Rate of *cyp51A* mutation in *Aspergillus fumigatus* among lung transplant recipients with targeted prophylaxis

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Objectives: The most common mechanism of azole (itraconazole and voriconazole) resistance in *Aspergillus fumigatus* is a mutation at the *cyp51A* locus. The aim of our study was to determine the rate of *cyp51A* mutations in lung transplant recipients (LTR) undergoing targeted antifungal prophylaxis with 12 weeks of voriconazole.

Methods: We conducted a prospective study that included 22 LTR with *A. fumigatus* between October 2008 and November 2011. Of those, 10 LTR were colonized with *A. fumigatus* and 12 had invasive pulmonary aspergillosis.

Results: Four patients were found to have *A. fumigatus* isolates with a *cyp51A* mutation, two had colonization and two had invasive pulmonary aspergillosis. The remaining 18 LTR were colonized with *A. fumigatus* and 12 had invasive pulmonary aspergillosis. All *A. fumigatus* isolates (except one due to mixed growth) were tested for antifungal susceptibility. A total of nine LTR were exposed to azoles prior to *A. fumigatus* isolation for a median duration of 249 (IQR 99–524) days. Azole exposure preceded the isolation of two mutant isolates and seven WT isolates. None of the *cyp51A* mutant isolates conferred phenotypic resistance to azoles.

Conclusions: Targeted antifungal prophylaxis in LTR did not lead to *cyp51A* resistance mutations in this cohort. Data on larger cohorts who receive universal antifungal prophylaxis are needed.

Keywords: transplantation, resistance, antifungal

Introduction

Invasive pulmonary aspergillosis is the most common invasive fungal infection in lung transplant recipients (LTR). Aspergillus fumigatus is the most common *Aspergillus* species isolated.1 Multiple reports of *A. fumigatus* strains with resistance to itraconazole, voriconazole, posaconazole and ravuconazole have emerged particularly after prolonged exposure to itraconazole and voriconazole.2,3 The most common mechanism of resistance is amino acid substitution in Cyp51A (the target protein in the ergosterol biosynthetic pathway) with amino acid modification most commonly at codons 98 (L98H) and 220.4 Previous surveillance studies of different populations reported a variable incidence (0.8%–75%) of *A. fumigatus* isolates harbouring *cyp51A* mutations.5–7

A recent surveillance study of fungal infection in solid organ transplant recipients showed a low rate of triazole resistance (1/181) in *A. fumigatus* isolates (MIC ≥ 4 mg/L for voriconazole and itraconazole and 0.25 mg/L for posaconazole). However, genetic sequencing of the *cyp51A* gene was not conducted.8 The rate of *cyp51A* mutations in LTR and their relationship to phenotypic antifungal resistance testing of *A. fumigatus* infections remain unknown. We therefore conducted this prospective study to assess the rate of *cyp51A* mutations in LTR receiving targeted antifungal prophylaxis.

Methods

A prospective observational study was conducted to assess the rate of *cyp51A* mutations in *A. fumigatus* isolates from LTR at Toronto General Hospital. Patients were recruited during the period October 2008–November 2011. The study was approved by the Research Ethics Board (approval number 09-0852-AE) University Health Network. Written consents were obtained from all included patients prior to enrolment.

Patients

Included patients were LTR who had post-transplant or peri-transplant (on the day of transplant) *A. fumigatus* colonization or infection. The follow-up period was 18 months from the date of *A. fumigatus* isolation.
Genotype testing and cyp51A sequencing

All samples were sent to the University of Manchester (Manchester, UK) for cyp51A sequencing. They were cultured on Sabouraud glucose agar and incubated at 37°C for 48 h. The DNA of isolates identified as A. fumigatus was extracted. The entire coding region of the cyp51A gene was amplified for both strands, using primers 210F and 2026R (Table S1, as Supplementary data at JAC Online). PCR was conducted as previously described.11

Sequences were aligned with an azole-susceptible strain sequence (GenBank accession number AF338659) and azole-susceptible clinical isolates as previously described and mismatches identified.11 Mutations were confirmed by repeating the extraction, PCR and sequencing of both strands using the closest two primers.

Phenotype testing

Susceptibility testing for A. fumigatus isolates was performed in the Public Health Ontario Laboratories, Toronto. Broth microdilution testing was adapted from CLSI M38-A2,12 using Sensititre YeastOne YO-9 broth microdilution panels (Trek Diagnostic Systems). The Aspergillus isolates were grown on potato dextrose agar slants for ≥48 h. Conidia were harvested in 3 mL of a 0.5% (v/v) Tween 20 solution in distilled water, which was applied to the culture slant. After gently probing the surface of the culture with a sterile Pasteur pipette and washing the surface several times to obtain a heavy homogeneous suspension, three drops of the resulting suspension were added to 5 mL of sterile water, capped and vortexed gently. The suspension was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard, using a nephelometer (from TREK auto-inoculator) at the uppermost limit on the scale (green colour). Fifty micro litres of this suspension was added to 11 mL of YeastOne RPMI, mixed and loaded onto the auto-inoculator with the panel. Prior to incubation, 10 μL was removed from the control well, spread onto a Sabouraud dextrose agar plate and incubated along with the panels at 35°C for 48 h until good growth was obtained in the control well. The final inoculum averaged 5×10^4 cfu/mL (range 2×8×10^4 cfu/mL). Endpoints were determined by visual read of turbidity and based on CLSI guidelines, i.e. 100% inhibition of visible growth for amphotericin B, itraconazole, voriconazole and posaconazole and minimum effective concentration (MEC) for the echinocandins.

Statistical analysis

Descriptive statistics were used to measure the distribution of all outcomes.

Results

A total of 27 LTR had A. fumigatus initially reported in a total of 33 BAL and 2 sputum samples in the microbiology laboratory of Toronto General Hospital. Five LTR were excluded: one received treatment prior to transplant, three were found to grow non- A. fumigatus Aspergillus species and one grew Penicillium species. A total of 22 LTR were included (Figure 1).

cyp51A mutation

All included LTR had a single positive A. fumigatus isolate except for two LTR who had two A. fumigatus isolated from different samples at two different timepoints. All isolates were sequenced at the cyp51A region. However, one isolate failed to grow upon phenotypic testing and another revealed mixed growth and therefore no susceptibility data are available on those isolates. The overall incidence of cyp51A mutation in the included LTR was 18% (4/22). One of the two LTR who had two A. fumigatus isolates had A9T cyp51A mutation on both occasions. There was no azole exposure prior to isolating the first isolate, while the second was isolated following 12 weeks of voriconazole as targeted prophylaxis. The second had a WT cyp51A in both isolates, despite previous exposure to voriconazole on both occasions.

Strains with cyp51A mutations harboured the following amino acid substitutions: N248K, L242V and two isolates from one patient harboured A9T. These four isolates were azole susceptible (Table 1). The fifth isolate harboured multiple amino acid substitutions (F46Y/M172V/N248T/D255E/E427K) as well as some silent mutations (which do not confer an amino acid alteration)—G89G, L358L and C454C. Susceptibility testing was not carried out for the latter isolate as the submitted sample revealed mixed growth at the time of susceptibility testing. The remaining 19 isolates from 18 LTR revealed WT cyp51A genes.

Azole exposure

While there was no documented history of previous azole exposure for the two cyp51A mutant isolates that harboured N248K and F46Y/M172V/N248T/D255E/E427K amino acid substitutions, previous azole exposure was noted for the remaining (2/4) LTR mutant cyp51A A. fumigatus isolates and 7/18 LTR WT cyp51A isolates. The median duration of exposure to voriconazole prior to A. fumigatus isolation was 249 (IQR 99–524) days. An LTR with a WT cyp51A A. fumigatus isolate revealed raised voriconazole and posaconazole MICs (voriconazole=2 mg/L, itraconazole=1 mg/L and posaconazole=0.5 mg/L).

The clinical courses of the included LTR are illustrated in Figure 1. Patients were followed for a median duration of 1222 days (IQR 825–1487). Four deaths were documented, all in patients with WT cyp51A A. fumigatus strains.

Discussion

Azole-resistant Aspergillus appears to be spreading globally, with recent reports of azole-resistant A. fumigatus isolates emerging from Europe, Asia and the Middle East.13,16 The high rate of azole exposure in LTR might place them at risk for emergence of azole resistance.
In our study, none of the isolates with cyp51A mutations had significantly higher MICs than the WT cyp51A group, suggesting that these alterations are SNPs that do not confer phenotypic resistance (Table 1). A9T, I242V as well as N248K amino acid substitutions were previously reported in azole-susceptible A. fumigatus strains as in our cases. The lack of documented azole exposure in two LTR with mutant cyp51A A. fumigatus isolates has been reported in previous studies. None of the cyp51A mutations detected in our study was in the hot spots identified in A. fumigatus isolates (at codons 54, 98 and 220) known to confer azole resistance.

Phenotypic testing was not performed for the fifth isolate with multiple amino acid substitutions as the sample revealed mixed growth upon susceptibility testing. Complete response to voriconazole suggests that the multiple amino acid substitutions may not have conferred voriconazole resistance as indicated in previous studies. Interestingly, the only A. fumigatus isolate in our cohort with MIC = 2 mg/L (intermediate voriconazole resistance) according to EUCAST lacked a cyp51A mutation. This points towards other mechanisms as possibly being responsible for the documented intermediate azole resistance such as efflux pumps, as reported previously in a large study that involved cyp51A genotype sequencing as well as susceptibility testing.

A limitation of the study is the small number of patients in this cohort, which limited our ability to draw any conclusions related to outcomes in these LTR. However, to our knowledge, this is the first study in LTR that prospectively collected data on cyp51A mutations and phenotypic resistance and has shown a lack of phenotypic resistance conferred by cyp51A mutations in the setting of targeted prophylaxis. Data on the prevalence of cyp51A
Susceptibility data are not available for the A. fumigatus isolate with amino acid substitutions F46Y/M172V/N248T/D255E/E427K, as A. fumigatus failed to grow from the sample submitted for susceptibility testing.

mutations in LTR from centres that employ routine azole universal prophylaxis are needed.

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Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References