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RESEARCH ARTICLE

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Isolation of triazole-resistant *Aspergillus fumigatus* harbouring *cyp51A* mutations from five patients with invasive pulmonary aspergillosis in Yunnan, China

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ABSTRACT

Invasive aspergillosis (IA) is the most severe type of *Aspergillus* infection. Yunnan has developed agriculture, and the proportion of triazole-resistant *A. fumigatus* induced by triazole fungicides is much higher than that in other regions of China. Inhalation of triazole-resistant *A. fumigatus* is one of the main factors inducing IA. We gathered five strains of *A. fumigatus* from the sputum or bronchoalveolar lavage fluid (BALF) of patients with IA in Yunnan. Subsequent testing showed that all of these strains were resistant to triazoles and harboured mutations in the tandem repeat sequence of the *cyp51A* promoter region, suggesting that they may be triazole-resistant *A. fumigatus* present in the environment.

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1. Introduction

Invasive aspergillosis (IA) is the most severe form of *Aspergillus* infection and has a mortality rate of over 30% (Garcia-Vidal et al. 2015). IA primarily affects immunocompromised individuals, such as cancer patients, organ transplant recipients, and people with acquired immunodeficiency syndrome (Latgé 1999). The primary pathogen causing IA is *A. fumigatus* (Kwon-Chung et al. 2013).

Currently, the recommended first-line treatments for IA are triazoles, including voriconazole (VRC), isavuconazole (ISZ), itraconazole (ITC), and posaconazole (POS) (Ullmann et al. 2018). In addition, polyene antifungal drugs such as liposomal amphotericin B (L-AMB) and echinocandins like caspofungin (CAS) used in combination or salvage therapy are also effective against IA. However, with the widespread use of triazole drugs and fungicides globally, there has been an increase in cases of IA caused by triazole-resistant strains of *Aspergillus*, particularly *A. fumigatus*. This has led to the classification of *A. fumigatus* as a critically important pathogen by the World Health Organization (WHO) according to the fungal priority pathogen list (WHO 2022).

The mechanism of triazole resistance mainly involves mutations in the *cyp51A* gene, which can be categorised into two types: one involves tandem repeat sequences in the promoter region and/or amino acid substitutions in *cyp51A* (TR34/L98H, TR34/L98H/S297T/F495I, TR46/Y121F/T289A, and TR53) (Garcia-Rubio et al. 2017). This mechanism is driven by the selective pressure exerted by triazole fungicides in the environment on *A. fumigatus* (Verweij et al. 2009). The other type involves individual amino acid substitutions in CYP51A, which are induced during long-term antifungal therapy (Arastehfar et al. 2021). Additionally, *hmg1* (Liang et al. 2021), *cyp51B* (Krishnan-Natesan et al. 2008), *hapE* (Camps et al. 2012) mutations and overexpression of efflux pump genes (Slaven et al. 2002), can also contribute to triazole resistance in *A. fumigatus*.

Here, we collected strains from the sputum and BALF of five IA patients at the First People's Hospital of Yunnan Province. These strains underwent species identification, antifungal susceptibility testing, and resistance-related gene sequencing. The results suggest that these strains may be resistant strains of

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A. fumigatus induced by triazole fungicides in the environment.

2. Materials and methods

2.1. Case presentation

The first patient was an 80-year-old female who had a persistent cough, difficulty breathing, and chest discomfort for 9 days. A chest computed tomographic (CT) scan showed a cavity in her right lung with nodules. She was diagnosed with probable IPA with a positive galactomannan test and sputum culture. She was given VRC (6 mg/kg IV BID followed by 4 mg/kg IV BID) for 5 days but didn't improve. Her treatment was changed to a combination of caspofungin (70 mg/day IV, followed by 50 mg/day IV) and posaconazole (300 mg IV BID, followed by 300 mg IV qd), but her condition worsened, leading to organ failure and cardiac arrest, and she unfortunately passed away.

The second patient, a 73-year-old male with relapsed acute myeloid leukaemia, underwent chemotherapy at the hospital (the chemotherapy plan is shown in Table 1). On the 12th day of chemotherapy, the patient's chest CT showed multiple nodular images in the left lower lobe, and *A. fumigatus* was detected in sputum culture. The diagnosis was probable IPA, and VRC was given (6 mg/kg IV BID followed by 4 mg/kg IV BID). After 3 weeks of treatment, the pulmonary infection remained unresolved, and AMB (3 mg/kg qd) was added for treatment. Unfortunately, the patient experienced severe bone marrow suppression, along with severe infection and dysfunction in multiple organs. The patient opted to discharge from the hospital and terminate the treatment voluntarily.

The third patient was a 60-year-old male who complained of fatigue and shortness of breath for 6 days. Upon admission, he was diagnosed with acute B-lymphocyte leukaemia and received chemotherapy. On the 8th day of chemotherapy, the patient developed a fever with a body temperature as high as 39 °C. Chest CT showed multiple small exudates in both lungs, (1,3)-β-d-glucan (G) test was positive and *A. fumigatus* was detected in sputum culture, so he was diagnosed with probable IPA. Administer VRC (6 mg/kg IV BID followed by 4 mg/kg IV BID). After one week of medication, the patient still had recurrent fever and severe pulmonary infection. The patient voluntarily gave up treatment and was discharged.

The fourth patient was a 55-year-old male who complained of fever, difficulty breathing, and productive cough with yellowish sputum for 17 days. After admission, a lung CT scan showed extensive

Table 1. Patient characteristics of invasive pulmonary aspergillosis.

No. of cases	1	2	3	4	5
Age[y]/sex	80/F	73/M	60/M	55/M	39/M
Associated underlying condition	T2DM	AML	ALL	SP	AHF
Immuno-suppressive therapy	Prednisone (40 mg po qd)	ANA and VIN	COVP	None	None
Imaging	Cavity in the right lung with nodules	Multiple nodular images in the left lower lobe	Multiple small exudates in both lungs	Extensive consolidations in both lungs	Diffuse ground glass-like shadows in both lungs
Sample	BALF	Sputum	Sputum	Sputum	Sputum
Prior antifungal	VRC	None	None	None	None
Anti-fungal therapy	VRC f/b POS + CAS	VRC f/b L-AMB	VRC	VRC	None
Outcome	Dead	Abandoned treatment	Abandoned treatment	Abandoned treatment	Abandoned treatment
Identified strain	*Aspergillus fumigatus*	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
Susceptibility (MIC/MEC) (μg/mL)	ITC: >16 VRC: 4 POS: 1 ISZ: 8 AMB: 2 CAS: 0.06	ITC: 4 VRC: >32 POS: 1 ISZ: 16 AMB: 2 CAS: 0.06	ITC: >16 VRC: 4 POS: 1 ISZ: 4 AMB: 2 CAS: 0.06	ITC: >16 VRC: 8 POS: 2 ISZ: 8 AMB: 1 CAS: 0.03	ITC: >16 VRC: 2 POS: 2 ISZ: 16 AMB: 1 CAS: 0.03
cyp51A mutation	TR34/L98H	TR46/Y121F/T289A	TR34/L98H	TR34/L98H	TR34/L98H/S297T/F495I

T2DM = type 2 diabetes mellitus, AML = acute myeloid leukaemia, ALL = acute lymphoblastic leukaemia, SP = severe pneumonia, AHF = acute heart failure, ANA = azarcytidine, VIN = vineclavone, COVP = cyclophosphamide + vincristine + prednisone and pirarubicin, f/b = followed by. Concentration Breakpoints (CBPs) and Epidemiological Cutoff Values (ECVs) for *in vitro* broth dilution susceptibility testing of *Aspergillus fumigatus* according to CLSI M59 document are as follows: ITC, ECV 1 μg/mL; VRC, ≤0.5 μg/mL, susceptible-dose dependent (SDD), ≥2 μg/mL resistance (R); AMB, ECV 2 μg/mL; CAS, ECV 0.5 μg/mL.

consolidation in both lungs. In addition, sputum culture detected *A. fumigatus*, and the G test was positive, so he was diagnosed with probable IA. He received VRC (6 mg/kg IV BID followed by 4 mg/kg IV BID) as treatment. However, his symptoms did not improve and respiratory failure occurred. He voluntarily gave up treatment and was discharged.

The fifth patient was a 39-year-old male with difficulty breathing and coughing for 7 days, unable to lie flat. CT showed diffuse ground glass-like shadows in both lungs. On the second day of admission, the patient developed acute heart failure and respiratory failure. The patient voluntarily gave up rescue and was discharged. On the third day of discharge, sputum culture reported the detection of *A. fumigatus*, and no antifungal drugs were used during this period.

2.2. Fungal purification and culture

The clinical strains obtained from the sputum samples of the aforementioned patients were named as BMU15486, BMU15487, BMU15488, BMU15489, and BMU15490. Due to the possibility that those strains may be mixed, we adopted a method of streaking onto the culture plate and randomly selected as many single colonies as possible for subsequent molecular identification and drug susceptibility testing. If all single colonies obtained from the strain showed the same results in appearance, growth rate, molecular identification, and drug susceptibility testing, we considered that strain to be pure. All single colonies were inoculated on YAG medium and followed by further incubation at 28 °C for 3–5 days.

2.3. Genomic DNA extraction and molecular identification

Genomic DNA extraction was performed using the Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Ltd., Hangzhou, China) following the manufacturer's instructions. PCR amplification was carried out targeting the internal transcribed spacers (ITS) region, β-tubulin gene, and calmodulin gene using the primers listed in Table S1, and the length of the PCR products were 621 bp, 436 bp, and 751 bp, respectively. The PCR products were then sent to BGI Genomics for sequencing. The sequences were analysed against the CBS database (https://wi.knaw.nl/page/Pairwise_alignment).

2.4. Antifungal susceptibility testing

Antifungal susceptibility testing was performed using ITC, VRC, POS, ISZ, CAS, and AMB against the fungal strains. The testing followed the guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) M38-A3 document (Clinical and Laboratory Standards Institute 2017). *A. flavus* (ATCC® 204304) served as the quality control strain. Interpretation of the results was conducted according to the CLSI-M59 guidelines (Clinical and Laboratory Standards Institute 2020). The MIC (minimum inhibitory concentration) of triazoles (VRC, ITC, POS, ISZ) and AMB was determined as the lowest concentration that completely inhibited growth compared to the control. For CAS, the MEC (minimum effective concentration) was determined as the lowest concentration that showed minimal, circular, compact hyphal growth compared to the control.

2.5. Sequencing of resistance-related genes for triazole-resistant A. fumigatus

To detect mutations in resistance-related genes in these five strains, the open reading frame (ORF) of the *cyp51A*, *cyp51B*, *hmg1*, and *hapE* genes, as well as the promoter region of *cyp51A* gene, were amplified with the primers listed in Table S1. The lengths of the PCR products of *cyp51A*, *cyp51B*, *hmg1*, and *hapE* genes were 2,200 bp, 2,136 bp, 3,570 bp, and 1,106 bp. The PCR products were sent to the BGI Company (Beijing, China) for sequencing. The sequences were analysed against those of reference strain (GenBank reference sequence No. *cyp51A* AF338659.1, *cyp51B* XM_744041.1, *hmg1* XM_744409.1, and *hapE* XM_742454.1) using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) to facilitate comparison.

3. Results

3.1. All the colonies were identified as A. fumigatus

On YAG medium, all single colonies isolated from each strain grew at a similar rate, growing well at 28 °C and maturing within 4 days. Fungal culture initially showed a white villous appearance, and after 3–4 days, the centre became blue-green fine powder, and the back was colourless.

We amplified and sequenced the ITS gene, β-tubulin gene, and calmodulin gene. The sequences

were aligned against the CBS database, and all single colonies were identified as *A. fumigatus*.

3.2. All the strains were resistant to triazoles, while susceptible to CAS and AMB

The MICs of ITC, VRC, POS, ISZ, and AMB against BMU15486 were 16 μg/mL, 4 μg/mL, 1 μg/mL, 8 μg/mL, and 2 μg/mL. The MEC of CAS against BMU15486 was 0.06 μg/mL. For BMU15487, the MICs of ITC, VRC, POS, ISZ, and AMB were 4 μg/mL, 32 μg/mL, 1 μg/mL, 16 μg/mL, and 2 μg/mL, and the MEC of CAS was 0.06 μg/mL. For BMU15488, the MICs of ITC, VRC, POS, ISZ, and AMB were 16 μg/mL, 4 μg/mL, 1 μg/mL, 4 μg/mL and 2 μg/mL, and the MEC of CAS was 0.06 μg/mL. The MICs of ITC, VRC, POS, ISZ, and AMB against BMU15489 were 16 μg/mL, 8 μg/mL, 2 μg/mL, 8 μg/mL and 1 μg/mL. The MEC of CAS against BMU15489 was 0.03 μg/mL. The MICs of ITC, VRC, POS, ISZ, and AMB against BMU15490 were 16 μg/mL, 2 μg/mL, 2 μg/mL, 16 μg/mL, and 1 μg/mL. The MEC of CAS against BMU15490 was 0.03 μg/mL.

According to the epidemiological cut-off values (ECVs) for *A. fumigatus* (Clinical and Laboratory Standards Institute 2020), all the strains were defined as resistant to VRC (ECV is 1 μg/mL), NWT to ITC (ECV is 1 μg/mL), POS (ECV is 0.5 μg/mL), ISZ (ECV is 1 μg/mL), WT to CAS (ECV is 0.5 μg/mL), and AMB (ECV is 2 μg/mL).

3.3. All these five strains harbored cyp51A gene mutation

In order to explore the triazole resistance mechanisms of these five strains, we sequenced the open reading frame of *cyp51A*, *cyp51B*, *hmg1*, and *hapE* genes, as well as the promoter region of *cyp51A* gene, and aligned against those of reference sequences. The results showed that all five strains harboured *cyp51A*-related mutations (Fisher et al. 2022), BMU15486, BMU15488, and BMU15489 harboured the TR34/L98H mutations, BMU15487 harboured TR46/Y121F/T289A mutation, and BMU15490 harboured TR34/L98H/S297T/F495I mutation. While *cyp51B*, *hmg1*, and *hapE* were all intact in these five strains. All results are shown in Table 1.

4. Discussions

Here, we conducted species identification, antifungal susceptibility testing, and sequencing of the triazole-resistance-related genes for *A. fumigatus* strains obtained from clinical specimens of five IA patients. The results showed that all strains were resistant to triazoles, with the identified mechanism as tandem repeat in the promoter region and point mutations in *cyp51A* gene (TR34/L98H, TR34/L98H/S297T/F495I, TR46/Y121F/T289A). In addition, the prognoses of these patients were unfavourable, resulting in either death or the abandonment of treatment. This emphasises that more attention should be paid to IA caused by triazole-resistant *A. fumigatus*.

In *A. fumigatus*, *cyp51A* gene-related mutations of triazole resistance arise in two main routines: one involves the individual amino acid substitutions in CYP51A, like G54R (Chen et al. 2005), G138C (Albarrag et al. 2011), and G448S (Krishnan Natesan et al. 2012), which is caused by long-term treatment with triazoles (Verweij et al. 2009). The other involves tandem repeat in the promoter region and/or point mutations in *cyp51A* gene (TR34/L98H, TR34/L98H/S297T/F495I, TR46/Y121F/T289A, and TR53) (Garcia-Rubio et al. 2017), which is caused by the selective pressure exerted by triazole fungicides in the environment (Verweij et al. 2009). As mentioned before, these five triazole-resistant strains all harboured tandem repeat in the promoter region and point mutations in the *cyp51A* gene. In consideration that four strains were isolated before treatment, although one strain was isolated from the patient after VRC treatment, we thus speculate that all five patients were affected with IA by inhaling triazole-resistant *A. fumigatus* spores from the environment.

The proportion of triazole-resistant *A. fumigatus* isolated from the environment of Yunnan, which has developed agriculture, is much higher compared to other regions in China (Chen et al. 2016, 2020; Ren et al. 2017). A survey on greenhouses around Kunming City revealed that about 80% of *A. fumigatus* strains in these greenhouses were resistant to at least one triazole drug, with more than 30% demonstrating cross-resistance to ITC and VRC (Zhou et al. 2021). In Yunnan, among *A. fumigatus* strains from 19 different geographical regions, the proportion of triazole-resistant *A. fumigatus* was 15.89% (58/365) (Zhou et al. 2022). In Lake Dian, the proportion of triazole-resistant *A. fumigatus* is even higher, reaching 42.9%. Together, these results suggested that agricultural and environmental fungicide usages were potential forces to drive triazole resistance in *A. fumigatus* in

non-clinical environments. Inhalation of triazole-resistant *Aspergillus* spores in the environment is regarded as the paramount factor triggering IA (Verweij et al. 2016). Considering that IA patients infected with triazole-resistant *A. fumigatus* tend to have unfavourable prognoses (Arastehfar et al. 2021). Therefore, in areas with a high proportion of environmental triazole-resistant *A. fumigatus*, more attention needs to be paid to the prevention of immune compromised populations and antifungal susceptibility testing for *A. fumigatus*.

In addition to *cyp51A*-related mutations, there are several other molecular mechanisms involved in the triazole resistance of *A. fumigatus*: (i) amino acid substitutions in Hmg1 result in dysregulation of the sterol pathway, leading to increased cellular ergosterol production and triazole resistance (Losada et al. 2015; Liang et al. 2021); (ii) the G457S substitution in another CYP51 isoenzyme, CYP51B, also leads to triazole resistance (Handelman et al. 2021); (iii) the P88L substitution in transcription factor HapE causes overexpression of CYP51A (Camps et al. 2012). Furthermore, additional mechanisms, such as biofilm formation (Fanning et al. 2012) and overexpression of drug efflux pumps (Coleman and Mylonakis 2009), also contribute to triazole resistance. Therefore, to identify the mechanisms of triazole resistance in *A. fumigatus* as soon as possible to guide clinical treatment, we should routinely detect these resistant-related genes described above, regardless of the presence or absence of mutations in the *cyp51A* gene.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The *cyp51A* gene sequences of BMU15486, BMU15487, BMU15488, BMU15489, and BMU15490 have been submitted to the GenBank database and the assigned accession numbers are OR906202–OR906206, respectively.

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