Original Article

Triazole Resistance in *Aspergillus fumigatus* Clinical Isolates Obtained in Nanjing, China

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Abstract

Background: During the past decades, the incidence of invasive aspergillosis (IA) caused by *Aspergillus fumigatus* has increased dramatically. The aims of this study were to investigate the susceptibility of clinical isolates of *A. fumigatus* to triazole and the underlying *cyp51A* mutations in triazole-resistant *A. fumigatus*.

Methods: A total of 126 *A. fumigatus* clinical isolates from 126 patients with proven or probable IA were obtained from four large tertiary hospitals in Nanjing, China, between August 2012 and July 2015. The determination of minimal inhibitory concentrations (MICs) for itraconazole, voriconazole, and posaconazole was performed by broth microdilution according to the European Committee on Antimicrobial Susceptibility Testing reference method.

Results: A total of 4 *A. fumigatus* isolates (3.17%) were confirmed to be itraconazole resistant, with MICs of ≥8 mg/L, and one isolate (0.8%) was confirmed to be voriconazole resistant and posaconazole resistant, with MICs of 4 mg/L and 0.5 mg/L, respectively. We found that two of the 4 isolates of triazole-resistant *A. fumigatus* had the L98H amino acid substitution in combination with a 34-base pair tandem repeat in the promoter region, one isolate had an M220I mutation, and another itraconazole-resistant isolate did not have a substitution in the *cyp51A* gene.

Conclusions: This study shows that triazole-resistant *A. fumigatus* clinical isolates are present in Nanjing, China, which is a new challenge to the clinical management of IA.

Key words: *Aspergillus fumigatus*; *cyp51A*; Minimal Inhibitory Concentrations; TR34/L98H; Triazole Resistance

Introduction

Invasive aspergillosis (IA) is a life-threatening infection in immunocompromised patients associated with severe mortality. *Aspergillus fumigatus* is the most common species recovered from cases of IA (90% of IA cases involving the lungs).[1,2] Triazole antifungals, such as itraconazole, posaconazole, and voriconazole, are first-line drugs in prophylaxis and treatment of IA.[3] However, during the past decades, a number of clinical failures of IA management due to triazole-resistant *A. fumigatus* have been reported,[4-8] which brings new challenges to the clinical treatment of IA.

A global survey in the year 2005 showed that there was no itraconazole-resistant *A. fumigatus* in the 331 isolates that were examined.[9] However, in the year 2009, 43 strains of *A. fumigatus* from a total of 637 (6.75%) isolates were determined to have an itraconazole minimum inhibitory concentrations (MICs) of ≥2 mg/L.[10] The major mechanism of triazole resistance in *A. fumigatus* involves mutations in the *cyp51A* gene encoding 14α-demethylase. Some specific point mutations, such as G54, M220, I266, S297, and L98H amino acid substitution in combination with a 34-base pair tandem repeat (TR) in the promoter region, have been identified as causes of triazole resistance.[11-14] TR/L98H, considered as an intrinsic resistance mechanism, has been identified mainly in Netherlands and China.[8,12,15]

At present, a few studies have focused on the prevalence of triazole resistance among *A. fumigatus* clinical isolates

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in China. Thus, we investigated the *in vitro* triazole susceptibilities of a large collection of *A. fumigatus* clinical isolates and analyzed *cyp51A* mutations in the triazole-resistant *A. fumigatus*.

Methods

Isolates

A. fumigatus clinical isolates were obtained from four large tertiary hospitals (Jinling Hospital, Medical School of Nanjing University; Drum Tower Hospital, Medical School of Nanjing University; the First Affiliate Hospital of Nanjing Medical University; and Zhongda Hospital, Southeast University) in Nanjing, China, between August 2012 and July 2015. All isolates were identified as *A. fumigatus* by macroscopic and micromorphological characteristics, thermotolerance at 48°C, and molecular identification. Conidia were stored in 10% glycerol broth at −80°C. The strains of *A. fumigatus* American Type Culture Collection 204305 were included as quality controls.

Antifungal agents

Itraconazole (Janssen Pharmaceutica NV, Turnhoutseweg, Belgium), voriconazole (Pfizer Inc., Dun Laoghaire, Ireland), and posaconazole (Merck Sharp & Dohme Pty. Ltd., New South Wales, Australia) were provided in powder form. Itraconazole, voriconazole, and posaconazole were dissolved in 100% dimethyl sulfoxide and stored at −80°C.

Susceptibility testing

The *in vitro* antifungal susceptibility testing was performed by broth microdilution according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method.[16] Itraconazole, voriconazole, and posaconazole were diluted to the required concentrations in RPMI with 2% of glucose stock solutions. Spore suspensions were prepared to a final working inoculum of 2–5 × 10^5 CFU/ml. Microdilution plates were incubated at 35°C for 48 h. The endpoints for MICs were defined as the lowest drug concentration that caused complete growth inhibition.

The EUCAST clinical breakpoints were used.[17,18] MICs>2 mg/L for itraconazole and voriconazole and >0.25 mg/L for posaconazole were considered resistant; MICs ≤1 mg/L for itraconazole and voriconazole and ≤0.125 mg/L for posaconazole were considered susceptible.

Sequence analysis

For resistant isolates, the entire *cyp51A* gene and its promoter were amplified as previously described.[13] The forward primer 5'-ATGGTGCCGATGCTATGG-3' and reverse primer 5'-AGTTTCAGGGACTCCTTTC-3' were used in polymerase chain reaction amplification. Sequence analysis was determined on an ABI 3730XL DNA sequencer (Applied Biosystems, Inc., Foster City, USA). Sequences were compared to a wild type *A. fumigatus* strain (GenBank Accession No. AF. 338659).

Results

Over a period of 4 years from August 2012 to July 2015, a total of 126 *A. fumigatus* clinical isolates from 126 patients with proven or probable IA were collected and analyzed. All the isolates were confirmed as *A. fumigatus sensu stricto*. Figure 1 shows the distribution of MIC values for itraconazole, voriconazole, and posaconazole according to the EUCAST broth microdilution procedure. The results for the quality control strains were in the acceptable range. A total of 4 *A. fumigatus* isolates (3.17%) were confirmed to be itraconazole resistant with MICs of ≥8 mg/L, and one isolate (0.8%) was confirmed to be voriconazole resistant and posaconazole resistant, with MICs of 4 mg/L and 0.5 mg/L, respectively.

The MIC values, *cyp51A* substitutions, and characteristics of triazole-resistant isolates of *A. fumigatus* are shown in Table 1. Two of the four isolates were demonstrated to contain the TR34/L98H substitution. One isolate from a chronic obstructive pulmonary disease (COPD) patient underwent itraconazole therapy had the M220I mutation. Moreover, another itraconazole-resistant isolate did not have a substitution in the *cyp51A* gene.

Discussion

In this study, we investigated the prevalence of triazole resistance and examined *cyp51A* mutations among *A. fumigatus* clinical isolates from patients with proven or probable IA in Nanjing, China. The percentage of triazole-resistant *A. fumigatus* isolates was 3.17% (4/126). This rate is similar to that reported in Germany (3.2%)[14] but lower than data from some previous studies: from a total of 497 *A. fumigatus* isolates, in a worldwide survey from 62 medical centers, 5.8% showed triazole resistance,[12] and similarly, a rate of 5.3% was shown in a nationwide multicenter study from Netherlands[15] and 5.5% in Copenhagen, Denmark.[19] Astonishingly, a rate of 14% and 20% of *A. fumigatus* clinical isolates were noted as having triazole antifungal resistance in the year 2008 and 2009, respectively, in Manchester.[20] Nevertheless, the prevalence rate in the present study is higher compared to Spain (2.5%)[21] and India (1.7%).[22]

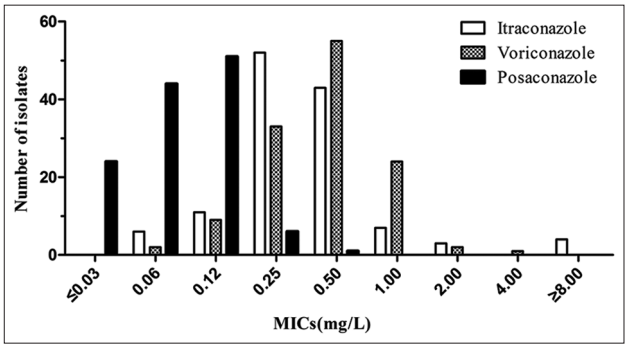

Figure 1: Minimal inhibitory concentrations distributions of itraconazole, voriconazole, and posaconazole in clinical isolates of *Aspergillus fumigatus*. MICs: Minimal inhibitory concentrations.

Table 1: Characteristics of triazole-resistant isolates of *Aspergillus fumigatus*

Isolates number	Patient age (years)/sex	Underlying condition	Disease	Prior azole treatment	Antifungal treatment	Outcome	MIC (mg/L)			Mutation(s) in *cyp51A* gene
							ITZ	VRZ	PSZ	
AF.28	56/male	COPD	IPA	ITZ	ITZ	Died	>8.00	0.50	0.25	M220I
AF.44	48/female	SLE	IPA	None	ITZ and CAS	Died	>8.00	4.00	0.50	TR/L98H
AF.98	63/female	NHL, BMD	IPA	None	No treatment	Died	>8.00	2.00	0.25	TR/L98H
AF.118	65/male	DM	IPA	None	VRZ and CAS	Cure	>8.00	2.00	0.25	WT

COPD: Chronic obstructive pulmonary disease; SLE: Systemic lupus erythematosus; NHL: Non-Hodgkin lymphoma; BMD: Bone marrow depression; DM: Diabetes mellitus; IPA: Invasive pulmonary aspergillosis; CAS: Caspofungin; MIC: Minimal inhibitory concentration; ITZ: Itraconazole; VRZ: Voriconazole; PSZ: Posaconazole; TR: Tandem repeat; WT: Wild type.

Mutations in the *cyp51A* gene encoding 14α-demethylase were the major mechanisms of triazole resistance in *A. fumigatus*. In the present study, two triazole-resistant isolates harbored the TR34/L98H mutation and one M220I mutation. Some studies suggested that the TR/L98H mutation acquired in the environment due to azole fungicides usage in agriculture.[23,24] We found that one itraconazole-resistant isolate harbored the TR34/L98H mutation was cross-resistance to both voriconazole and posaconazole. Similarly, multiazole-resistant isolates with TR34/L98H mutations were detected in Asia as well as in Europe.[12,14] In this study, the itraconazole-resistant isolate with the M220I mutation was considered as acquired resistance as a result of prolonged treatment with itraconazole. Other specific point mutations, such as G54, I266, and S297, have been identified as primary causes of triazole-acquired resistance.[11-14] However, in this study, one itraconazole-resistant isolate was without substitution in the *cyp51A* gene. Tashiro *et al*.[11] showed that 43% of triazole-resistant isolates did not contain a substitution in the *cyp51A* gene. Therefore, *cyp51A* gene mutation is not the only mechanism of triazole resistance in *A. fumigatus*. Other possible mechanisms (e.g., *HapE* mutation, efflux pumps, cholesterol import by *A. fumigatus*) might contribute to triazole resistance.[25]

There are a series of limitations in this study. We only investigated *A. fumigatus* clinical isolates from patients with proven or probable IA in four hospitals in Nanjing; therefore, the results might not be representative. The epidemiological data on the resistance of triazole to *A. fumigatus* were still insufficient, and the mechanisms of triazole resistance have not been fully investigated.

In conclusion, this study confirmed the occurrence of triazole-resistant *A. fumigatus* clinical isolates, with a 3.17% resistance rate in Nanjing, China. Triazole-resistant *A. fumigatus* might develop through different mechanisms, which brings new challenges to the clinical treatment of IA. Larger studies are needed to better investigate triazole-resistant *A. fumigatus* in different regions of China and elucidate the underlying mechanisms of triazole resistance.

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Conflicts of interest

There are no conflicts of interest.

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