

Azole-resistant *Aspergillus fumigatus* isolates carrying TR₃₄/L98H mutations in Taiwan

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Summary

Cumulative evidence described the emergence and geographical expansion of azole-resistant *A. fumigatus* associated with azole treatment failure. To investigate the status of azole resistance in *A. fumigatus* in Taiwan, we studied 38 *A. fumigatus* clinical isolates cultivated from 31 patients at two teaching hospitals from 2011 to 2014. Three isolates obtained from respiratory samples of two azole-naïve patients with pulmonary aspergillosis were found to display multi-azole resistance and cross resistance to agricultural azole fungicides, and all carried TR₃₄/L98H mutations in *cyp51A* gene. The prevalence rates of azole resistance were 7.9% and 6.5% based on isolates and patients respectively. A phylogenetic analysis suggested genetic diversity of the TR₃₄/L98H isolates in Taiwan, including a unique genotype distinct from strains outside Taiwan. The result underlines the emergence of such isolates in Taiwan as well, emphasising the importance of further surveillance for azole-resistant *A. fumigatus* and implementation of strategies that prevent fungicide-driven resistance selection.

Key words: Azole resistance, *Aspergillus fumigatus*, Taiwan, TR₃₄/L98H, azole fungicide.

Introduction

Aspergillus fumigatus is the leading pathogen causing invasive mould diseases in humans, and voriconazole and other triazole antifungals, the competitive inhibitor of *cyp51A*, are the recommended primary therapies for aspergillosis in international guidelines and in Taiwan.^{1–3} So far, cumulative evidence described the emergence and geographical expansion of azole-resistant *A. fumigatus* associated with voriconazole treatment failure.^{4–7} *Cyp51A*-related mutations are the principal resistance mechanisms recognised in azole-resistant *A. fumigatus*.⁴ Multiple amino acid substitutions in the *cyp51A* gene have been described to be

associated with azole resistance that emerged during azole treatment, while a resistance mechanism consisting a 34-bp tandem repeat in the promotor region of *cyp51A* in combination of a substitution at codon 98 (TR₃₄/L98H) has been linked to the agricultural use of azole fungicides in Europe.^{5,7,8} A recent report also demonstrated the TR₃₄/L98H resistance mechanism in *A. fumigatus* isolates from China and India, two highly populated countries in Asia.⁹

Taiwan is a subtropical island country situated in Eastern Asia, where clinical azole-resistant *A. fumigatus* isolates have been reported in 2003¹⁰ though the molecular mechanisms contributing to azole resistance have not been delineated. Considering the potential of geographic migration of resistant isolates and to investigate the current status of azole resistance in *A. fumigatus* in Taiwan, we examined clinical isolates from two teaching hospitals and described the genetic relationship between resistant isolates and global strains.

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Material and methods

Patients and isolates

Thirty eight clinical *A. fumigatus* isolates cultivated from 31 patients at National Cheng-Kung University Hospital located in southern Taiwan and National Taiwan University Hospital located in northern Taiwan during 2011–2014 were studied, retrospectively. The strain identification was based on the morphological characteristics and the sequence analysis of the internal transcribed spacer region, β -tubulin and the calmodulin gene, as previously described.¹¹ Clinical data of patients with isolation of non-wild-type *A. fumigatus* were reviewed.

In vitro antifungal susceptibility testing

Of all *A. fumigatus* isolates, the minimal inhibitory concentrations (MICs) of medical azoles (itraconazole, voriconazole and posaconazole) were determined using the Sensititre YeastOne broth microdilution system (YO10, Trek Diagnostic Systems, Ltd., East Grimstead, U.K.)^{12,13} and MICs of azole fungicides [tebuconazole and penconazole (Chem Service, Inc., Pennsylvania, USA)] were determined using the Clinical Laboratory Standard Institute (CLSI) M38-A2 broth microdilution method.¹⁴ An isolate with an itraconazole, voriconazole or posaconazole MIC of ≥ 1 , ≥ 1 , or ≥ 0.5 mg/L determined by the YeastOne plate respectively was re-examined for MICs of itraconazole, voriconazole, posaconazole and amphotericin B (all Sigma-Aldrich, Saint Louis, MO, USA) using the CLSI method.¹⁴ Based on CLSI-proposed epidemiological cut-off values, an isolate with an itraconazole, voriconazole or posaconazole MIC of >1 , >1 or >0.5 mg/L was considered 'non-wild-type' and also described as 'resistant' herein.¹⁵

Mutation analysis

Non-wild-type *A. fumigatus* isolates were selected for further detection of *cyp51A* mutations. Of the selected isolates, the full sequences of *cyp51A* gene along with the promoter regions were amplified with previously described PCR primers and conditions¹⁶ and the amplified products were sequenced with Applied Biosystems sequencer (ABI 3130, Foster City, CA, USA). The DNA sequences of the non-wild-type *A. fumigatus* strains were compared with the wild-type susceptible *A. fumigatus* reference strain (GenBank AF338659).

Microsatellite genotyping

The genotypic relationship in non-wild-type strains and wild-type strains in this study and strains from other countries were investigated using microsatellite genotyping method, which was based on an cluster analysis of nine short tandem repeat loci of *A. fumigatus*.¹⁷ *A. fumigatus* BCRC 32836 (i.e. CBS 487.65) was included as a reference strain and allelic ladders were introduced for interlaboratory standardization of the genotyping assay.^{17,18} The repeat numbers of nine markers of all isolates were repeated thrice. The strains from outside Taiwan for comparison were from the following countries: India (clinical susceptible [$n = 2$] and resistant [1]; environmental susceptible [1]), Iran (environmental susceptible [1] and resistant [1]), France (clinical resistant [1]), China (clinical resistant [4]), Australia (clinical resistant [2]), Germany (clinical resistant [1]), the Netherlands (clinical resistant [2]; environmental susceptible [1] and resistant [2]) and Kuwait (clinical resistant [1]).^{19–21} The dendrogram based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm was generated using the BioNumerics v6.0 software (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Among the 38 isolates, three isolates (A31, B44 and B51) obtained from two azole-naïve patients displayed multi-azole resistance, that is. simultaneous resistance to itraconazole, voriconazole and posaconazole, and higher MICs of tebuconazole and penconazole (≥ 16 mg/L) (Table 1). The prevalence rates of azole resistance were 7.9% and 6.5% based on isolates and patients respectively. TR₃₄/L98H mutations in *cyp51A* gene were present in all three isolates, but not in the remaining 35 azole-susceptible isolates. Additional substitutions at S297T and F495I were detected in strains B44 and B51.

Strain A31 was obtained from bronchoalveolar lavage of a patient residing in southern Taiwan who had lung cancer and underwent lobectomy 5 weeks ago. Proven invasive pulmonary aspergillosis was diagnosed based on the histopathological examination of the bronchoscopic biopsy according to the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group.²² The patient was treated with posaconazole and voriconazole sequentially, and died of progressive pneumonia with bacterial co-infection. Strains B44 and B51 were obtained 2 days apart from sputum of a 66-year-old

Table 1 Characteristics of 3 azole-resistant and 35 azole-susceptible *Aspergillus fumigatus* clinical isolates from Taiwan.

Patient, sex/age(y)	Isolate	Sample type	Underlying diseases	<i>Aspergillus</i> diseases	Treatment	Outcome	<i>cyp51A</i> mutations	Minimum inhibitory concentrations, mg/L					
								ITZ	VCZ	POS	AMB	TBZ	PEN
Azole-resistant isolates, <i>n</i> = 3													
1, M/59	A31	BAL	Lung cancer	IPA, proven	PSC, VOR	Died	TR ₃₄ /L98H	>16 (>16 ²)	4 (4 ²)	1 (0.5 ²)	0.5	32	>32
2, F/66	B44	sputum	Chronic hepatitis C, cirrhosis of liver, diabetes	IPA ¹	VOR, AMB	Died	TR ₃₄ /L98H	>16 (>16 ²)	2 (1 ²)	1 (0.5 ²)	0.5	16	>32
	B51	Sputum	without control and adrenal insufficiency				TR ₃₄ /L98H	>16 (>16 ²)	2 (1 ²)	1 (0.5 ²)	0.5	16	>32
Azole-susceptible isolates, <i>n</i> = 35													
							ND	0.06/0.12 [0.03–0.25] ^{2,3}	0.25/0.5 [0.12–1] ^{2,3}	0.03/0.06 [0.015–0.06] ^{2,3}	–	1/2 [0.5–4] ³	2/2 [0.5–4] ³

The minimum inhibitory concentrations (MICs) of medical azoles and azole fungicides were determined using the Clinical Laboratory Standard Institute broth microdilution method and the Sensititre YeastOne system.

¹This patient could not be classified according to the EORTC/MSG consensus definitions.

²The MICs were determined with the Sensititre YeastOne system.

³MIC_{50/90} [MIC range].

AMB, amphotericin B; BAL, bronchoalveolar lavage; IPA, invasive pulmonary aspergillosis; ITZ, itraconazole; ND, not detected; PEN, penconazole; POS, posaconazole; VCZ, voriconazole; TBZ, tebuconazole; PEN, penconazole.

woman with multiple underlying diseases residing in northern Taiwan (Table 1). She was transferred from a regional hospital due to progressive shortness of breath and productive cough in the past 3 weeks and sudden onset of high fever despite antibacterial therapy. Computed tomography of the chest showed multiple nodular lesions over bilateral lungs. The patient died 1 week after admission despite voriconazole and amphotericin B initiated based on one positive serum galactomannan antigen assay (0.788) and no other identifiable aetiology.

The results of microsatellite genotyping showed that strain A31 was closely related to the azole-resistant TR₃₄/L98H isolates from Australia, the Netherlands, Iran and India, while strains B44 and B51 clustered with wild-type clinical isolates in this study but not with strains from outside Taiwan (Fig. 1).

Discussion

In the present study, we reported an azole resistance rate of 6.5% in clinical *A. fumigatus* isolates and TR₃₄/L98H mutations as the responsible resistant

mechanism. Further phylogenetic analysis demonstrated genetic diversity of TR₃₄/L98H isolates in Taiwan, which might come from overseas (strain A31) or result from the evolution of local strains (strains B44 and B51). Currently, the global prevalence of azole resistance in *Aspergillus* appears to be 3–6% and TR₃₄/L98H mutation is the most frequently reported resistance mechanism.⁴ TR₃₄/L98H *A. fumigatus* isolates have been isolated from many azole-naïve patients, exhibited resistance to both medical azoles and azole fungicides, as seen in our cases, and was presumed to be associated with environmental use of azole fungicides.^{5,7} So far, environmental or clinical TR₃₄/L98H *A. fumigatus* isolates have been reported from many European countries, Tanzania, Kuwait, Iran, India, China and Australia.^{5,9,19–21,23,24} Molecular epidemiology studies indicated that TR₃₄/L98H isolates might have a common ancestor and have subsequently migrated widely through airborne conidia and ascospores, as observed across Europe, or may be an adaptive recombinant progeny that developed locally, as observed in India where a unique genotype distinct from the Chinese, Middle East and

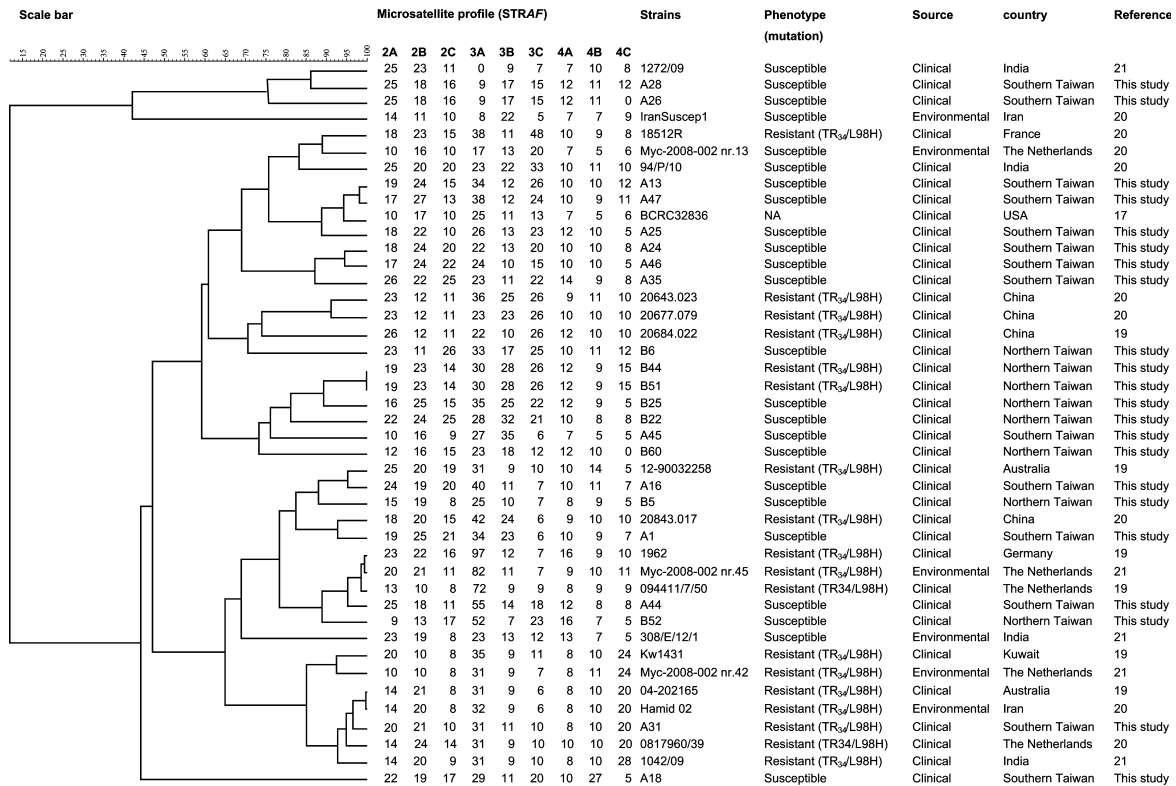


Figure 1 Genotypic relationship in *Aspergillus fumigatus* clinical isolates from Taiwan, including 3 azole-resistant strains (A31, B44, and B51), and published clinical and environmental isolates from other countries determined by microsatellite genotyping.

European TR₃₄/L98H strains was identified.^{5,7,21} Our results added to the speculation that resistance due to TR₃₄/L98H mutation among the *A. fumigatus* strains across Asia might have evolved from separate local strains.⁹

The widespread application of azole fungicides and their persistence in the environment are significant selective forces for the emergence and spread of azole-resistant *A. fumigatus*.^{5,7,8} From a global perspective, the western European and the Asia-Pacific regions account for 61% of the global market share of agricultural fungicides.²⁵ Among five azole fungicides (difenoconazole, tebuconazole, propiconazole, epoxiconazole and bromuconazole) with a very similar structure to medical azoles and the highest potential to select for the TR₃₄/L98H genotype,⁸ the former four agents (49 705 kg sold in 2013) and penconazole (152 kg in 2013) have been widely used in Taiwan for at least 10 years.²⁶ Our previous study found genetic clustering of *Candida tropicalis* isolates exhibiting reduced susceptibility to both fluconazole and azole fungicides recovered from epidemiologically unrelated patients and environmental samples.²⁷ Together with the identification of TR₃₄/L98H isolates from azole-naïve patients and the presence of a unique genotype distinct from other countries, these observations raised the concern that TR₃₄/L98H *A. fumigatus* isolates might have already existed in the environment in Taiwan. On the other hand, no TR₃₄/L98H mutation was detected in 1,026 clinical *A. fumigatus* isolates from the United States, and the observed low azole-resistance rate has been postulated to be related to the difference in the extent of *A. fumigatus* exposure to azole fungicides.²⁸

The spectrum of patients at risk of invasive pulmonary aspergillosis has expanded in recent years because of an increase in patient population without haematological disorders, as our two patients.²⁹ Resistance threatens the outcome of patients with azole-resistant invasive aspergillosis with a reported case-fatality rate of 88%.⁶ Without antifungal susceptibility testing result for therapeutic guidance, clinical condition of our first case deteriorated on azole therapy. Facing the worldwide emergence of azole-resistant *A. fumigatus* and substantial geographical variation in the prevalence of resistance, it is important to consider local drug resistance rate to devise local guidelines.³⁰ Furthermore, in areas where resistant isolates are prevalent, antifungal susceptibility testing should be undertaken routinely for all clinically relevant *A. fumigatus* isolates to guide antifungal therapy.¹⁹

In conclusion, this study constitutes the first report of TR₃₄/L98H azole-resistant *A. fumigatus* isolates in Taiwan which was in line with the emergence of such isolates in Asian countries. In the era of increasing azole resistance, systematic and periodic surveillance of antifungal resistance in environmental and clinical *A. fumigatus* strains are important. In addition, agricultural fungicide usage strategies contributing to a lower resistance selection pressure should be investigated.

Acknowledgments

This work was supported by research grants from the National Health Research Institutes, Taiwan (IV-101-PP-10, IV-102-PP-09, IV-103-PP-09, IV-102-PP-33 and IV-103-PP-33). We thank Jacques F. Meis for providing invaluable suggestions, Hung-Mo Chen, Jau-Yn Wan, and Li-Fang Chen for their laboratory assistance, and Huan Lai for preparing figure artwork.

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