In Vivo Selection of a Unique Tandem Repeat Mediated Azole Resistance Mechanism (TR\textsubscript{120}) in Aspergillus fumigatus cyp51A, Denmark

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We report a fatal aspergillosis case in which STR\textsubscript{A}f typing and whole-genome sequencing substantiated in vivo emergence of an azole-resistant Aspergillus fumigatus with a 120-bp tandem repeat in the promoter region of cyp51A. This event, previously restricted to the environment, challenges current understanding of azole resistance development in A. fumigatus.

Azole antifungal drug resistance in Aspergillus fumigatus is a concern for patients with aspergillosis because of increased risk for disease and death (1). Two routes of acquiring azole resistance have been identified: 1) in vivo, as a consequence of long-term azole treatment; and 2) ex vivo, in the environment, resulting from the use of azole fungicides in crop protection. The underlying mechanisms are primarily linked to structural changes or upregulation of the azole target lanosterol 14α-demethylase encoded by cyp51A (1). Most environmentally induced resistance mechanisms involve tandem repeats (TRs) in the promoter region of cyp51A coupled with nonsynonymous mutations, TR\textsubscript{L98H} and TR\textsubscript{Y1211/289A} (1). However, in vivo resistance development has primarily been associated with nonsynonymous mutations in cyp51A-inducing amino acid substitutions of hot spots (e.g., G54, G138, M220, and G448) or non-cyp51A-mediated mechanisms, but not a tandem repeat (1). We describe a clinical case of infection with azole-resistant A. fumigatus that acquired a 120-bp tandem repeat (TR\textsubscript{120}) resistance mechanism during long-term azole treatment. The finding was substantiated by whole-genome sequencing (WGS).

The Study
In 2013, a 69-year-old man who was a former smoker with chronic obstructive pulmonary disease (COPD) and severe airflow obstruction sought care at the University Hospital in Århus, Denmark, because of gradually worsening dyspnea, cough, and expectoration. Previously, in 2011, imaging (Figure 1, panel A) and 2 thoroscopies had been conducted because of suspicion of malignant mesothelioma. Further histopathologic examination and cultures revealed inflammation but no malignancy or mold infection. Subsequently, in 2012, a fistula between pleura and skin led to a persistent air-containing pleural cavity in the right side (Figure 1, panel B). In 2014, a fungus ball in the pleural cavity was found (Figure 1, panel C). Aspergillus IgG titer was 1:25,600 (reference range ≤1:200), and azole-susceptible A. fumigatus was cultured from sputum (P-1, May 2014). Voriconazole (200 mg 2×d) was given, alternating with posaconazole (300 mg/d) for 2 years until clinical failure, and 2 azole-resistant A. fumigatus isolates were cultured from a new sputum sample (P-2 and P-3, June 2016). Despite amphotericin B inhalations followed by liposomal amphotericin B (3 mg/kg 1×d), the patient died because of severe hemoptysis 1 year later in 2017.

Three A. fumigatus patient isolates (P-1, P-2, and P-3) were available for confirmatory species verification, reference susceptibility testing defined by the European Committee on Antimicrobial Susceptibility Testing using protocol for molds (E.Def 9.3), cyp51A Sanger sequencing (using wild-type reference sequence AF338659), and genotyping using the short tandem-repeat Aspergillus fumigatus (STRAf) assay (2,3) (Table). We included 4 A. fumigatus isolates representing relevant cyp51A profiles as control strains (SSI-3614 [wild-type], SSI-7828 [TR\textsubscript{L98H}], SSI-5197 [TR\textsubscript{Y1211/289A}], and SSI-5197 [F46Y/M172V/E427K]). We detected 3 common Cyp51A variants (F46Y, M172V, and E427K) in the susceptible patient isolate P-1 (GenBank accession no. MG972984). Pan-azole resistance was observed for P-2 and P-3, and both shared cyp51A profiles with P-1 but also harbored a TR\textsubscript{120} mechanism (GenBank accession no. MG972983) in the promoter region (Table). All patient isolates had identical STR\textsubscript{A}f genotypes suggesting that they were isogenic (Table 1) (4). Furthermore, the STR\textsubscript{A}f profile was unique among A. fumigatus isolates genotyped in Denmark (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/3/18-0297-App1.pdf).
We performed WGS for P-1, P-3, and all control strains to investigate relatedness and other potential mechanisms conferring azole resistance. We subjected total DNA (≈10 ng/μL) to WGS (NextSeq 550; Illumina, https://www.illumina.com) by using Nextera DNA library preparation kit (Illumina) and following the manufacturer’s instructions. We used NASP (5) to detect single-nucleotide polymorphisms (SNPs) after removal of duplicated regions in the *A. fumigatus* strain Af293 chromosomes (http://www.aspergillusgenome.org, genome version s03-m05-r09) using NUCmer (6). We inferred relatedness by using FastTree version 2.1.5 (7) and a 77.69% core genome (Table; Figure 2). To increase resolution, we conducted a subanalysis for P-1 and P-3 (core genome 79.71%), which identified 41 SNP differences; 6 of the SNPs were nonsynonymous in genes with no previous reported association to azole resistance (Appendix Table 1), and 35 were either synonymous or in noncoding regions (Appendix Table 2).

**Conclusions**

WGS revealed 41 SNP differences between the susceptible and the resistant patient *A. fumigatus* isolates that evolved during 2 years, similar to a previously described case of in-host microevolution of *A. fumigatus* (4). This finding substantiated an isogenic relationship between P-1 and P-3 and demonstrated that the TR$_{120}$ resistance mechanism emerged from P-1, probably during long-term azole therapy. Furthermore, WGS results supported the conclusion that the TR$_{120}$ was the sole mechanism of azole resistance in the azole-resistant patient isolates.

To our knowledge, the TR$_{120}$ is a novel azole-resistance mechanism in *A. fumigatus*, and the in vivo selection of a tandem repeat in the promoter of *cypr51A* is unique. The de novo acquisition of a TR has not previously been shown in vitro or in the environment (i.e., no isolates with L98H or Y121F+T289A combined with wild-type promoters have been reported). However, triplication of an existing TR$_{46}$ on tebuconazole exposure was selected in vitro, and a novel variant, TR$_{46}^{3}$, found in clinical and environmental samples, has been derived from sexual mating between TR$_{46}$ parents (8,9).

Azole resistance involving TRs in the promoter region has been associated exclusively with environmental fungicide selection pressure in *A. fumigatus* and other plant pathogens. Furthermore, although asexual propagation of *A. fumigatus* with TR$_{46}^{-}$/L98H or TR$_{46}^{-}$/Y121F/T289A resistance mechanisms is widespread in the environment, the extent of de novo selection of TR$_{46}^{-}$/L98H and TR$_{46}^{-}$/Y121F/T289A is unclear (10). One hypothesis describes both environmental resistance mechanisms as being derived from single events of sexual reproduction (in environmental

**Table. Aspergillus fumigatus strain characteristics, antimicrobial susceptibility, and molecular data, Denmark, 2013**

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Susceptibility MICs, mg/L</th>
<th>Sanger sequencing: Cyp51A profile</th>
<th>STR/Af assay genotyping data</th>
<th>WGS data‡ SNP differences compared with P-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>VRZ 1 0.5 0.125</td>
<td>F46Y/M172V/E427K</td>
<td>10–13–10–17–13–8–7–5–6</td>
<td>0</td>
</tr>
<tr>
<td>P-2</td>
<td>VRZ 4 16 0.5</td>
<td>TR$_{120}$/F46Y/M172V/E427K</td>
<td>10–13–10–17–13–8–7–5–6</td>
<td>NA</td>
</tr>
<tr>
<td>P-3</td>
<td>VRZ 4 &gt;16 0.5</td>
<td>TR$_{120}$/F46Y/M172V/E427K</td>
<td>10–13–10–17–13–8–7–5–6</td>
<td>41</td>
</tr>
<tr>
<td>SSI-7413</td>
<td>VRZ 0.5 0.25 0.125</td>
<td>WT</td>
<td>21–25–19–28–12–6–20–10–8</td>
<td>105,900</td>
</tr>
<tr>
<td>Af293 (13)</td>
<td>VRZ 1 0.5 0.06</td>
<td>F46Y/M172V/N248T/E427K</td>
<td>26–18–18–46–21–33–11–10–8</td>
<td>102,727</td>
</tr>
<tr>
<td>SSI-5946</td>
<td>VRZ 4 &gt;16 0.5</td>
<td>TR$_{46}$/L98H</td>
<td>20–21–12–84–10–7–8–9–10</td>
<td>108,901</td>
</tr>
<tr>
<td>SSI-5717</td>
<td>VRZ &gt;4 0.5 0.25</td>
<td>TR$_{46}$/Y121F/T289A</td>
<td>26–21–16–32–9–10–8–14–10</td>
<td>108,882</td>
</tr>
</tbody>
</table>

‡Reference genome coverage ranged from 88.5% to 90.93%. Sequencing depth based on all assembled contigs >1,000 bp ranged from 57.2x to 80.7x; 71.1x for P-1; and 66.3x for P-3.

§Sanger sequencing was performed as previously described (3). Underlined STR/Af markers are shared with P-1.

**Figure 1.** Sequential thoracic computed tomography scan images illustrating the gradual progression from pleural thickening to cavity formation and development of an aspergilloma in a patient with Aspergillus fumigatus infection, Denmark, 2013. A) 2011, B) 2012, C) 2014, D) 2016.
in Aspergillus fumigatus cyp51A, Denmark

habitats) combining the TR with a cyp51A mutant. In addition, sexual reproduction might have led to a high genetic diversity among environmental azole-resistant A. fumigatus, which otherwise might have indicated multiple origins (10). Our finding might challenge the perception that TR azole-resistance mechanisms are exclusive to the environment and might warrant the question of whether TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A derive from single events. Hypothetically, the patient might initially have inhaled isogenic isolates with and without TR<sub>120</sub>, the resistant one being undetected. However, a patient being co-infected de novo by a susceptible and an isogenic resistant strain has not been previously reported and is considered highly unlikely.

Long-term and subtherapeutic antifungal treatment might facilitate selection of resistance (11). Therapeutic drug monitoring was performed once in this patient but without information if the sample was taken according to guidelines as a trough level (lowest level after dosage). Thus, despite a concentration of 4.3 mg/L (within the recommended trough range), potential subtherapeutic levels during the 200 mg 2×d dosing scheme cannot be ruled out. The F46Y/M172V/E427K substitutions in Cyp51A, found in both susceptible and resistant isolates, have been suggested to play no role or only a minor role in reducedazole susceptibilities (12,13). TRs in the promoter region of cyp51A have previously been linked to increased cyp51A gene expression and MICs because of duplicated srbA transcription factor binding motifs (SRE1 and SRE2), leading to increased expression of cyp51A (14,15). Taken together, our data suggest that TR<sub>120</sub> alone is an important driver of pan-azole resistance at a level comparable to that known to be mediated by the TR<sub>34</sub>/L98H mechanism.

Our WGS results might obviate the desire for in vitro experiments testing the TR<sub>120</sub> mechanism in laboratory-engineered mutants. Further dissection of the WGS data can help elucidate potential genetic drivers of TR acquisition and add further knowledge as to whether the TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A resistance genotypes derived from a single origin. This report adds another piece to the complex picture of emerging azole-resistant A. fumigatus and might serve to stimulate further research.

**Acknowledgments**

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**About the Author**

Dr. Hare is a molecular biologist at the Mycology Laboratory at Statens Serum Institut, Copenhagen, Denmark, where he completed his PhD on antifungal drug resistance in 2016. Besides antifungal resistance, his main research interests are molecular fungal diagnostics.

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