (genotype A), which contained 11 isogenic or

nearly isogenic clinical (n=3) and environmental (n=8) TR_{34}/L98H/ S297T/F495I isolates recovered from four regions across Taiwan. Genetic clustering of clinical and environmental TR_{34}/L98H isolates was also observed with the genotype B2, C, D, and E isolates. Interestingly, allele-sharing of 3 to 6 of the nine microsatellite markers was found between the susceptible isolates and the TR_{34}/L98H isolates, such as KP264A1 with AN0106-6 (6 alleles), KP264A1 with SD246A1 (5 alleles), TD133-1 with SD246A1 (4 alleles), and TY182-1 with SD202A3/SD202A2 (5 alleles). Isolates of mixed microsatellite genotypes or STR patterns may co-exist in the same sample, including HL102-3 and HL102-1 (genotypes B1 and B2), SD202A1 and SD202A2/SD202A3 (genotypes A and C), and TY366B-2 and TY366B-3 (genotype D). Five resistant isolates without *cyp51A* mutations did not cluster. Notably, the low-level azole-resistant isolate KP183A1 shared an identical STR pattern and *cyp51A* polymorphism with susceptible isolate KP189A1, which was recovered from the same geographical area (Tables 1 and 2).

Growth assays revealed similar growth rates for the TR_{34}/L98H isolates with different microsatellite genotypes, the resistant isolates without *cyp51A* mutations, except for SD116A3, and the susceptible control strains, except for F02411 (Figure S2). All of the tested strains had apparent sporulation at 48 hours.

Discussion

This study represents one of the largest nationwide environmental surveillance studies of azole-resistant *A. fumigatus* in Asia. The prevalence rates of azole resistance, 10.9% and 7.5% based on *A. fumigatus*-positive samples and isolates, respectively, are within the range of environmental resistance rates reported for other Eurasian countries as well as for Colombia (6% to 14%) (Snelders *et al.*, 2009; Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Bader *et al.*, 2015; Alvarez-Moreno *et al.*, 2017;

Tangwattanachuleeporn *et al.*, 2017), but they are higher than the environmental resistance rate in China (1.4%) (Chen *et al.*, 2016a). TR_{34}/L98H mutations constitute the major resistance mechanism found in this study, which is similar to the abovementioned reports, except for in Colombia, where the TR_{46}/Y121F/T289A mutations, which were mostly recovered from the azole fungicide-containing soils of flower crops, dominated (Alvarez-Moreno *et al.*, 2017). As expected, the TR_{34}/L98H isolates exhibited resistance to both itraconazole and posaconazole and reduced susceptibility to the azole fungicides difenoconazole and tebuconazole (Snelders *et al.*, 2012; van Ingen *et al.*, 2015). The lower voriconazole resistance rate (37.9%) found here might be explained by the inclusion of a higher portion of TR_{34}/L98H/ S297T/F495I isolates that were associated with lower voriconazole MICs than TR_{34}/L98H isolates without S297T/F495I substitutions observed here and in an earlier study (Abdolrasouli *et al.*, 2015).

The first occurrence of TR_{34}/L98H isolates can be traced to 1998 in the Netherlands (Verweij *et al.*, 2016). Studies have suggested that a common ancestral lineage exists for TR_{34}/L98H isolates across Europe, and a distinct local microsatellite genotype exists for TR_{34}/L98H isolates across India (Camps *et al.*, 2012a; Chowdhary *et al.*, 2012). In contrast to Europe and India, our data revealed both the global spread of TR_{34}/L98H isolates and the occurrence of TR_{34}/L98H/S297T/F495I isolates belonging to local microsatellite genotypes, a phenomenon similar to that observed in China (Chen *et al.*, 2016a). In addition, to the best of our knowledge, this is the first study to demonstrate the clonal expansion of TR_{34}/L98H/S297T/F495I isolates that belong to the locally-evolved microsatellite genotype A; these isolates have become widely disseminated and been recovered from patients. Two genotype A isolates, B44 and B51, caused fatal pulmonary aspergillosis in an azole-naïve patient with diabetes and cirrhosis in 2012 (Wu *et al.*, 2015). Notably, most of the TR_{34}/L98H isolates in Taiwan have evolved into several local microsatellite genotypes that are distinct from overseas isolates, including the clones prevalent in India and China (Chowdhary *et al.*,

2012; Chen *et al.*, 2016a). Recent sexual crossing experiments demonstrated that TR_{34}/L98H isolates could outcross with azole-susceptible isolates of different genetic backgrounds, and TR_{46}/Y121F/T289A isolates of opposite mating types could outcross with each other, resulting in a generation of new or microvariant microsatellite genotypes (Camps *et al.*, 2012a; Zhang *et al.*, 2017). The occurrence of sexual crossing is supported by observations of shared alleles at 3 to 6 of the nine microsatellite markers between the susceptible isolates and TR_{34}/L98H isolates in this study. After sexual reproduction, the repeat numbers of microsatellite markers may undergo subtle changes during asexual reproduction (Camps *et al.*, 2012a), which provides a possible explanation for the minor differences observed here for the repeat numbers in microsatellite markers between resistant isolates within each microsatellite genotype or within the same sample. Collectively, the genetic diversity of TR_{34}/L98H isolates in Taiwan may have resulted from continuous sexual and asexual cycles in the field over the years. Moreover, adverse fitness costs were not demonstrated for TR_{34}/L98H isolates of different microsatellite genotypes, which has been previously reported (Verweij *et al.*, 2016), facilitating their survival in the environment and enables their successful dissemination through a large number of easily dispersed airborne spores. The genetic clustering of clinical and environmental TR_{34}/L98H isolates further underlines the risk of acquiring azole-resistant aspergillosis among azole-naïve susceptible hosts.

The absence of TR_{46}/Y121F/T289A isolates in this surveillance may be due to their recent appearance compared to TR_{34}/L98H isolates (Verweij *et al.*, 2016). Another plausible explanation is that nearly all soil samples here were not from compost heaps. A recent Dutch study reported that TR_{46} isolates were overwhelmingly abundant over TR_{34} isolates in heat-shock treated compost heaps with residual azole fungicides, which serve as evolutional incubators that allow the emergence of TR_{46} isolates with new genotypes through sexual recombination (Zhang *et al.*, 2017). Though not found here, TR_{53} and G54 mutations, two

resistance mechanisms that have arisen from the environment, have been identified in different countries, suggesting their adaptation to the environment (Sharma *et al.*, 2015; Alvarez-Moreno *et al.*, 2017; Tangwattanachuleeporn *et al.*, 2017). Given the history of TR_{34}/L98H isolate dissemination, continuous surveillance is warranted to monitor whether these resistance mechanisms occur.

Five azole-resistant isolates harbored *cyp51A*-independent resistance mechanisms. Although *cyp51B* overexpression and consistent *hapE* or *srbA* mutations were not identified, overexpression of drug efflux transporters, *atrF* in SD116A3 and *AfuMDR3* in SD359, was observed. Fungi can overcome intracellular toxin accumulation with efflux transporters, which include two main categories: ATP-binding cassette (ABC) proteins and major facilitator superfamily (MFS) pumps (Cannon *et al.*, 2009). AtrF, an ABC transporter, has been reported to be constitutively upregulated in an itraconazole-resistant *A. fumigatus* clinical isolate, AF72, and two of the four itraconazole-resistant *A. fumigatus* environmental isolates (Slaven *et al.*, 2002; Meneau *et al.*, 2016). The MFS transporter AfuMDR3 has been reported to be constitutively upregulated in itraconazole-resistant *A. fumigatus* ultraviolet-irradiated mutants with or without co-upregulation of *AfuMDR4* as well as in a clinical isolate (AF2) recovered from a patient chronically treated with itraconazole (Nascimento *et al.*, 2003; Chen *et al.*, 2005). However, the role of *AfuMDR3* in AF2 is unclear because of the coexistence of an M220I mutation in *cyp51A*. The SD359 strain isolated here is valuable because it appears to be the first naturally occurring azole-resistant *A. fumigatus* with significant *AfuMDR3* overexpression that is not accompanied by *cyp51A* mutations or co-upregulation of *AfuMDR4*. These findings provide additional evidence supporting the potential roles of *atrF* and *AfuMDR3* in azole resistance. Future studies validating the phenotypic roles ascribed to *atrF* and *AfuMDR3* and delineating the extent to which *atrF* and *AfuMDR3* overexpression contribute to clinical and environmental resistance are warranted.

Although the role of transporters in KP183A1 resistance is inconclusive, it is interesting to find that low-level posaconazole-resistant KP183A1 is isogenic to the susceptible isolate KP189A1 collected from the same geographical area, suggesting that KP183A1 might have evolved from a local susceptible strain but has not yet achieved full resistance, which might require the accumulation of additional mutations.

Since the 1970s, azole fungicides have been widely used for crop protection against phytopathogenic molds in many countries (Stensvold *et al*, 2012.*)*, which have the potential to increase the emergence of azole-resistant *A. fumigatus* threatening human health (Chowdhary *et al.*, 2013). In Taiwan, of the five azole fungicides that have the highest potential to select for the TR_{34}/L98H genotype (Snelders *et al.*, 2012), propiconazole and difenconazole have been used since 1987 and 1993, respectively, and tebuconazole, bromuconazole, and epoxiconazole have been used since 1999 (Taiwan Crop Protection Industry Association, 1962-2016). The annual consumption of these 5 azole fungicides in Taiwan was approximately two-thirds that used in the Netherlands in 2008 (Kleinkauf *et al.*, 2013) and has increased since 2012 (20,031 kg, 47,095 kg, and 57,396 kg in 2008, 2012, and 2016, respectively) (Table S3). Azole fungicides can persist in soil for a long time and exert selective pressure, driving the development of azole resistance among not only phytopathogenic molds, such as *Mycosphaerella graminicola* (Cools and Fraaije, 2008), but also human pathogenic fungi, such as *A. fumigatus* (Snelders *et al.*, 2012). In this study, the higher prevalence rates of azole resistance were observed in central, southern, and eastern Taiwan, which is consistent with their higher agricultural activities and crop yield than that in northern Taiwan, a highly urbanized area surrounded by hills and mountains, according to the data from the Council of Agriculture in Taiwan. In addition, the isolates of *Fusarium fujikuroi* in Taiwan, the causative agent of bakanae disease in rice, have already expressed reduced susceptibility to tebuconazole (Chen *et al.*, 2016b). Recent reports revealed the genetic clustering of *Candida tropicalis*

isolates exhibiting reduced susceptibility to both fluconazole and azole fungicides recovered from epidemiologically unrelated patients and soil samples (Yang *et al.*, 2012). Together with the diverse TR_{34}/L98H clonality and resistance mechanisms found in this study, it is speculated that for both human and plant pathogenic fungi, resistance traits may emerge and be maintained and then subsequently evolve in fields where azole fungicides have been applied. Hence, studies on reversing the increasing resistance rates by restricting key agricultural azoles might be helpful to identify appropriate public health measures (Kleinkauf *et al.*, 2013). To minimize the risk of fungicide resistance in phytopathogenic molds, farmers in Taiwan are advised to use tebuconazole in rotation with alternative fungicides that have different modes of action (Chen *et al.*, 2016b), which would theoretically reduce the selective pressure exerted by azole fungicides on bystander human pathogenic fungi as well. Resistance trends should continue to be monitored to evaluate the effects of fungicide rotation.

Given the environmental resistance rate (7.5%) found in this study, for patients with invasive aspergillosis in Taiwan, strenuous efforts should be made to obtain a positive culture, followed by accurate species identification and susceptibility testing of causative isolates (Verweij *et al.*, 2015). For empirical therapy, decisions between the current primary agent voriconazole versus a voriconazole-echinocandin combination or liposomal amphotericin B monotherapy should depend on individual clinical conditions. The latter two regimens are particularly considered for critically ill or neutropenic patients, as a delay in initiating effective therapy most likely compromises their survival (Chowdhary *et al.*, 2017).

In conclusion, this study presented both the global spread and local evolution of azole-resistant *A. fumigatus* isolates carrying TR_{34}/L98H mutations on this island and the potential role of efflux transporters in azole resistance. Because resistant isolates were not rare in the environment and were genetically clustered with clinical isolates, periodic environmental surveillance at the molecular level is thus emphasized in regions where azole fungicides are

applied to detect epidemiological trends and aid the development of treatment recommendations for human aspergillosis. To retain clinical use of azoles, agricultural fungicide management strategies that generate less selective pressure for antifungal resistance should also be investigated and implemented.

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Transparency declarations

All of the authors declare no conflicts of interest. This study was presented in part at the 27th European Congress of Clinical Microbiology and Infectious Diseases in Vienna, Austria, from April 22-25, 2017.

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Table 1: Minimum inhibitory concentrations (MICs) of antifungal agents and triazole fungicides for *A. fumigatus* environmental strains isolated in Taiwan.

Azole susceptibility, *cyp51A* characteristics (no.)	MIC or MIC_{50}/ MIC_{90} (Range) (mg/L)					
	Amphotericin B	Itraconazole	Posaconazole	Voriconazole	Difenoconazole	Tebuconazole
Azole-resistant, all (n=34)	0.5/0.5 (0.25-0.5)	>8/>8 (0.5->8)	1/2 (0.5-2)	2/4 (1-4)	>32/>32 (4->32)	32/>32 (8->32)
TR_{34}/L98H, all (n=29)	0.5/0.5 (0.25-0.5)	>8/>8 (>8)	2/2 (1-2)	2/4 (1-4)	>32/>32 (16->32)	32/>32 (16->32)
TR_{34}/L98H (n=12)	0.5/0.5 (0.5)	>8/>8 (>8)	1/2 (1-2)	4/4 (2-4)	32/>32 (16->32)	32/>32 (16->32)
TR_{34}/L98H, S297T, F495I (n=17)	0.5/0.5 (0.25-0.5)	>8/>8 (>8)	2/2 (1-2)	2/2 (1-4)	>32/>32 (32->32)	32/>32 (16->32)
Wild-type or polymorphisms[a] (n=5)	0.5/0.5 (0.5)	1/>8 (0.5->8)	0.5/1 (0.5-1)	4/4 (2-4)	16/32 (4-32)	16/32 (8-32)
Azole-susceptible, all (n=45)	0.5/1 (0.25-1)	0.5/0.5 (0.12-1)	0.12-0.25 (0.06-0.25)	0.5/1 (0.25-1)	2/4 (0.5-8)	4/8 (2-16)
Wild-type (n=37)	0.5/1 (0.25-1)	0.5/0.5 (0.12-1)	0.25/0.25 (0.06-0.25)	0.5/1 (0.25-1)	2/4 (1-4)	4/8 (2-8)
F46Y, G89G, M172V, N248T, D255E, L358L, E427K, C454C (n=5)[b]	0.25/1 (0.25-1)	0.5/1 (0.5-1)	0.25/0.25 (0.06-0.25)	1/1 (1)	4/8 (4-8)	8/16 (8-16)
D262Y (n=1)	0.5	0.12	0.06	0.5	0.5	4
F46Y, G89G, M172V, D262Y, L358L, E427K, C454C (n=1)	0.25	0.25	0.12	1	1	4
S297T, F495I (n=1)	0.5	0.25	0.06	1	4	8

[a] for details, see Table 2.

[b] includes KP189A1, which had itraconazole, posaconazole, voriconazole, difenoconazole, and tebuconazole MICs of 0.5, 0.12, 1, 8, and 8 mg/L, respectively.

Table 2. Laboratory characteristics of 5 azole-resistant *A. fumigatus* isolates without *cyp51A* mutations as well as control strains evaluated for *cyp51A*-independent resistance mechanisms.

Strain	Source	*cyp51A* substitution(s)[a]	Azole susceptibility phenotype	MIC (mg/L)					*srbA* substitution(s)[a]
				Itraconazole	Posaconazole	Voriconazole	Difenoconazole	Tebuconazole	
Study isolates									
SD359	E	I242V	R	>8	1	4	32	32	V37D
SD116A3	E	WT	R	0.5	0.5	4	4	8	V37D
TN061-2	E	WT	R	>8	0.5	4	32	16	V37D, L68F
KP158A2	E	WT	R	1	0.5	2	16	32	V37D
KP183A1	E	polymorphism[b]	R	1	0.5	2	16	16	V37D, E957D
Control strains									
ATCC MYA-3626	NA	I242V	S	0.25	0.12	0.5	4	8	V37D
ATCC 16903	C	polymorphism[c]	S	0.25	0.12	0.5	2	8	V37D, S82P
F2509	C	WT	S	0.25	0.12	0.5	2	8	V37D
F02411	C	polymorphism[b]	S	0.12	0.12	0.5	2	4	A865 V
YL1	C	polymorphism[b]	S	0.25	0.12	1	4	4	E957D
PE10A1	E	TR34/L98H, S297T, F495I	R	>8	1	1	>32	32	V37D
A31	C	TR_{34}/L98H	R	>8	1	4	32	32	V37D

C, clinical; E, environmental; MIC, minimum inhibitory concentration; NA, not available; S, susceptible; R, resistant; and WT, wild-type.

[a] The reference sequences for *cyp51A* and *srbA* are GenBank accession numbers AF338659 and AFUA_2g01260, respectively.

[b] Strains KP183A1, F02411, and YL1 have F46Y/G89G/M172V/N248T/D255E/L358L/E427K/C454C substitutions in *cyp51A*.

[c] ATCC16903 has F46Y/G89G/M172V/L358L/E427K/C454C substitutions in *cyp51A*.

Figure 1. Prevalence of azole-resistant *A. fumigatus* isolates in the environment of Taiwan. The numbers indicate the prevalence rate (%) of azole-resistance based on *A. fumigatus*-positive samples and *A. fumigatus* isolates, respectively. Counties where TR_{34}/L98H isolates have been recovered are illustrated by a dot pattern. The triangles indicate microsatellite genotype A TR_{34}/L98H isolates (▲, environmental isolate, and △, clinical isolate).

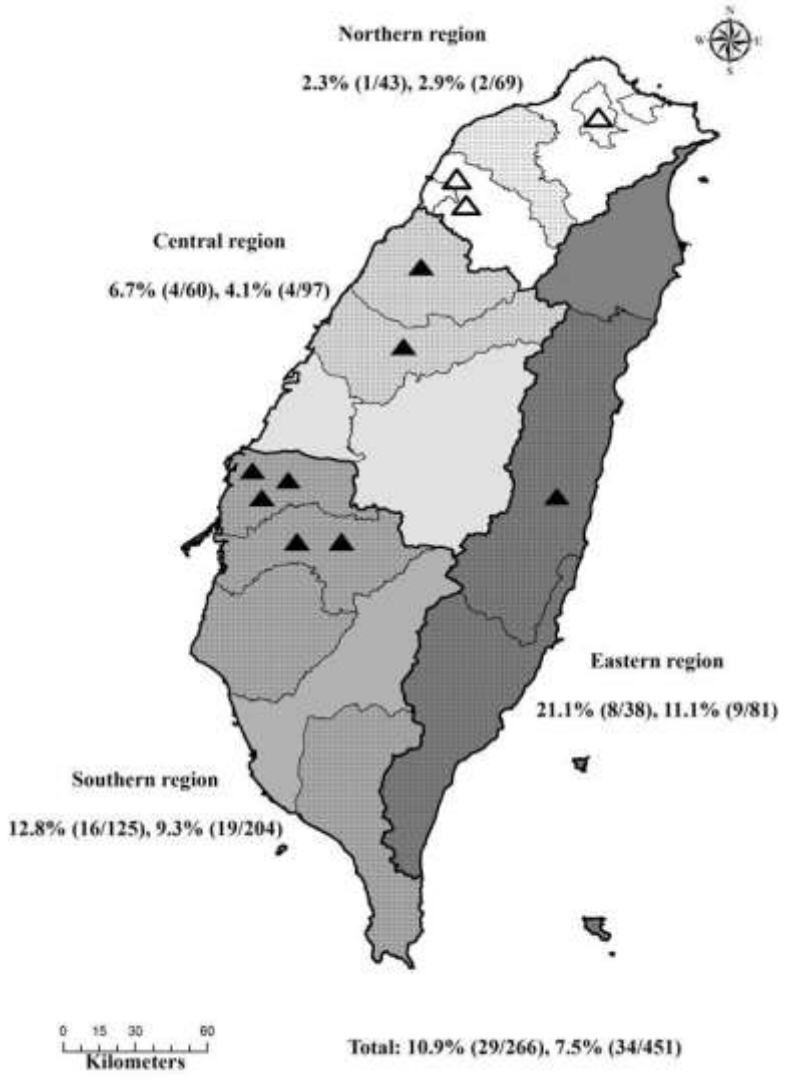
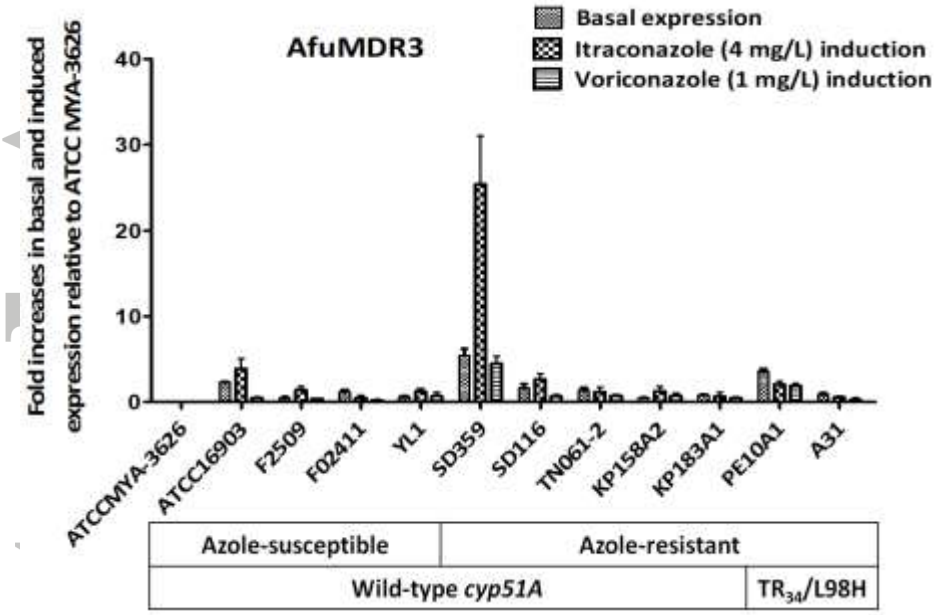


Figure 2. Expression levels of drug efflux transporter genes (a) *Afu*MDR3 and (b) *atrF* in *A. fumigatus* isolates. Expression was evaluated in triplicate biological samples with three technical replicates. The expression levels were normalized to the β-tubulin levels of *A. fumigatus* ATCC MYA-3626. The error bars represent standard deviation. The wild-type *cyp51A* category includes *cyp51A* polymorphisms.

(a)



(b)

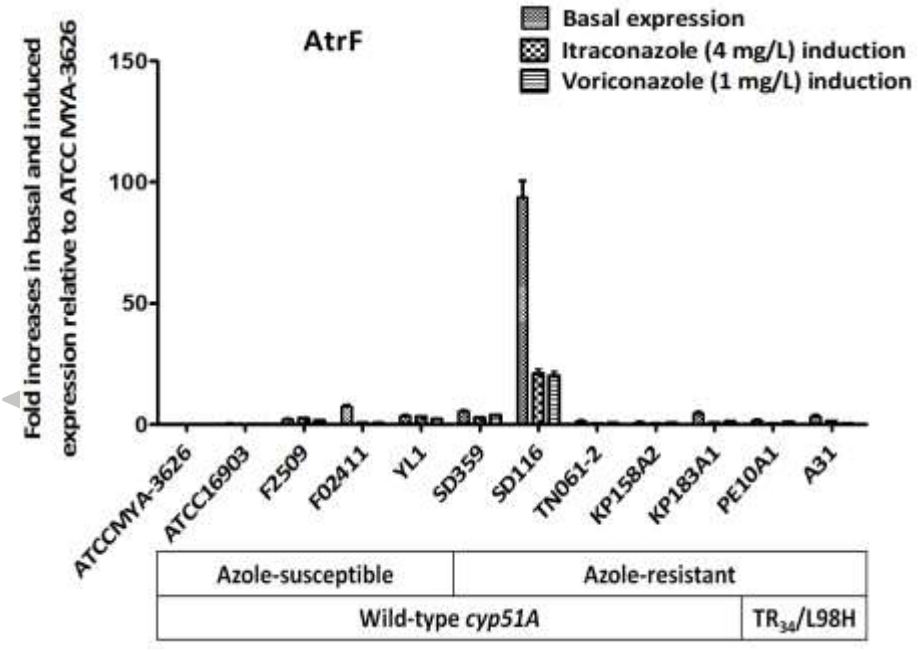


Figure 3. Genetic relationships between *A. fumigatus* clinical and environmental isolates from Taiwan and published isolates from other countries determined by microsatellite genotyping. The scale bar indicates the percentage identity. Microsatellite genotypes (A-E) were assigned to isolates from Taiwan with >90% similarity based on short tandem repeat patterns if there were three or more isolates in that cluster; O, other genotypes distinct from A-E. Abbreviations: C, clinical isolate; E, environmental isolate; R, resistant; S, susceptible; and TW: Taiwan.