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

Rasmus K. Hare, Jan B. Gertsen, Karen M.T. Astvad, Kristine B. Degn, Anders Løkke, Marc Stegger, Paal S. Andersen, Lise Kristensen, Maiken C. Arendrup

We report a fatal aspergillosis case in which STR*Af* 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 14 α-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_{34}/L98H and TR_{46}/Y121F/T289A (*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_{120}) resistance mechanism during long-term azole treatment. The finding was substantiated by whole-genome sequencing (WGS).

Author affiliations: Statens Serum Institut, Copenhagen, Denmark (R.K. Hare, K.M.T. Astvad, M. Stegger, P.S. Andersen, M.C. Arendrup); Åarhus University Hospital, Åarhus, Denmark (J.B. Gertsen, K.B. Degn, A. Løkke, L. Kristensen); Rigshospitalet, Copenhagen (M.C. Arendrup); University of Copenhagen, Copenhagen (M.C. Arendrup)

DOI: https://doi.org/10.3201/eid2503.180297

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 thoracoscopies 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* (STR*Af*) assay (*2*,*3*) (Table). We included 4 *A. fumigatus* isolates representing relevant *cyp51A* profiles as control strains (SSI-3614 [wild-type], SSI-7828 [TR_{34}/L98H], SSI-5717 [TR_{46}/Y121F/T289A], 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_{120} mechanism (GenBank accession no. MG972983) in the promoter region (Table). All patient isolates had identical STR*Af* genotypes suggesting that they were isogenic (Table 1) (*4*). Furthermore, the STR*Af* profile was unique among *A. fumigatus* isolates genotyped in Denmark (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/3/18-0297-App1.pdf).

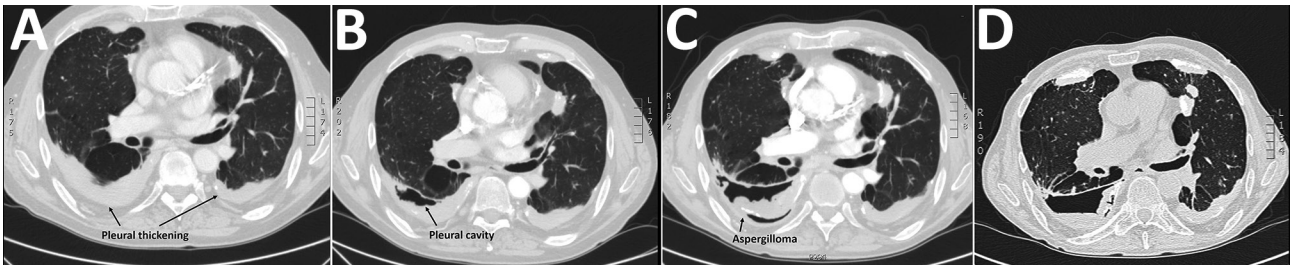


Figure 1. Sequential thoracic computed tomography scan images illustrating the gradual progression from pleural thickening to cavity formation and development of an aspergilloma in a patient with *Aspergillus fumigatus* infection, Denmark, 2013. A) 2011, B) 2012, C) 2014, D) 2016.

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 *cyp51A* 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_{34} 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_{34}/L98H or TR_{46}/Y121F/T289A resistance mechanisms is widespread in the environment, the extent of de novo selection of TR_{34}/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*

Isolate no.	EUCAST-based susceptibility MICs, mg/L			Sanger sequencing: Cyp51A profile§	STR*Af* assay genotyping data:† 2A-2B-2C-3A-3B-3C-4A-4B-4C	WGS data:‡ SNP differences compared with P-1
	VRZ	ITZ	POS			
P-1	1	0.5	0.125	F46Y/M172V/E427K	10–13–10–17–13–8–7–5–6	0
P-2	4	16	0.5	TR_{120}/F46Y/M172V/E427K	10–13–10–17–13–8–7–5–6	NA
P-3	4	>16	0.5	TR_{120}/F46Y/M172V/E427K	10–13–10–17–13–8–7–5–6	41
SSI-5197	1	1	0.125	F46Y/M172V/E427K	10–15–10–28–13–11–7–5–6	4,968
SSI-7413	0.5	0.25	0.125	WT	21–25–19–28–12–6–20–10–8	105,900
Af293 (13)	1	0.5	0.06	F46Y/M172V/N248T/D255E/E427K	26–18–18–46–21–23–11–10–8	102,727
SSI-5946	4	>16	0.5	TR_{34}/L98H	20–21–12–84–10–7–8–9–10	108,901
SSI-5717	>4	0.5	0.25	TR_{46}/Y121F/T289A	26–21–16–32–9–10–8–14–10	108,882

*ITZ, itraconazole; NA, not available; POS, posaconazole; SNP, single-nucleotide polymorphism; STR*Af*, short tandem repeat *Aspergillus fumigatus*; VRZ, voriconazole (isavuconazole MICs were equivalent, data not shown); WGS, whole-genome sequencing; WT, wild-type.
†STR*Af* genotyping was performed as previously described (*3*). Underlined STR*Af* markers are shared with P-1.
‡Reference genome coverage ranged from 88.5% to 90.93%. Sequencing depth based on all assembled contigs >1,000 bp ranged from 57.2× to 80.7×; 71.1× for P-1; and 66.3× for P-3.
§TR_{34}: GAATCACGCGGTCCGGATGTGTGCTGAGCCGAAT, TR46: **AGTTGTCTA**GAATCACGCGGTCCGGATGTGTGCTGAGCCGAAT**GAA**, TR_{120}: TTCTCCTCTAGAAAAAACTCATGAGTGAATAATCGCAGCACCACTCCAG**AGTTGTCTA**GAATCACGCGGTCCGGATGTGTGCTGAGCCGAAT **GAA**AGTTGCCTAATTACTAAGGTGTAGT. GenBank accession numbers are MG972983 with TR_{120} and MG972984 without TR_{120}.

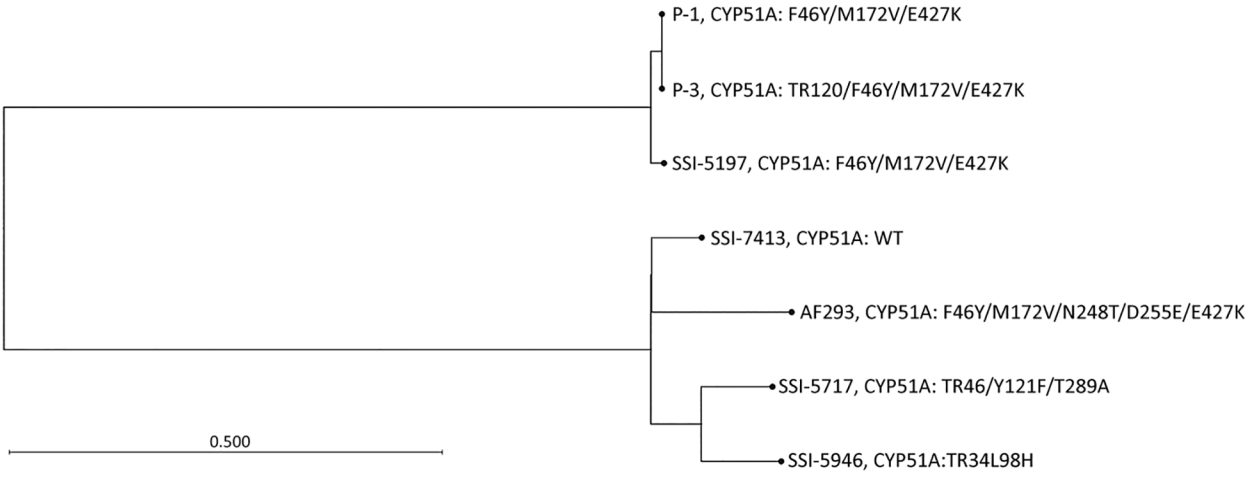


Figure 2. Unrooted phylogenetic tree based on whole-genome sequencing of 2 patient isolates (P-1 and P-3) and 5 reference strains to highlight relatedness between *Aspergillus fumigatus* isolates, Denmark, 2018. We inferred relatedness by using FastTree version 2.1 (*7*) based on a 77.69% core genome. Whole-genome sequencing identified 41 single-nucleotide polymorphism (SNP) differences between P-1 and P-3. We observed subtle differences (<5,000 SNPs) between unrelated patient isolate SSI-5197 and P-1/P-3, whereas >100,000 SNPs differed from P-1/P-3 to the other control strains and Af293. WT, wild-type. Scale bar indicates nucleotide substitutions per site.

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_{34}/L98H and TR_{46}/Y121F/T289A derive from single events.

Hypothetically, the patient might initially have inhaled isogenic isolates with and without TR_{120}, 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 reduced azole 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_{120} alone is an important driver of pan-azole resistance at a level comparable to that known to be mediated by the TR_{34}/L98H mechanism.

Our WGS results might obviate the desire for in vitro experiments testing the TR_{120} 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_{34}/L98H and TR_{46}/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

We thank the entire Mycology Laboratory at Statens Serum Institut for their invaluable work in culturing, identification, and antimicrobial susceptibility testing. We acknowledge the staff responsible for the WGS workflow for their expedited service. We also thank laboratory technicians involved in PCR and DNA analyses, including Nissrine Abou-Chakra, for their invaluable aid and expertise in molecular biology.

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.

References

1. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. Philos Trans R Soc Lond B Biol Sci. 2016;371:20150460.
2. Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. EUCAST technical note on the method for the determination

of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. Clin Microbiol Infect. 2008;14:982–4. http://dx.doi.org/10.1111/j.1469-0691.2008.02086.x
3. de Valk HA, Meis JF, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CHW. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol. 2005;43:4112–20. http://dx.doi.org/10.1128/JCM.43.8.4112-4120.2005
4. Ballard E, Melchers WJG, Zoll J, Brown AJP, Verweij PE, Warris A. In-host microevolution of *Aspergillus fumigatus*: a phenotypic and genotypic analysis. Fungal Genet Biol. 2018; 113:1–13. http://dx.doi.org/10.1016/j.fgb.2018.02.003
5. Sahl JW, Lemmer D, Travis J, Schupp JM, Gillece JD, Aziz M, et al. NASP: an accurate, rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. Microb Genom. 2016;2:e000074. http://dx.doi.org/10.1099/mgen.0.000074
6. Delcher AL, Salzberg SL, Phillippy AM. Using MUMmer to identify similar regions in large sequence sets. Curr Protoc Bioinformatics 2003;00:10.3.1–10.3.18.
7. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS One. 2010;5:e9490. http://dx.doi.org/10.1371/journal.pone.0009490
8. Snelders E, Camps SMT, Karawajczyk A, Schaftenaar G, Kema GHJ, van der Lee HA, et al. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus.* PLoS One. 2012;7:e31801. http://dx.doi.org/10.1371/journal.pone.0031801
9. Zhang J, Snelders E, Zwaan BJ, Schoustra SE, Meis JF, van Dijk K, et al. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. MBio. 2017;8:e00791–17.
10. Abdolrasouli A, Rhodes J, Beale MA, Hagen F, Rogers TR, Chowdhary A, et al. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome s equencing. MBio. 2015;6:e00536.
11. Verweij PE, Zhang J, Debets AJM, Meis JF, van de Veerdonk FL, Schoustra SE, et al. In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. Lancet Infect Dis. 2016;16:e251–60. http://dx.doi.org/10.1016/S1473-3099(16)30138-4
12. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJG. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. Antimicrob Agents Chemother. 2010;54:2425–30. http://dx.doi.org/10.1128/AAC.01599-09
13. Garcia-Rubio R, Alcazar-Fuoli L, Monteiro MC, Monzon S, Cuesta I, Pelaez T, et al. Insight into the significance of *Aspergillus fumigatus cyp51A* polymorphisms. Antimicrob Agents Chemother. 2018;62:e00241–18. http://dx.doi.org/10.1128/AAC.00241-18
14. Snelders E, Camps SMT, Karawajczyk A, Rijs AJMM, Zoll J, Verweij PE, et al. Genotype-phenotype complexity of the TR46/Y121F/T289A *cyp51A* azole resistance mechanism in *Aspergillus fumigatus.* Fungal Genet Biol. 2015;82:129–35. http://dx.doi.org/10.1016/j.fgb.2015.06.001
15. Gsaller F, Hortschansky P, Furukawa T, Carr PD, Rash B, Capilla J, et al. Sterol biosynthesis and azole tolerance is governed by the opposing actions of SrbA and the CCAAT binding complex. PLoS Pathog. 2016;12:e1005775. http://dx.doi.org/10.1371/journal.ppat.1005775

Address for correspondence: Maiken C. Arendrup, Statens Serum Institut, Building 43-317, Artillerivej 5, 2300, Copenhagen S, Denmark; email: maca@ssi.dk; Rasmus K. Hare, Statens Serum Institut, Building 43-315, Artillerivej 5, 2300, Copenhagen S, Denmark; email: rmj@ssi.dk

Article DOI: https://doi.org/10.3201/eid2503.180297

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

Appendix

Appendix Table 1. Insight in six non-synonymous SNPs found in the azole resistant isolate P-3 compared to P-1

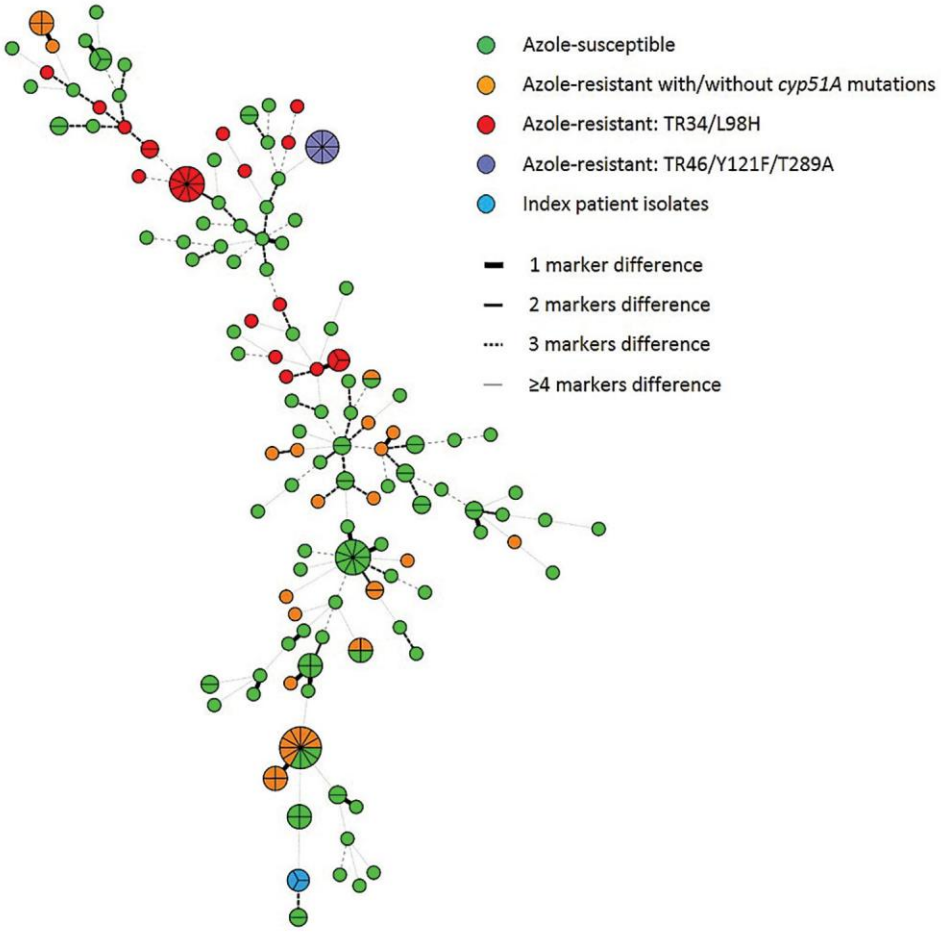
Gene product	Protein ID AF293	Non-synonymous SNP	Amino acid substitution	Comment
Uncharacterized protein. 4-coumarate-CoA ligase	AFUA_1G13110	G1183T	V373*STOP	Precursor of AMP + diphosphate + 4-coumaroyl-CoA–>Coumaroyl-coenzyme A is a central intermediate in the biosynthesis of polyketides such as flavonoids (pigmentation) and other natural organic compounds. Mutation leads to a truncated protein from 566 to 373 aa
Uncharacterized protein. Has domain(s) with predicted nucleus localization	AFUA_2G04780	A1873T	D602G	Ortholog in *C. albicans* is *ENP2*, encodes an essential protein and a heterozygous mutation confers resistance to 5–fluorocytosine (5-FC) and 5-fluorouracil (5-FU). Amino acid position is not conserved but in a conserved region compared to *A. niger* and *A. nidulans* but less for *C. albicans* (12/15 shared amino acids) and the protein sequences have 70%–82% homology.
FLUG	AFUA_3G07140	C650A	Q216*STOP	Upstream activator of conidiation; required for proper regulation of *brlA* transcript levels; calcium induced. White phenotype in *A. nidulans* but not necessarily in *A. fumigatus* (*1*). Mutation leads to a truncated protein from 861 to 216 aa. *A. fumigatus* patient isolate P-3 had a white phenotype.
Uncharacterized protein	AFUA_3G08260	A3192T	K1029M	Orthologs (e.g., *smc1* in *C. albicans*). Has cohesin ATPase activity, double-stranded DNA binding, topological DNA entrapment activity, role in mitotic cohesin loading and condensed nuclear chromosome, centromeric region, nuclear mitotic cohesin complex localization. Amino acid position is in a conserved region compared to other *Aspergilli* (26/31 shared amino acids) and the protein sequences have 84% homology.
Uncharacterized protein. Putative sensor histidine kinase	AFUA_8G06150	G96T	M32I	Ortholog in *A. nidulans* (AN4815). Amino acid position in non-conserved region compared to *A. nidulans* and *A. oryzae* and have 20%–21% protein sequence homology.
Uncharacterized protein. Putative sensor histidine kinase	AFUA_8G06150	C119T	A40V	Same as above. Amino acid position in non-conserved region compared to *A. nidulans* and *A. oryzae* and have 20%–21% protein sequence homology.

*Missense mutation, premature stop-codon leading to a truncated gene product.

Appendix Table 2. Insight in 35 SNPs in non-coding/intergenic regions found in the resistant isolate P-3 compared to P-1. The positions listed are in relation to any annotated gene with the closest proximity, upstream or downstream

SNP position in Af293 (Chromosome::position)	SNP	SNP position in relation to annotated gene (AF293)	Protein function	Comment
Chr1::184219	A → G	571 bp upstream, AFUA_1G00580 (PacG/VIB-1)	Putative DNA binding transcription factor	Intergenic/Promoter region
Chr1::184234	A → G	566 bp upstream, AFUA_1G00580 (PacG/VIB-1)	Same as above	Intergenic/Promoter region
Chr1::38596	G → A	1121 bp upstream, AFUA_1G00200	Uncharacterized	Intergenic
Chr1::38605	A → G	1130 bp upstream, AFUA_1G00200	Uncharacterized	Intergenic
Chr1::51920	G → A	2439 bp downstream, AFUA_1G00220	Uncharacterized	Intergenic
Chr1::964093	T → C	717 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964119	A → G	691 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964126	C → T	684 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964130	C → T	680 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964137	A → G	673 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964148	A → G	662 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964154	A → G	656 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr1::964159	G → C	651 bp upstream, AFUA_1G03330	Uncharacterized	Intergenic/Promoter region
Chr2::4375395	G → T	344 bp downstream of start codon, AFUA_2G16480	Uncharacterized. Has domain(s) with predicted zinc ion binding activity.	Synonymous SNP
Chr3::1025902	C → T	576 bp downstream, AFUA_3G03740	Uncharacterized. Putative protein kinase	Intergenic/Promoter region
Chr3::1126737	G → A	398 bp downstream, AFUA_3G03950	Uncharacterized (*ste7* in *S. cerevisiae*)	Intergenic/Promoter region. In Af293, this position is an A.
Chr3::1445547	G → A	590 bp downstream startcodon, AFUA_3G05900	MAP kinase kinase	Intron region
Chr3::2483009	G → A	419 bp downstream startcodon, AFUA_3G09710	Uncharacterized (*uga4* in *S. cerevisiae*)	Intron region. In Af293, this position is an A.
Chr3::3962289	C → T	177 bp upstream, AFUA_3G15030	Uncharacterized. Has domains with predicted oxidoreductase, zinc binding dehydrogenase family.	Intergenic/Promoter region
Chr3::686389	A → G	7931 bp upstream, AFUA_3G02670	Uncharacterized (*lys2* in *S. cerevisiae*). Protein similar to nonribosomal peptide synthases (NRPS-like), encoded in a predicted secondary metabolite gene cluster.	Intergenic region
Chr3::686394	T → C	7926 bp upstream, AFUA_3G02670	Same as above.	Intergenic region
Chr4::1902849	T → C	In intron, 702 bp downstream start codon: AFUA_4G07320	Uncharacterized.	Intron region
Chr5::2055153	C → T	Synonymous SNP: 21 bp downstream start codon: AFUA_5G08120	*argJ*, Arginine biosynthesis bifunctional protein.	SrbA-regulated during hypoxia.
Chr5::3843678	A → G	163 bp upstream AFUA_5G14865,	Uncharacterized	Intergenic/Promoter region
Chr5::3843680	T → C	161 bp upstream AFUA_5G14865, conserved hypothetical protein	Uncharacterized	Intergenic/Promoter region
Chr5::3843698	G → T	143 bp upstream AFUA_5G14865, conserved hypothetical protein	Uncharacterized	Intergenic/Promoter region
Chr5::3843704	C → T	137 bp upstream AFUA_5G14865, conserved hypothetical protein	Uncharacterized	Intergenic/Promoter region
Chr5::3856240	A → G	1110 bp upstream, AFUA_5G14920	Uncharacterized	Intergenic region
Chr6::2315039	T → C	735 bp upstream, AFUA_6G09500	Uncharacterized (*caj1* in *S. cerevisiae*).	Intergenic/Promoter region

SNP position in Af293 (Chromosome::position)	SNP	SNP position in relation to annotated gene (AF293)	Protein function	Comment
Chr6::2508784	T → C	828 bp downstream, AFUA_6G10140	Uncharacterized. Has domain(s) with predicted DNA binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, zinc ion binding activity and role in regulation of transcription, DNA-templated, transcription, DNA-templated.	Intergenic/Promoter region
Chr6::2509191	G → A	1235 bp downstream, AFUA_6G10140	Same as above	Intergenic region
Chr7::1555469	G → A	212 bp downstream, AFUA_7G06350	*phoE*, Putative phosphate transporter, phoB-regulated	In Af293, this position is an A.
Chr7::1644584	C → G	259 bp downstream, AFUA_7G06770 AND 378 bp upstream, AFUA_7G06760	AFUA_7G06770: Conserved protein of unknown function; protein abundantly expressed in conidia; transcript induced in conidia exposed to neutrophils and to human airway epithelial cells. AFUA_7G06760: Uncharacterized.	Intergenic/promoter region
Chr7::2037815	C → T	5870 bp upstream, AFUA_7G08640	Uncharacterized.	Intergenic region
Chr8::1465035	C → T	G111A–>synonymous. Afu8g06150	Uncharacterized. Putative sensor histidine kinase	Synonymous SNP



Appendix Figure. Minimum spanning tree created using BioNumerics (v. 7.6, Applied Maths, Sint-Martens-Latem, Belgium) showing Danish STR*Af* genotypes with the three index patient isolates colored in light blue. A total of 122 unique genotypes among 191 clinical and environmental isolates, of which 120 genotypes have previously been described (*2*).

References

1. Mah JH, Yu JH. Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. Eukaryot Cell. 2006;5:1585–95. PubMed http://dx.doi.org/10.1128/EC.00192-06

2. Jensen RH, Hagen F, Astvad KMT, Tyron A, Meis JF, Arendrup MC. Azole-resistant *Aspergillus fumigatus* in Denmark: a laboratory-based study on resistance mechanisms and genotypes. Clin Microbiol Infect. 2016;22:570.e1–9. PubMed http://dx.doi.org/10.1016/j.cmi.2016.04.001