MYCOBIOLOGY
2020, VOL. 48, NO. 2, 148–152
https://doi.org/10.1080/12298093.2020.1744955

RESEARCH NOTE

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Antifungal Susceptibility Tests and the *cyp51* Mutant Strains among Clinical *Aspergillus fumigatus* Isolates from Korean Multicenters

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ABSTRACT

We investigated the antifungal susceptibilities and the *cyp51* mutant strains among *Aspergillus fumigatus* clinical isolates obtained from 10 university hospitals in Korea. Of the 84 isolates examined, two itraconazole-resistant isolates were found with no amino acid substitution in the *cyp51A/cyp51B* genes. However, 19 (23.2%) azole-susceptible isolates harbored amino acid substitutions: Nine isolates harbored one to five mutations in *cyp51A* with high polymorphism, and 11 isolates exhibited the same Q42L mutation in *cyp51B*. Overall, a low azole resistance rate and high frequency of *cyp51A/cyp51B* amino acid substitutions were observed in the azole-susceptible *A. fumigatus* isolates in Korea.

ARTICLE HISTORY
Received 24 February 2020
Revised 16 March 2020
Accepted 16 March 2020

KEYWORDS
Aspergillus fumigatus; antifungal susceptibility; cyp51A/cyp51B; multicentre

Invasive fungal disease caused by *Aspergillus* species has increased in recent years and can be problematic associated with significant morbidity and mortality especially in the immunocompromised patients. *Aspergillus fumigatus* is the major causative agent of aspergillosis, and triazole antifungals are recommended as the primary medication for prophylaxis and treatment [1]. Itraconazole-resistant *A. fumigatus* was first reported in 1997 and the azole resistance has been increasingly reported worldwide: the main mechanism of this resistance is changes in the amino acid sequence of the Cyp51 protein [2–5]. Global surveillance has revealed diversity in the frequencies of triazole-resistance and *cyp51A* mutations [6–8]. In Korea, only one azole-resistant *A. fumigatus* clinical isolate with *cyp51A* mutations was reported in 2018 [9], however, surveillance data on the prevalence of azole resistance remains lacking. Here, we investigated the antifungal susceptibilities and mutations in the *cyp51A/cyp51B* genes of *A. fumigatus* clinical isolates from a nationwide multicentre study conducted in Korea.

In total, 84 *A. fumigatus* clinical isolates were collected from 10 university hospitals and subjected to screening for azole resistance. All isolates were obtained from clinical specimens using routine culture methods between January 2012 and August 2013. Only one isolate from each patient was included. All submitted isolates were subcultured on the potato dextrose agar at 30 °C for three days. After phenotypical identification, the isolates were finally identified by partial sequencing of the β-tubulin and calmodulin genes [10]. *In vitro* susceptibility testing for itraconazole, voriconazole, posaconazole, and amphotericin B was performed using the reference broth microdilution method, according to Clinical and Laboratory Standards Institute (CLSI) document M38-A2 [11]. The minimum inhibitory concentration (MIC) endpoint is the lowest drug concentration that results in complete growth inhibition after 48 h of incubation. Quality control was performed using *A. flavus* ATCC 204304, and *A. fumigatus* MYA-3626. Isolates with MICs exceeding epidemiological cutoff values (ECVs) (1, 1, 0.5, and 2 µg/mL for itraconazole, voriconazole, posaconazole, and amphotericin B, respectively) were considered to be resistant [12]. The target genes, *cyp51A/cyp51B,* and their promoter regions were sequenced for all 84 isolates, as described previously [13]. The sequence from *A. fumigatus* strain 237 (GenBank accession no. AF338659) was used as the wild type. Data from patients' medical records, including relevant information regarding underlying disease, previous or current antifungal use, and prognosis, were collected in accordance with the guidelines of, and with the

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Table 1. Amino acid substitutions in *cyp51A, cyp51B* and *in vitro* antifungal susceptibility testing results for 84 *A. fumigatus* clinical isolates in Korea.

	Minimum inhibitory concentration (μg/mL)				Amino acid substitutions in *cyp51* genes		
Category	Itraconazole	Voriconazole	Posaconazole	Amphotericin B	*cyp51A*	*cyp51B*	No. (%) of isolates
Azole-resistant isolates ($N = 2$)							
	2	0.25–1	0.25	0.5–1	None	None	2 (2.4)
Azole-susceptible isolates ($N = 82$)							
	0.5	0.25–0.5	0.25–0.5	0.5	F46Y/M172V/E427K	None	2 (2.4)
	0.5	0.5	0.5	2	F46Y/M172V/N248T/D255E/E427K	Q42L	1 (1.2)
	0.5	0.5	0.25	0.5	F46Y/N248T/D255E/E427K	None	1 (1.2)
	0.5	0.25–1	0.06–0.5	0.5	N248K	None	2 (2.4)
	0.5	0.5	0.125	0.5	M39I	None	1 (1.2)
	0.5	0.25	0.125	0.5	D343N	None	1 (1.2)
	1	0.25	0.25	0.5	G408V	None	1 (1.2)
	0.25–1	0.25–0.5	0.125–0.25	0.25–4	None	Q42L	10 (11.9)
	0.25–1	0.25–1	0.06–0.5	0.125–4	None	None	63 (75.0)
Total	0.25-2	0.25–1	0.06–0.5	0.125–4			84 (100.0)

approval of, the Institutional Review Board of Chonnam National University Hospital (IRB CNUH-2014-290), to elucidate the clinical relevance of isolates harboring any *cyp51A/cyp51B* mutation.

Of the 84 isolates, 72 (85.7%) were obtained from respiratory specimens, and the remaining 12 were obtained from pus and other fluid specimens. The total of 84 isolates showed following MIC ranges: 0.25–2 μg/mL for itraconazole, 0.25–1 μg/mL for voriconazole, 0.06–0.5 μg/mL for posaconazole, and 0.125–4 μg/ml for amphotericin B (Table 1). Two (2.4%) and three (3.6%) isolates were resistant to itraconazole and amphotericin B, respectively. All isolates were susceptible to voriconazole and posaconazole. Sequence analysis of the *cyp51A/cyp51B* genes revealed that the two itraconazole-resistant isolates did not harbor any amino acid substitution, whereas 19 of 82 (23.2%) azole-susceptible isolates exhibited one or more substitutions in the *cyp51A* or *cyp51B* genes, as follows: F46Y/M172V/E427K and/or N248T/D255E were frequently found ($n = 4$), followed by N248K ($n = 2$), D343N ($n = 1$), M39I ($n = 1$) and G408V ($n = 1$) in *cyp51A*; Q42L was observed in *cyp51B* ($n = 11$), respectively. Clinical information associated with the treatment and prognosis of the 14 patients from whom *A. fumigatus* isolates with the *cyp51A/cyp51B* mutations were recovered is summarized in Table 2. Six strains harboring the mutations G408V, M39I, N248K, or Q42L were associated with probable invasive aspergillosis, and all but one patient had poor outcome, due to mainly underlying disease (haematological malignancies or idiopathic pulmonary fibrosis) and irrespective of antifungal therapy (amphotericin B and/or azoles). Eight strains (those with D343N, N248K, combinations of three or more mutations, or Q42L) were obtained from colonized patients and only two of these patients, who had the severe underlying disease (idiopathic pulmonary fibrosis or bacterial peritonitis), died. Previous antifungal exposure was observed in 4 of 14 patients.

Until now, no surveillance data on the azole resistance of *A. fumigatus* clinical isolates in Korea has been available. We found that the azole resistance rate of the *A. fumigatus* clinical isolates was low (2.4%) and seemed to be similar to the current global prevalence of 3.2%, based on screening conducted in 22 centers in 19 countries [14]. Moreover, we found no mutation of the *cyp51A/cyp51B* genes in the two itraconazole-resistant isolates, raising the possibility that other resistance mechanisms, such as efflux pump, exist in these isolates [15]. However, Lee et al. [9] reported the first Korean case of azole-resistant *A. fumigatus* harboring *cyp51A* mutations in 2018, suggesting the possibility of the further emergence of azole resistance since our data were collected. Our 2-year multicentre data reflect the nationwide epidemiology at that time; therefore, continuous surveillance is warranted. Notably, several amino acid substitutions in the *cyp51A* gene were found in nine susceptible isolates, rather than in the resistant isolates examined in this study. Four studies have been conducted to compare *cyp51A* mutations between azole-susceptible and -resistant *A. fumigates* clinical isolates [7,8,16,17]. In those studies, several substitutions in position 54, 138, 220 or a duplication in tandem of a 34-bp fragment in the *cyp51A* promoter combined with a substitution of leucine at position 98 for histidine were suggested to be related to azole resistance, although they were not found in our study. Instead, the frequency of *cyp51A* mutations in azole-susceptible isolates in those studies ranged widely from 5.5% to 25.1% [7,8,16,17]. In agreement with our results, F46Y/M172V/E427K was the most commonly found mutation in the U.S. surveillance (5.5%) and Spainish studies (14.0%) [7,16]. In China, however, N248K (13.1%) was the most common mutation; F46Y/M172V/N248T/D255E/E427K (1.3%) and D343N (1.3%) were relatively uncommon [8]. The diversity of polymorphisms according to geographical distribution may be due to the genetic diversity of environmental and airborne isolates [18].

Table 2. Clinical information of the patients with *Aspergillus fumigatus* isolates harboring amino acid substitutions in *cyp51A* or *cyp51B*.

A. fumigatus isolate					Patient					
No	AAS in Cyp51A	AAS in Cyp51B	Year	Source	Hos	Age (yr)	Sex (M/F)	Underlying diseases	Previous antifungals use	Post-antifungal therapy	Outcome
Strains associated with probable invasive aspergillosis[a]											
1	G408V	None	2012	Sputum	A	46	F	AML	ITR, FLU, CAS	AMB, VOR	Dead
2	M39I	None	2012	Bronchial wash	A	62	M	AML	N/A	AMB, VOR	Dead
3	N248K	None	2012	Sputum	A	37	F	Bronchiolitis obliterans	FLU	AMB, VOR	Survive
4	None	Q42L	2012	Sputum	A	48	M	HL, Peripheral T cell lymphoma	FLU	ITR, AMB	Dead
5	None	Q42L	2013	Pus	B	77	F	IPF, DM, HTN	AMB	AMB	Dead
6	None	Q42L	2012	Sputum	A	66	M	HL	N/A	FLU	Dead
Strains associated with colonization											
7	D343N	None	2012	Sputum	A	70	M	IPF, Pulmonary HTN	N/A	No	Dead
8	F46Y,N248T, D255E,E427K	None	2012	Sputum	A	69	M	Salmonella sepsis	N/A	No	Survive
9	F46Y,M172V,E427K	None	2013	Urine	C	75	F	HTN	No	No	Survive
10	F46Y,M172V,E427K	None	2013	Sputum	D	41	M	TB	No	No	Survive
11	N248K	None	2012	Bronchial wash	A	53	M	TB	N/A	No	Survive
12	F46Y,M172V, N248T,D255E, E427K	Q42L	2013	Sputum	C	59	M	DM, CKD	No	No	Survive
13	None	Q42L	2012	Sputum	A	46	F	Liver cirrhosis, bacterial peritonitis	N/A	AMB	Dead
14	None	Q42L	2012	Sputum	A	62	M	NSCLC, Glottic cancer	N/A	No	Survive

[a]Probable invasive aspergillosis was classified according to criteria from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) [20].

AAS: amino acid substitution; AML: acute myeloid leukemia; CAS: caspofungin; CKD: chronic kidney disease; DM: diabetes mellitus; FLU: fluconazole; HL: Hodgkin's lymphoma; Hos: hospital; HTN: hypertension; IPF: idiopathic pulmonary fibrosis; ITR: itraconazole; N/A: not available; NSCLC: non-small cell lung cancer; TB: tuberculosis.

Although we did not conduct genotyping of the isolates, Escribano et al. suggested that several polymorphisms are linked to certain genotypes [5]. Recent research using whole genome sequencing has revealed that the AF293 reference genome belongs to a cluster of strains with the F46Y/M172V/N248T/D255E/E427K mutation, whereas the A1163 reference genome belongs to a cluster of wild-type strains [13]. We found no relationship between the azole MIC and specific strains harboring any muation in the *cyp51A* gene. Overall, caution should be necessary when interpreting the significance of muations in the *cyp51A* gene, considering the background heterogeneity of *A. fumigatus* isolates. Further research may help to elucidate the significance of the mutations newly reported in this study, such as M39I and G408V. Few studies have addressed mutations in the *cyp51B* gene from azole-susceptible isolates, although Diaz-Guerra et al. reported the Q42L amino acid change in two itraconazole-resistant isolates [19]. In the present study, 13.4% of the azole-susceptible isolates, but no azole resistant isolate, harbored Q42L mutations. This finding suggests that the Q42L amino acid change is not directly involved in the azole resistance of *A. fumigatus* and that *cyp51B* mutations are less diverse than those of *cyp51A*. We found no relationship between any clinical prognosis and *cyp51A/cyp51B* mutation type. Previous antifungal exposure was also not correlated strongly with the presence of isolates harboring any mutation in the *cyp51A/cyp51B* genes, suggesting that these mutations are caused by environmental antifungal use, rather than by exposure to azole *in vivo*.

The current study offers epidemiological data on antifungal susceptibilities and the occurrence of mutations in *cyp51A/cyp51B* in clinical isolates of *A. fumigatus* from Korea. Overall, the rate of occurrence of antifungal resistance in *A. fumigatus* remains low in Korea. Caution should be taken, however, in accepting the interpretation that azole resistance is conferred by mutation of the *cyp51A* gene, considering the high degree of polymorphism found among azole-susceptible isolates. Epidemiological surveillance of antifungal resistance of *A. fumigatus* should be continued.

Authors' contributions

This study was designed by SHK. EJW, MYJ, DL, MK, YP collected and analyzed the data. EJW and SHK wrote the initial draft of the manuscript. MGS and JHS supervised the project. All authors revised the manuscript.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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