Molecular Characterization of a Voriconazole-Resistant, Posaconazole-Susceptible *Aspergillus fumigatus* Isolate in a Lung Transplant Recipient in the United States

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Molecular characterization of *cyp51A* from the azole-resistant *Aspergillus fumigatus* isolate 50593 from a lung transplant patient showed Y121F/T289A changes coupled with a 46-bp tandem repeat (TR46) on the promoter, whereas *cyp51A* from the pre-therapy isolate, *A. fumigatus* 47381, showed no changes. This is the first reported case of *A. fumigatus* azole resistance due to Y121F/T289A/TR46 in the United States, suggesting that multiple mutational alterations of *cyp51A* resulting in high-level azole resistance could occur during prolonged antifungal therapy.

Triazole resistance among environmental and clinical isolates of *Aspergillus fumigatus* is a major problem in certain regions of Europe, particularly in the United Kingdom, France, Spain, Belgium, and the Netherlands (1–3). The major azole resistance mechanism in *A. fumigatus* involves point mutations of the *cyp51A* gene coding for cytochrome P450 14 α -sterol demethylase A resulting in amino acid alterations in critical regions of Cyp51A protein. A recent analysis of available data on drug target modification-dependent azole resistance in *A. fumigatus* identified 18 mutations on 14 different loci on *cyp51A* (3). The amino acid residues often involved in triazole resistance are G54, L98, G138, P216, F219, M220, E427, G434, and G448.

Single or multiple amino acid changes in Cyp51A caused by mutations confer resistance to azoles with various patterns of cross-resistance. *A. fumigatus* isolates harboring amino acid alterations at position G54 are resistant to itraconazole (Itz) and posaconazole (Pcz) but remain susceptible to voriconazole (Vcz) (4, 5). Amino acid variations at M220 leads to pan-azole resistance (6), whereas alterations at E427 in association with another amino acid change(s) confer resistance to Itz and Vcz but not to Pcz (3). G448S amino acid alterations confer high-level resistance to Vcz but only low-level resistance to Itz and Pcz (7, 8). In most cases, a single amino acid change is sufficient for conferring resistance in the strain carrying the variant protein. However, in some cases, high-level resistance results from nucleotide changes in the promoter region coupled with amino acid changes, as in TR34/L98H and TR46/Y121F/T289A (where TR34 and TR46 represent 34- and 46-bp tandem repeats, respectively) (9, 10).

Although two genes coding for cytochrome P450 14 α -sterol demethylase are present in *A. fumigatus* (11), Cyp51A is primarily responsible for the removal of the methyl group on carbon 14 of the lanosterol molecule. The goal of this study was to evaluate the Cyp51A mutation-dependent mechanism of resistance in a Vcz-resistant *A. fumigatus* isolate (AF50593) that developed secondary Vcz resistance during therapy for invasive aspergillosis (IA) in a lung transplant recipient. We hypothesized that an amino acid variation(s) at a critical region(s) of Cyp51A protein may be at least partly responsible for the reduced *in vitro* susceptibility of the clinical isolate AF50593 to Vcz.

Case report. A 66-year-old white male (retired firefighter) was referred to the infectious diseases department of a large Midwest-

ern hospital for fever and an infiltrate 9 months after a bilateral lung transplant for pulmonary fibrosis. The patient had received a bilateral lung transplant with a primary cytomegalovirus (CMV) mismatch. Since his transplant, he had several episodes of rejection and was receiving mycophenolate (500 mg every 12 h [q12h]), prednisone (20 mg once a day [q.d.]), and tacrolimus (2 mg q.d.). On admission, a computed tomography (CT) scan of the chest showed multifocal pneumonia and large bilateral pleural effusions. A bronchoscopy with a lung biopsy revealed invasive *A. fumigatus*. In addition, a thoracentesis of the bilateral pleural effusions revealed exudates which eventually grew *Mycobacterium chelonae* subsp. *abscessus*. Treatment with voriconazole (300 mg twice a day [BID]), linezolid (600 mg BID), azithromycin (500 mg q.d.), and cefoxitin (2 g q6h) was started, and the patient was discharged home in stable condition.

One month later, he was readmitted with increased dyspnea and a reaccumulation of pleural fluid. A repeat bronchoscopy with bronchoalveolar lavage (BAL) again revealed *A. fumigatus*. A repeat thoracentesis also revealed *M. chelonae* subsp. *abscessus*. Because of the persistent effusions, bilateral pigtail catheters were inserted for drainage. Voriconazole and the other antimicrobials were all continued. After 2 months of therapy, he was again admitted with dyspnea and diffuse interstitial infiltrates. A repeat bronchoscopy and BAL revealed *A. fumigatus* and cytomegalovirus pneumonia. Ganciclovir was initiated; voriconazole, azithromycin, imipenem, and linezolid were all continued for 2 months. At that point, a repeat thoracentesis was culture negative, and linezolid, cefoxitin, and ganciclovir were discontinued. However, a repeat bronchoscopy and BAL still revealed *A. fumigatus*. Voriconazole and azithromycin were continued. Three months later,

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FIG 1 Effects of Vcz on the growth of AF47381 and AF50593. (A) Photograph showing the colony growth of AF47381 and AF50593 on SD agar plates for 4 days in the presence of different concentrations of Vcz. (B) Growth of AF47381 and AF50593 in the presence (0.5 µg/ml Vcz) and absence (0 µg/ml Vcz) of Vcz for 4 days. (C) Effects of various concentrations of Vcz on the growth of AF47381 and AF50593 for 4 days. Vcz, voriconazole; AF47381, *A. fumigatus* isolate 47381; AF50593, *A. fumigatus* isolate 50593.

he was readmitted with new multifocal pulmonary infiltrates. A repeat bronchoscopy and BAL revealed *A. fumigatus* and *Pseudomonas aeruginosa*, while acid-fast bacilli (AFB) stains and cultures were negative, as was PCR for CMV. On this admission, the patient developed acute respiratory distress syndrome (ARDS) and septic shock and expired. On autopsy, *A. fumigatus* was cultured from the respiratory tract. In addition, both lungs and pleural cavities showed fibrotic changes with a fibrinous exudate but were culture negative.

A. fumigatus 50593 (AF50593), the last clinical isolate obtained from the patient on his final hospitalization after 8 months of Vcz therapy, and *A. fumigatus* 47381 (AF47381), the initial *Aspergillus* strain recovered ≈8 months prior to the isolation of AF50593, were used in this study. The *in vitro* susceptibilities of AF50593 and AF47381 to various antifungals were determined in RPMI 1640 by the M38-A2 technique (12). The MIC was defined as the lowest concentration of the drug that yielded no visible growth for the triazoles and amphotericin B (AMB). For the echinocandins, the minimum effective concentrations (MECs) (the lowest concentrations of the drugs that produced stunted mycelial growth) were determined (13). Drug concentrations ranging from 0.015 to 16 µg/ml were used. Each MIC determination was repeated once, and the results were either the same or plus or minus one 2-fold dilution. *Candida albicans* ATCC 90028 and *A. fumigatus* ATCC 36607 were used as quality control and reference strains, respectively.

To study the effect of various concentrations of Vcz on growth, AF50593 and AF47381 were grown on Sabouraud's dextrose (SD) agar for 4 days at 35°C, and from the edge of the colony, 5-mm diam-

eter agar blocks containing actively growing hyphae were excised aseptically. The SD agar blocks thus obtained were placed at the center of an SD agar plate (one agar block per plate) containing various concentrations (0 to 16 µg/ml) of Vcz. The plates were incubated at 35°C, and the diameter of the fungal colony was measured every 24 h. The mean of duplicate colony diameters of two independent experiments was plotted against various Vcz concentrations.

For the characterization of *cyp51A* and *cyp51B* genes, genomic DNA from AF50593 and AF47381 was isolated, purified, and analyzed using a DNeasy plant/fungus minikit (14). The complete transcriptional units or parts thereof of *cyp51A* and *cyp51B* genes were amplified using eight sets of oligonucleotide primers by the HotStarTaq master mix PCR protocol (initial denaturation step [15 min, 95°C], melting [0.5 min, 94°C], annealing [1 min, 53 to 55°C], extension [2 min, 72°C], and termination [10 min, 72°C] for 30 cycles) (15). The amplified DNA fragments were purified, and the nucleotide sequences of the amplicons carrying the *cyp51A* and *cyp51B* genes were determined by the Applied Biosystems (ABI) BigDye chain termination reaction followed by sample analysis on an ABI Prism (model 3730) DNA sequencer (16). Both the protein coding and the noncoding strands of the DNA fragment from multiple PCRs were sequenced to minimize sequencing and Taq polymerase-induced errors.

The DNA and the protein sequences were analyzed using online analytical tools provided by the European Bioinformatics Institute (ClustalW2; available at <http://www.ebi.ac.uk>) and the National Center for Biotechnology and Information (Basic BLAST; available at <http://www.ncbi.nlm.nih.gov>).

Our *in vitro* susceptibility studies showed that voriconazole

FIG 2 Molecular analysis of *cyp51A* and *cyp51B* genes from AF47381 and AF50593. (A) Amplification of the complete *cyp51A* and *cyp51B* genes or parts of the *cyp51A* and *cyp51B* genes of AF47381 and AF50593. Lane 1, 1-kb ladder; lanes 2 to 5, AF47381; lanes 6 to 9, AF50593. (B) Comparison of the amino acid sequences of Cyp51A from AF47381 and AF50593. The asterisks (*) denote identity, whereas the colon (:) indicates conserved substitutions which result in Y121F and T289A (shown in large bold letters). (C) A 46-bp repeat on the 5' regulatory region of AF50593. The 485-bp region upstream to the ATG codon of *cyp51A* from AF47381 was compared with the same region from AF50593. The 5' regulatory region of the *cyp51A* gene from AF50593 contains a 46-bp repeat from bp -284 to -329 (shown in bold with a gray background). (D) Schematic illustration of the possible formation of TR46 from TR34 or *vice versa*. The TR34/TR46 sequences are highlighted in bold with a gray background. The suspected 12-nucleotide insertion sequence (GAAA GTTGTCTA) is shown in italics.

had no meaningful *in vitro* activity against AF50593 with a considerably high MIC (MIC of ≥ 16 $\mu\text{g/ml}$) but remained susceptible to AMB (MIC of 0.5 $\mu\text{g/ml}$), to the echinocandins (MECs of 0.03 $\mu\text{g/ml}$), and to Pcz (MIC of 0.5 $\mu\text{g/ml}$). In contrast, AF47381 recovered approximately 8 months earlier was wild type for all antifungals, including Vcz (MIC of 0.25 $\mu\text{g/ml}$), suggesting that emergence of resistance in AF50593 occurred during Vcz therapy. As shown in Fig. 1, growth of the pretreatment isolate AF47381 was almost completely inhibited in the presence of 0.5 $\mu\text{g/ml}$ Vcz, whereas no reduction of growth occurred for AF50593 at 0.5 $\mu\text{g/ml}$ Vcz (Fig. 1A and B). In fact, AF50593 growth was inhibited only by about 43% in the presence of Vcz at 16 $\mu\text{g/ml}$ (Fig. 1C).

For PCR amplification of the *cyp51A* and *cyp51B* genes from AF50593 and AF47381, we used four sets of PCR primers each. Each set of primers was designed such that the PCR would produce amplicons of different sizes representing various regions of the protein coding and noncoding regulatory regions of the gene, confirming that the amplification produced genuine PCR products and not artifacts. The PCR primer pairs used were expected to yield amplicons of 2,210 bp, 1,723 bp, 1,324 bp, and 844 bp for

cyp51A and 1,831 bp, 1,413 bp, 1,033 bp, and 630 bp for *cyp51B* (Fig. 2A) genes. The sizes of the amplification products obtained were identical to what was predicted based on the oligodeoxynucleotide primers used for PCR.

The complete protein coding regions (including the introns) of the *cyp51A* (1,723 bp) and *cyp51B* (1,831 bp) genes of AF47381 and AF50593 were sequenced, and the protein coding sequences were assembled. The 1,545-bp protein coding sequence of AF47381 was compared with that obtained from AF50593 by nucleotide alignment. Two nucleotide changes (362A→T and 865A→G) resulting in Y121F and T289A amino acid changes were obtained for the *cyp51A* gene from AF50593, compared to that for the wild-type isolate AF47381 (Fig. 2B).

The amino acid sequences of Cyp51A deduced from the initial isolate AF47381 were identical to those of *A. fumigatus* ATCC 36607 and ATCC 208996 (data not shown) and three other previously published sequences (GenBank accession no. [AF338659](#) and [AF222068](#)). On the other hand, alignment of the amino acid sequences of Cyp51A from AF293 (the *A. fumigatus* isolate used for the genome sequence determination) and AF47381 showed Y46F,

V172M, T248N, E255D, and K427E variations (data not shown). Amino acid variations at F46, M172, N248, D255, and E427 have been reported to play significant roles in conferring azole resistance (MICs ranged from 4 µg/ml [Vcz] to >8 µg/ml [Itz]) in *A. fumigatus*. The fact that AF50593 remained susceptible to Pcz is not surprising since there is no amino acid change at the G54 locus, the primary amino acid alteration that has had an impact on the Pcz susceptibility of *A. fumigatus* isolates. Changes on other amino acids such as L98, G138, P216, F219, M220, E427, G434, and G448 associated with triazole resistances were undetected in AF50593.

The *cyp51B* gene of *A. fumigatus* is 1,574 bp long, coding for a polypeptide containing 524 amino acid residues. A comparison of the deduced amino sequences of AF47381 and AF50593 revealed no amino acid variations, suggesting that it is highly unlikely that the *cyp51B* gene of AF50593 plays any significant role in conferring resistance to Vcz.

A 485-bp region upstream to the ATG codon of *cyp51A* from AF47381 was compared with the same region from AF50593. As shown in Fig. 2C, the 5' regulatory region of the *cyp51A* gene from AF50593 contains a 46-bp tandem repeat (TR46) from bp –284 to –329. A comparison of the 5' regulatory region of AF50593 with two previously published sequences (GenBank accession no. AF338659 and AF222068) revealed that the TR46 was found only in the regulatory region of the AF50593 *cyp51A* gene. However, a comparison of the promoter region (386 bp) of EU626235 carrying the TR34 with 398 nucleotides upstream to the translational initiation ATG codon of *cyp51A* from AF50593 revealed that TR46 is a derivative of TR34 and the two promoters differed by the 12-nucleotide sequence GAAAGTTGTCTA missing from EU626235. Insertion of the missing sequence at the junction of the TR34 repeat together with the adjacent flanking sequences would generate a perfect 46-bp repeat producing TR46 (Fig. 2D). Conversely, a deletion of the 12-nucleotide stretch from TR46 would give rise to TR34. Which one of these two possibilities occurred first in nature is unclear.

The case we describe here is the first documented case of TR46/Y121F/T289A-mediated *A. fumigatus* resistance to Vcz in the United States developed during therapy in an isolate that remained susceptible to Pcz. This combination of changes in the regulatory sequence in conjunction with amino acid alterations encoded by the *cyp51A* gene of *A. fumigatus* is known to have originated in environmental isolates exposed to excessive azole demethylase inhibitors that were used as fungicides for agricultural crop protection (1, 17). Interestingly, we found the TR46/Y121F/T289A changes in the clinical isolate AF50593 obtained from a lung transplant recipient in Michigan who had previously received almost 8 months of Vcz therapy.

It is important to note that molecular modeling experiments revealed that Y121 is part of a molecular pocket involved in the initial docking of the incoming triazole molecule prior to binding to the active site (18). Thus, any significant changes in the docking site would have serious implications in the binding of the drug to the target, thus affecting its bioactivity. Even more intriguing is that the TR46/Y121F/T289A changes on the *cyp51A* gene confer resistance to Vcz (MIC of >16 µg/ml), yet the Itz MIC remained relatively low (MIC of 2 µg/ml), and the isolate was wild type for Pcz (MIC of 0.5 µg/ml). Our studies on the mechanism of voriconazole resistance in AF50593 obtained from a lung transplant recipient after prolonged voriconazole therapy for invasive asper-

gillosis suggest that complex mutational alterations of *cyp51A* leading to high-level drug resistance could occur during prolonged antifungal therapy.

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