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The T788G Mutation in the *cyp51C* Gene Confers Voriconazole Resistance in *Aspergillus flavus* Causing Aspergillosis

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With voriconazole (VRC) being approved as the first choice in treating invasive aspergillosis (IA) and its increasing use in treatment, a VRC-resistant strain of *Aspergillus flavus*, the second leading cause of IA after *Aspergillus fumigatus*, has emerged. The VRC-resistant strain of *A. flavus* was isolated for the first time from the surgical lung specimen of an IA patient with no response to VRC therapy. In order to ascertain the mechanism of VRC resistance, the azole target enzyme genes in this strain of *A. flavus* were cloned and sequenced, and 4 mutations generating amino acid residue substitutions were found in the *cyp51C* gene. To further determine the role of this mutated gene for VRC resistance in *A. flavus*, an *Agrobacterium tumefaciens*-mediated gene replacement approach was applied. Consequently, the mutated *cyp51C* gene from this *A. flavus* strain was proven to confer the VRC resistance. Finally, to discern the one out of the four mutations in the *cyp51C* gene that is responsible for contributing to VRC resistance, a site-directed gene mutagenesis procedure combined with a gene replacement method was performed. As a result, the T788G missense mutation in the *cyp51C* gene was identified as responsible for VRC resistance in *A. flavus*. These findings indicated that the detection of this mutation in *A. flavus* could serve as an indicator for physicians to avoid the use of VRC during IA treatment. Further comprehensive surveillance for antifungal susceptibility, as well as intensive study on the mechanism of azole resistance in *A. flavus* causing IA, would be required to fully understand this mechanism.

Invasive aspergillosis (IA) mainly occurs in severely immunocompromised patients such as those with prolonged neutropenia, advanced AIDS, and chronic granulomatous disease. It is also a severe complication in patients with hematologic malignancies, allogeneic hematopoietic stem cell transplantation (HSCT), and solid organ transplantation (23). During IA treatment, voriconazole (VRC), an azole drug, is the first choice due to its favorable responses (11, 31). However, with the wide use of azoles in clinical practice, azole-resistant aspergillosis including that having multiple-triazole resistance is becoming a prominent clinical problem, resulting in the poor prognosis and high mortality (2, 28, 29). Azole resistance in *Aspergillus fumigatus* has become a new challenge in the management of IA (25). Although azole-resistant aspergillosis was reported for the first time in an *A. fumigatus* infection in 1997 (5), until now, there has been no report of azole resistance in *Aspergillus flavus*-infected patients, even though *A. flavus* is the second most common IA pathogen (7, 9, 14, 23, 31, 32). Recently, a patient with IA resistant to VRC therapy appeared in our practice. From the lung surgical specimen of this patient, we isolated a VRC-resistant *A. flavus* strain, from which we then determined that VRC resistance in this *A. flavus* strain resulted from the T788G missense mutation in the *cyp51C* gene encoding azole target enzyme. This mutation was different from that in the *cyp51A* gene of *A. fumigatus* (2), which was responsible for azole resistance.

MATERIALS AND METHODS

Isolation of VRC-resistant *A. flavus* strain from the patient. The pathogenic fungal strain isolated from the culturing of the lung surgical specimen of the IA patient was used to extract the genomic DNA according to the procedure described previously (33). This strain was identified by macroscopic and microscopic characteristics after being grown on both Czapek agar and malt extract agar at 25°C for 10 days (20). The *actin* and *calmodulin* genes and the internal transcribed spacer of this strain were then amplified (12, 22) and identified by alignment online using the recognized *Aspergillus* species database (http://www.cbs.knaw.nl). This strain was identified and named *A. flavus* BMU29791. To verify whether *A. flavus* BMU29791 is resistant to the commonly used antifungal drugs, the Clinical and Laboratory Standards Institute (CLSI) M38-A2 method (4, 30) and Etest (27) were used to assay antifungal susceptibility in *A. flavus* BMU29791.

Expression level and sequencing analysis of the azole target enzyme genes in *A. flavus* BMU29791. Since overexpression in azole target enzyme gene *cyp51A* could contribute to azole resistance in *A. fumigatus* (2), we hypothesized that this is also the case in this VRC-resistant strain, *A. flavus* BMU29791. We searched the homologue of the *A. fumigatus cyp51A* gene in *A. flavus* using the nucleotide databank NCBI BLAST (http://www.ncbi.nlm.nih.gov). Three gene homologues of the *A. fumigatus cyp51A* gene, namely, *cyp51A* (NCBI accession number XM_002375082.1), *cyp51B* (NCBI accession number XM_002379089.1), and *cyp51C* (NCBI accession number XM_002383890.1), were found in *A. flavus*. We then downloaded the whole sequences of the open reading frames (ORFs), flanked by both the upstream and downstream regions with a size of about 1,000 bp, of these three genes from the *A. flavus* genome (http://www.broadinstitute.org). According to these sequences, the primers for cloning as well as the TaqMan probes (Table 1) for measuring the expression levels of the *cyp51A*, *cyp51B*, and *cyp51C* genes by quantitative real-time PCR were designed. Relative quantitative real-time PCR (19) to measure expression levels of genes for the azole target enzyme in *A. flavus*

Received 7 August 2011 Returned for modification 10 November 2011
Accepted 31 January 2012
Published ahead of print 6 February 2012
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doi:10.1128/AAC.05477-11

TABLE 1 Primers and probes used in the present study

Primer/TaqMan probe	Sequence[e]	Target
CYP51AF[a]	5′-ATCCCGGGATAAACTCTATACATCATAG-3′ (SmaI)	*cyp51A* gene of *A. flavus*
CYP51AR[a]	5′-ATGCGGCCGCGACCTTACATGGAACGCCTT-3′ (NotI)	
CYP51BF[a]	5′-ATGCGGCCGCGCCCGTGGGTTGCTGTCTTT-3′ (NotI)	*cyp51B* gene of *A. flavus*
CYP51BR[a]	5′-ACGCATGCTTTTCGGTTCCTATCCACCC-3′ (SphI)	
CYP51CF1[a]	5′-ATCCCGGGCACGTACACCAAACACTGCT-3′ (SmaI)	*cyp51C* gene of *A. flavus*
CYP51CR1[a]	5′-ATGCGGCCGCAGCTGGAAGCTCTTCCGCTA-3′ (NotI)	
AflaF1[b]	5′-GCGCGCATGAGGGAGAT-3′	*cyp51A* gene of *A. flavus*
AflaR1[b]	5′-CAATGCATGAGGTTCCAGATCA-3′	
ALF1-TaqMan[b]	FAM-5′-TCATTAACGAGCGCCGCAAGAACC-3′-TAMRA	
AflaF2[b]	5′-ATTCGACTCGACATTTGCTGAA-3′	*cyp51B* gene of *A. flavus*
AflaR2[b]	5′-GCATCACGCTTGCGGTTAT-3′	
ALF2-TaqMan[b]	FAM-5′-CATGATCTCGACATGGGTTTTGCCC-3′-TAMRA	
AflaF3[b]	5′-GTGACAACGTCCCGGAGAAG-3′	*cyp51C* gene of *A. flavus*
AflaR3[b]	5′-ATCAGCAGCGTGATCATGATG-3′	
ALF3-TaqMan[b]	FAM-5′-ATGCAGTGCACCTACAAGAACGGACAGC-3′-TAMRA	
AflaActinF[b]	5′-TGGTTCCAATCTACGAAGGTTTC-3′	*actin* gene of *A. flavus*
AflaActinR[b]	5′-ATCTCGTGCTCGGCAGATGT-3′	
ALFActin-TaqMan[b]	FAM-5′-CTATGCCACACGCTATCGCTCGGATG-3′-TAMRA	
AflaCYP51CF2[c]	5′- TCGAGCTCCACGTACACCAAACACTGCT-3′ (SacI)	*cyp51C* gene of *A. flavus*
AflaCYP51CR2[c]	5′- AACCCGGGAGCTGGAAGCTCTTCCGCTA-3′ (SmaI)	
F-161[d]	5′-AGCACAATCCACTATGGAACGGATCCGTAC-3′	Recombinant plasmid pDHt/sk-3357C-161
R-161[d]	5′-GTTCCATAGTGGATTGTGCTGCCGATGAAT-3′	
F-788[d]	5′-CTGCCCATGCTCGGATGCGTGCTATCTACAT-3′	Recombinant plasmid pDHt/sk-3357C-788
R-788[d]	5′-CACGCATCCGAGCATGGGCAGCATCGCGTTT-3′	
F-1325[d]	5′-CCGGTGGGAAACACAGGCAACTCAGGAAAA-3′	Recombinant plasmid pDHt/sk-3357C-1325
R-1325[d]	5′-TTGCCTGTGTTTCCCACCGGTGCGGGTCCCA-3′	
F-1337[d]	5′-CACAGGCACCTCAGGAAAACGATAAGGATGA-3′	Recombinant plasmid pDHt/sk-3357C-1337
R-1337[d]	5′-CGTTTTCCTGAGGTGCCTGTGTTTCCCACCG-3′	

[a] Primers used in cloning of the genes *cyp51A*, *cyp51B*, and *cyp51C* for sequencing analysis and construction of the *A. flavus* transformants with extra copies of these genes from *A. flavus*.
[b] Primers and probes used for real-time PCR assaying.
[c] Primers used for amplification of *cyp51C* for both gene replacement and site-directed mutagenesis.
[d] Primers used for construction of plasmids pDHt/sk-3357C-161, pDHt/sk-3357C-788, pDHt/sk-3357C-1325, and pDHt/sk-3357C-1337 during site-directed mutagenesis experiment.
[e] Underlining indicates the sites for the restriction enzymes indicated in parentheses or site-directed mutations (single underlined bases). FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

BMU29791 was performed by using an ABI 7500 PCR device. The primers and TaqMan probes are listed in Table 1.

Construction of *A. flavus* transformants with extra copies of azole target enzyme genes. In an attempt to assess whether VRC resistance in *A. flavus* was the result of increased copies of the *cyp51A*, *cyp51B*, or *cyp51C* gene, we used an approach described for *A. fumigatus* (16) to study gene-based dose-dependent resistance to antifungals. Primers were designed (Table 1) to amplify the *cyp51A*, *cyp51B*, and *cyp51C* genes, including the whole coding region with 1 kb of genomic DNA upstream and downstream of each gene, from the genomic DNA (33) of *A. flavus* NRRL3357 (obtained from the Fungal Genetics Stock Center [17]). The PCR amplicons were cloned into the pRG3-AMA1-NotI plasmid (16), and the recombinant plasmids (Table 2) pRG3-AMA1-NotI-A, pRG3-AMA1-NotI-B, and pRG3-AMA1-NotI-C were further transformed into the protoplast of the uracil prototroph *A. flavus* strain 3357-5 (kind gift of Zhumei He, School of Life Sciences, Sun Yat-Sen University, Guangzhou, People's Republic of China) (Table 2) (8) to obtain the corresponding transformants, namely, AF-A, AF-B, and AF-C. The transformant AF-E with the empty plasmid pRG3-AMA1-NotI was used as a control.

We further tested the antifungal susceptibility in these 3 transformants with 2 independent methods. First, a broth microdilution method according to the CLSI M38-A2 guidelines was used. Selective liquid minimal medium (MM; 70 mM $NaNO_3$, 7 mM KCl, 2 mM $MgSO_4$, 12 mM KPO_4 [pH 6.8], trace elements, 1% glucose) minus uracil was used to replace the recommended RPMI 1640 broth medium in order to maintain the selection of the $pyrG^+$ plasmid in these transformants. AF-E, the *A. flavus* transformant with extra copies of the empty plasmid pRG3-AMA1-NotI, was used as a control. Second, the Etest was performed. Similarly, MM plates minus uracil were used to maintain the selection of the $pyrG^+$ plasmid in these transformants.

Sequence analysis of azole target enzyme genes in *A. flavus* BMU29791. Because azole resistance in *A. fumigatus* is mainly the result of mutations and a 34-bp duplicate in the promoter region in *cyp51A* (2), we speculated that this would also be the case in *A. flavus* BMU29791.

We hence amplified the whole coding regions of the *cyp51A*, *cyp51B*, and *cyp51C* genes as well as the 1-kb genomic DNA fragment upstream and downstream of each gene in *A. flavus* BMU29791 by using primers (Table 1) designed according to the azole target enzyme gene sequences in *A. flavus*. After cloning, each PCR product was sequenced. The PCR amplicons were then cloned into the pMD20-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) (21) for sequencing. These DNA sequences were aligned online

TABLE 2 Strains and plasmids used in the present study

Strain or plasmid	Relevant characteristic	Source
Strains		
A. flavus 3357-5	*A. flavus* lacking *pyrG*	8
NRRL3357	Standard *A. flavus* strain	17
BMU29791	VRC-resistant *A. flavus*	Present study
AF-A	*A. flavus* 3357-5 transformed with plasmid pRG3-AMA1-NotI-A	Present study
AF-B	*A. flavus* 3357-5 transformed with plasmid pRG3-AMA1-NotI-B	Present study
AF-C	*A. flavus* 3357-5 transformed with plasmid pRG3-AMA1-NotI-C	Present study
AF-E	*A. flavus* 3357-5 transformed with plasmid pRG3-AMA1-NotI	Present study
AflavC	*A. flavus* NRRL3357 transformant with *cyp51C* gene from *A. flavus* BMU29791	Present study
AflavC-3357	*A. flavus* BMU29791 transformant with *cyp51C* gene from *A. flavus* NRRL3357	Present study
AflavC-161	*A. flavus* NRRL3357 transformant with T161C mutation in *cyp51C* gene	Present study
AflavC-788	*A. flavus* NRRL3357 transformant with T788G mutation in *cyp51C* gene	Present study
AflavC-1325	*A. flavus* NRRL3357 transformant with C1325A mutation in *cyp51C* gene	Present study
AflavC-1337	*A. flavus* NRRL3357 transformant with A1337G mutation in *cyp51C* gene	Present study
E. coli DH10B	*E. coli*	Invitrogen
A. tumefaciens EHA105	*A. tumefaciens*	26
Plasmids		
pRG3-AMA1-NotI	Shuttle plasmid allowing autonomous nonintegrating replication of itself in life cycle of *Aspergillus* spp.	16
pRG3-AMA1-NotI-A	Recombinant plasmid of pRG3-AMA1-NotI with complete *cyp51A* gene of *A. flavus*	Present study
pRG3-AMA1-NotI-B	Recombinant plasmid of pRG3-AMA1-NotI with complete *cyp51B* gene of *A. flavus*	Present study
pRG3-AMA1-NotI-C	Recombinant plasmid of pRG3-AMA1-NotI with complete *cyp51C* gene of *A. flavus*	Present study
pDHt/sk	Binary plasmid	26
pDHt/sk-29791C	Recombinant plasmid of pDHt/sk with complete *cyp51C* gene of *A. flavus* BMU29791	Present study
pDHt/sk-3357C	Recombinant plasmid of pDHt/sk with complete *cyp51C* gene of *A. flavus* NRRL3357	Present study
pDHt/sk-3357C-161	Recombinant plasmid of pDHt/sk with *A. flavus cyp51C* gene carrying T161C mutation	Present study
pDHt/sk-3357C-788	Recombinant plasmid of pDHt/sk with *A. flavus cyp51C* gene carrying T788G mutation	Present study
pDHt/sk-3357C-1325	Recombinant plasmid of pDHt/sk with *A. flavus cyp51C* gene carrying C1325A mutation	Present study
pDHt/sk-3357C-1337	Recombinant plasmid of pDHt/sk with *A. flavus cyp51C* gene carrying A1337G mutation	Present study
pMD20-T vector	For T-A clone	TaKaRa Biotechnology

(http://www.ebi.ac.uk) with those from *A. flavus* NRRL3357, and four mutations were found in the *cyp51C* gene of *A. flavus* BMU29791.

***cyp51C* gene replacement in *A. flavus*.** In order to ascertain whether the mutated *cyp51C* gene in *A. flavus* BMU29791 accounts for VRC resistance, we used in *A. flavus* an *Agrobacterium tumefaciens*-mediated gene transformation system as described previously for *A. fumigatus* (18) with minor modifications to replace the *cyp51C* gene of *A. flavus* NRRL3357 with the corresponding gene from *A. flavus* BMU29791. Primers were designed (Table 1) to amplify the *cyp51C* gene from the genomic DNA (33) of *A. flavus* BMU29791, including the whole coding region, 1 kb of genomic DNA upstream of the gene, and 1 kb of genomic DNA downstream of the gene. The PCR amplicon was cloned into the pDHt/sk vector (Table 2) (18, 26) to obtain the recombinant plasmid pDHt/sk-29791C, which was further transformed into *A. tumefaciens* EHA105 (a gift from K. J. Kwon-Chung, Laboratory of Clinical Infection, National Institutes of Health) (Table 2) (26) to obtain the resulting *A. tumefaciens* strain EHA105-29791C. Then, the *A. tumefaciens* EHA105-29791C suspension (200 μl) was mixed with an equal volume of *A. flavus* NRRL3357 conidia (1×10^7/ml), and the mixture was spread on the induction medium plates (26) containing 0.5 μg/ml VRC for incubation at 24°C for 48 h followed by another 48 h at 37°C. In our pilot experiment, it was proven that 200 μl of the *A. flavus* NRRL3357 conidium suspension (1×10^7/ml) could be killed on the induction medium with VRC at concentration of 0.5 μg/ml, without any VRC-resistant *A. flavus* strains grown. The colonies of the *A. flavus* transformants were selected up and cultured on the MM plates containing 200 μg/ml cefotaxime sodium and 0.5 μg/ml VRC at 37°C to verify the purity. After amplification by PCR, the *cyp51C* gene in the *A. flavus* transformant (named AflavC) was sequenced to confirm the successful replacement of the *cyp51C* gene. Finally, the expression levels of the *cyp51A*, *cyp51B*, and *cyp51C* genes of the transformant AflavC preexposed to VRC at 0.25 μg/ml were assayed by using real-time PCR as described above.

At the same time, the mutated *cyp51C* gene in *A. flavus* BMU29791 was also replaced with the one from *A. flavus* NRRL3357 per the procedure described above with a minor modification: primers were designed (Table 1) to amplify the *cyp51C* gene from the genomic DNA (33) of *A. flavus* NRRL3357, including the whole coding region, 1 kb of genomic DNA upstream of the gene, and 1 kb of genomic DNA downstream of the gene. The PCR amplicon was cloned into the pDHt/sk vector (Table 2) (18, 26) to obtain the recombinant plasmid pDHt/sk-3357C, which was further transformed into *A. tumefaciens* EHA105 (Table 2) (26) to obtain the resulting *A. tumefaciens* strain, EHA105-3357C. Then, the *A. tumefaciens* EHA105-3357C suspension (200 μl) was mixed with an equal volume of the *A. flavus* BMU29791 conidia (1×10^7/ml), and the mixture was spread on the induction medium plates (26) for incubation at 24°C for 48 h followed by another 48 h at 37°C. The *A. flavus* transformant colonies were selected and cultured on the MM plates containing 200 μg/ml cefotaxime sodium to kill *A. tumefaciens*. Each of the *A. flavus* transformant colonies was further subcultured on MM both without and with 0.5 μg/ml VRC at 37°C. Then, 48 to 72 h later, *A. flavus* transformant colonies which were not able to grow on MM with 0.5 μg/ml VRC despite being grown on MM without VRC were used for sequencing analysis after PCR amplification. Finally, the *A. flavus* transformant (named AflavC-3357) containing the *cyp51C* gene from *A. flavus* NRRL3357 was confirmed.

Site-directed mutagenesis of the *cyp51C* gene in *A. flavus*. To further discern the one out of the four mutations in *cyp51C* which was responsible

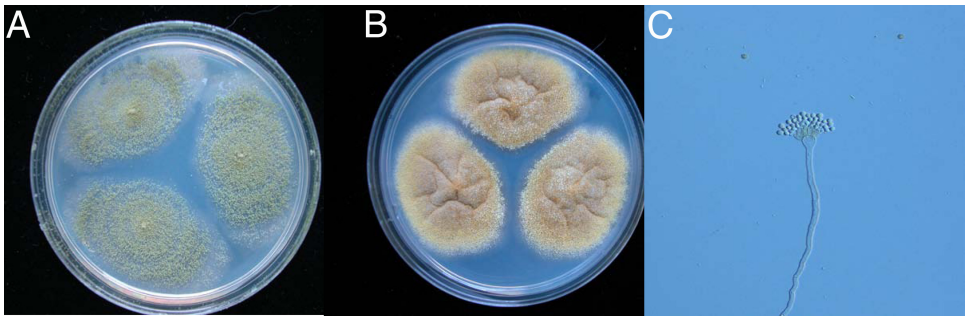

FIG 1 Morphological observation for *A. flavus* BMU29791 following 10 days of culture at 25°C. (A) Macroscopic appearance on MEA plate: velvet-like, yellow-green colony. (B) Macroscopic appearance on CZA plate: cottony and yellow colony. (C) Microscopy: radiate conidial heads with uniseriate conidiogenous cells.

for contributing to VRC resistance in *A. flavus* BMU29791, we performed a site-directed gene mutagenesis procedure in *A. flavus*. Primers for site-directed mutagenesis were designed (10) (Table 1) to amplify the recombinant plasmid pDHt/sk-3357C, a derivative of the plasmid pDHt/sk, which contained the *A. flavus* NRRL3357 *cyp51C* gene. The PCR amplicon, an entire circular plasmid anticipated to be about 9,500 bp in size, was then transformed into bacterial competent cells (Beijing TransGen Biotech Co., Ltd.) for sequencing. The corresponding plasmids pDHt/sk-3357C-161, pDHt/sk-3357C-788, pDHt/sk-3357C-1325, and pDHt/sk-3357C-1337 (Table 2), containing the *A. flavus cyp51C* gene with respective mutated sites T161C (M54T), T788G (S240A), C1325A (P419T), and A1337G (N423D), were confirmed by alignment online (http://www.ebi.ac.uk). These 4 recombinant plasmids were then transformed into *A. tumefaciens* EHA105, and the suspension of resulting *A. tumefaciens* transformants (named EHA105-3357C-161, EHA105-3357C-788, EHA105-3357C-1325, and EHA105-3357C-1337, respectively) was further coincubated with *A. flavus* NRRL3357 conidia as described above. The *cyp51C* gene in the corresponding *A. flavus* transformants was further analyzed by PCR sequencing and alignment online (http://www.ebi.ac.uk) to confirm the success of replacement. Consequently, 4 transformants carrying each of the 4 mutations in the *cyp51C* gene, namely, AflavC-161, AflavC-788, AflavC-1325, and AflavC-1337, were obtained. The expression levels of the *cyp51A*, *cyp51B*, and *cyp51C* genes of the transformants AflavC-161, AflavC-788, AflavC-1325, and AflavC-1337 preexposed to VRC at 0.25 μg/ml were also assayed by using real-time PCR as described above.

RESULTS

IA patient unresponsive to VRC treatment. The patient, a 14-year-old girl, was diagnosed with acute myeloid leukemia (AML-M2) in December 2007, and she was treated with chemotherapy consisting successively of dexamethasone and epirubicin; arabinosylcytosine, dexamethasone, and epirubicin; harringtonine and arabinosylcytosine; idarubicin, arabinosylcytosine, and epirubicin; and idarubicin and arabinosylcytosine. Twelve months later, her bone marrow examination indicated a relapse of leukemia. Allogeneic HSCT was performed in February 2009. Two days after the transplantation, the patient coughed and had expectoration with back pain, and *Staphylococcus aureus* was isolated from her sputum. Then, she was treated with vancomycin and azythromycin. After a short period of remission, symptoms emerged again during antibiotic treatment. VRC was given orally 200 mg every 12 h to control the possible fungal infection. Her symptoms were alleviated only for a short period of time, and her condition did not improve after antifungal treatment for 2 months. Radiological examination showed localized masses in the upper lobe of her right lung, and pulmonectomy was conducted on 23 September 2009. Her infection and respiratory symptoms disappeared after the operation. Histopathological examination showed branched hyphae with septa in the lung tissue. Culture of the surgical specimen found mold, which was further identified as *A. flavus* by morphological (Fig. 1) and molecular experiments and was named *A. flavus* BMU29791. Therefore, this patient was diagnosed with IA.

Etiologic organism *A. flavus* BMU29791 was resistant to VRC. The MICs of VRC, itraconazole (ITC), and amphotericin B (AMB) against *A. flavus* BMU29791 were 8 μg/ml, 2 μg/ml, and 1 μg/ml (Table 3), respectively. According to the proposed interpretative breakpoints (4, 30), *A. flavus* BMU29791 was defined as resistant to VRC, intermediate to ITC, and susceptible to AMB. The minimal effective concentration (MEC) of caspofungin (CAS) for *A. flavus* BMU29791 was 0.25 μg/ml. An Epsilometer test (Etest; AB Biodisk, Solna, Sweden) for antifungal susceptibility in this strain showed similar results (data not shown).

VRC resistance in *A. flavus* BMU29791 was not related to the expression level of azole target enzyme genes. The expression level of the *cyp51C* gene, excluding those for *cyp51A* and *cyp51B*, was slightly increased in *A. flavus* BMU29791 in comparison with what was seen for the standard strain, *A. flavus* NRRL3357. Compared to control transformant AF-E, however, the *A. flavus* transformant AF-C with extra copies of the *A. flavus cyp51C* gene showed that it was susceptible to VRC despite being resistant to ITC (Table 3). Moreover, *A. flavus* transformant AF-A or AF-B with extra copies of the *A. flavus cyp51A* or *cyp51B* gene exhibited *in vitro* resistance to both ITC and VRC (Table 3). The Etest assay showed similar results (data not shown).

Mutations in the *cyp51C* gene in *A. flavus* BMU29791 were detected. Comparing with the standard strain, *A. flavus* NRRL3357, no abnormal sequences in the upstream regions of the coding sequences in these 3 genes were observed. The *cyp51A* and *cyp51B* genes in *A. flavus* BMU29791 were also intact. However, four missense mutations, the T161C, T788G, C1325A, and A1337G mutations, were found in the *cyp51C* gene in the *A. flavus* BMU29791 strain. These four mutations result in amino acid residue substitutions of M54T, S240A, P419T, and N423D, respectively, in the CYP51C protein.

The mutated *cyp51C* gene from *A. flavus* BMU29791 contributed to VRC resistance. Using an *A. tumefaciens*-mediated gene transformation system, the *cyp51C* gene in *A. flavus* NRRL3357, the standard *A. flavus* strain susceptible to VRC, was replaced with the one from *A. flavus* BMU29791. At the same time, the *cyp51C* gene in *A. flavus* BMU29791 was also replaced with the one from

TABLE 3 MIC or MEC of antifungal drugs against *Aspergillus* sp. strains[l]

Strain	MIC/MEC (μg/ml) of:				Portion of sequence of *cyp51C* gene containing mutation[k]
	ITC	VRC	AMB	CAS[j]	
BMU29791[a]	2	8	1	0.25	ATGCGTGCTATCTAC (240A)
AflavC[b]	2	8	1	0.25	ATGCGTGCTATCTAC (240A)
AflavC-3357[c]	1	0.25	1	0.25	ATGCGTTCTATCTAC (240S)
AflavC-788[d]	2	8	1	0.25	ATGCGTGCTATCTAC (240A)
NRRL3357[e]	1	0.25	1	0.25	ATGCGTTCTATCTAC (240S)
AF-A[f]	≥16	4	0.5	0.03	NA
AF-B[g]	≥16	4	0.5	0.03	NA
AF-C[h]	≥16	1	0.5	0.03	NA
AF-E[i]	0.5	0.5	0.5	0.03	NA

[a] VRC-resistant *A. flavus* strain isolated from the lung tissue of the patient.
[b] *A. flavus* NRRL3357 transformant carrying the *cyp51C* gene of *A. flavus* BMU29791.
[c] *A. flavus* BMU29791 transformant carrying the *cyp51C* gene of *A. flavus* NRRL3357.
[d] *A. flavus* NRRL3357 transformant carrying the *cyp51C* gene with T788G missense mutation.
[e] VRC-susceptible *A. flavus* strain obtained from the Fungal Genetics Stock Center.
[f] *A. flavus* transformant with recombinant plasmid pRG3-AMA1-NotI-A.
[g] *A. flavus* transformant with recombinant plasmid pRG3-AMA1-NotI-B.
[h] *A. flavus* transformant with recombinant plasmid pRG3-AMA1-NotI-C.
[i] *A. flavus* transformant with the empty plasmid pRG3-AMA1-NotI.
[j] The antifungal activity of CAS was determined by using MEC.
[k] Codons (underlined) and corresponding amino acid residues (in parentheses) are shown. NA, not available.
[l] MEC, minimal effective concentration; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B; CAS, caspofungin.

A. flavus NRRL3357. The antifungal susceptibility assay showed that the *A. flavus* transformant AflavC containing the mutated *cyp51C* gene from *A. flavus* BMU29791 achieved the specific resistance to VRC, while being intermediate to ITC and susceptible to AMB and CAS (Table 3). Moreover, the *A. flavus* transformant AflavC-3357 containing the cured allele of the *cyp51C* gene (from *A. flavus* NRRL3357) showed the same antifungal susceptibility spectrum as *A. flavus* NRRL3357 (Table 3).

In the transformants AflavC, AflavC-161, AflavC-788, AflavC-1325, and AflavC-1337 preexposed to VRC at 0.25 μg/ml, no significant change was observed in the expression levels of the *cyp51A*, *cyp51B*, and *cyp51C* genes, compared to those in *A. flavus* NRRL3357 (data not shown).

The T788G mutation in the *cyp51C* gene confers VRC resistance in *A. flavus*. Using a site-directed gene mutagenesis procedure combined with the gene replacement method mentioned above, the *cyp51C* gene in *A. flavus* NRRL3357 was respectively replaced by each mutated *cyp51C* gene containing individual mutations observed in *A. flavus* BMU29791. Consequently, 4 transformants of *A. flavus* carrying each of the 4 mutations in the *cyp51C* gene were identified, which were named AflavC-161, AflavC-788, AflavC-1325, and AflavC-1337. After the antifungal susceptibility assay, we found that an *A. flavus* transformant harboring a mutated *cyp51C* gene with the T788G missense mutation (Table 3, strain AflavC-788) was resistant to VRC, intermediate to ITC, and susceptible to AMB, while the transformants carrying other point mutations, including T161C, C1325A, and A1337G, were all still susceptible to these antifungal drugs, suggesting that the T788G missense mutation (amino acid change of S240A) in the *cyp51C* gene endows *A. flavus* including the strain *A. flavus* BMU29791 with the ability to resist VRC treatment.

DISCUSSION

According to the clinical practice guideline by the Infectious Diseases Society of America (31) and the definitions of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group (6), the patient described in the present study was diagnosed as having IA. She failed to respond to antifungal treatment with VRC. After the isolation of fungus from the surgical specimen of her pulmonary tissue, we first ascertained that the colonies originated from a single *Aspergillus* strain by a random-amplification polymorphism DNA approach (data not shown), since mixed infections by different microbes, such as fungi and bacteria (1), *Candida albicans* and non-*albicans Candida* (13), or *A. fumigatus* and *A. flavus* (3, 11), happen occasionally in the clinical environment. This strain was further identified as *A. flavus* by using the standard molecular and morphological approaches for *Aspergillus* species identification and was named *A. flavus* BMU29791.

A. flavus, the second leading cause of IA (7, 9, 14, 31, 32), is widely distributed in the environment, including soil, water, and air (7, 9, 14, 32). Individuals with severely impaired immune systems are prone to IA after inhaling *A. flavus* spores (9, 14, 23, 31). *A. flavus* is usually susceptible to the aforementioned antifungal drugs, including VRC. However, *A. flavus* strain BMU29791, isolated from the IA patient in the present study, was *in vitro* resistant to VRC, intermediate to ITC, and susceptible to both AMB and CAS (Table 3). To the best of our knowledge, this is the first description of VRC-resistant IA caused by the *A. flavus* strain with specific resistance to VRC.

Since ITC resistance was first described by Denning and colleagues in 1997 (5) in an IA patient infected by *A. fumigatus*, azole-resistant IA, all of which is caused by *A. fumigatus* as reported previously, has become a serious clinical issue (2, 24, 28, 29). Azole resistance in *A. fumigatus* has become a new challenge for IA treatment (25). Studies on the resistance mechanisms have shown that amino acid residue substitution derived from mutations in the azole target enzyme gene *cyp51A*, overexpression of this gene and drug efflux genes, and upregulation of homeostatic stress response

pathways contribute to azole resistance in *A. fumigatus* (2, 24, 28, 29). Different from these reports, our data from the VRC-resistant *A. flavus* BMU29791 demonstrated that no abnormal sequences in the *cyp51A* and *cyp51B* genes and the upstream region of the coding sequence in the *cyp51C* gene were observed, whereas 4 missense mutations causing amino acid residue substitutions were detected in the *cyp51C* gene, a member of the azole target enzyme gene family in *A. flavus*. These findings are also different from the report by Krishnan-Natesan et al. (15) that the VRC-resistant strain of *A. flavus*, selected *in vitro* by preexposure with VRC in the laboratory, had mutations in both the *cyp51A* and *cyp51B* genes but not in the *cyp51C* gene. In addition, we also demonstrated that the expression levels of the azole target enzyme genes *cyp51A*, *cyp51B*, and *cyp51C* were not related to VRC resistance in *A. flavus* BMU29791, even though extra copies of the *A. flavus cyp51A* or *cyp51B* gene conferred reliable azole resistance in *A. flavus* (Table 3).

We then confirmed that the mutated *cyp51C* gene from *A. flavus* BMU29791 was responsible for VRC resistance by an *A. tumefaciens*-mediated gene replacement method. Furthermore, we determined that the T788G (S240A) missense mutation in the *cyp51C* gene in *A. flavus* accounted for the specific resistance to VRC (Table 3) by using site-directed gene mutagenesis combined with gene replacement approaches. However, other mutations in genes *cyp51C*, *cyp51A*, and *cyp51B*, in addition to the T788G (S240A) mutation in the *cyp51C* gene as we presented here, may also correlate with VRC resistance in *A. flavus*.

In conclusion, with the wide use of VRC as the first choice in IA treatment (11, 31), VRC-resistant IA caused by an *A. flavus* strain with a T788G missense mutation in the *cyp51C* gene is now emerging as a potentially common cause of IA in clinics. Detection of this mutation in *A. flavus* will be a good indicator to avoid VRC use during IA treatment. Further comprehensive surveillance for antifungal susceptibility, as well as intensive study on the mechanism of azole resistance in *A. flavus* causing IA, is certainly required to gain full understanding.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China grants 30970131 (to Wei Liu) and 30930006 (to Ruoyu Li), by Beijing Natural Science Foundation grant 7102149 (to Wei Liu), and by the Program for New Century Excellent Talents in University grants NCET-10-0198 (to Wei Liu) and BMU20110158 (to Wei Liu). The funders had no role in the study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

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