A novel Y319H mutation in *CYP51C* associated with azole resistance in *Aspergillus flavus*

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Abstract

The study was aimed to explore any mutation in the *CYP51* gene conferring azole resistance in *Aspergillus flavus*. Two voriconazole resistant and 45 susceptible isolates were included in the study. Sequence analysis demonstrated a T1025C nucleotide change in *CYP51C* resulting in amino acid substitution Y319H in one resistant isolate. However the earlier described T788G mutation in *CYP51C* conferring voriconazole resistance in *A. flavus* isolates, was present in all isolates irrespective of their susceptibility status.

Aspergillus flavus is the second leading cause of invasive aspergillosis in immunocompromised patients and predominant causative agent of fungal rhinosinusitis and fungal eye infections (endopthalmitis and keratitis) in tropical countries like India, Sudan, Kuwait and Iran (1-8). Voriconazole is used primarily to treat infections caused by *Aspergillus flavus.* Long term azole therapy may predispose *A. flavus* to acquire resistance to azoles including voriconazole

Lanosterol 14 α demethylase (LDM) which catalyses the rate limiting step in the ergosterol biosynthetic pathway serves as the primary target for azole antifungal drugs. The mechanism of azole resistance in *Aspergillus fumigatus* is well studied. Missense mutations and alteration of *cis* regulatory regions in the LDM coding gene *CYP51A* have been found as the dominant mechanism of azole resistance in *Aspergillus fumigatus* (9-12), whereas studies to evaluate the mechanism of azole resistance in *A. flavus* are sparse (13-15). The present study is an attempt to understand the mechanism of azole resistance in *A. flavus.*

Two non-wild type (non-WT) clinical isolates of *A. flavus,* NCPPF 761157 and NCCPF 760815 having higher MIC values for voriconazole than the respective wild type (WT) cut-off value and 4 WT isolates were initially used (Table1). The wild type and non-wild type were defined on the basis of epidemiological cut-off values (ECV); the non-WT

having voriconazole MIC >1µg/mL and WT with voriconazole MIC ≤1 µg/mL (16). The non-WT strain, NCCPF 761157 was isolated from sputum of a patient with chronic obstructive pulmonary disease and NCCPF 760815 from the nasal tissue of a patient from India having granulomatous fungal rhinosinusitis. Forty-five additional WT *A. flavus* clinical isolates were included to screen and validate the mutations (SNPs and Indels). Identification of the isolates was done by sequencing of partial β-tubulin and calmodulin genes using primers bt2a (GGTAACCAAATCGGTGCTGCTTTC); bt2b (ACCCTCAGTGTAGTGACCCTTGGC) and cmdA7 (GCCAAAATCT TCATCCGTAG) cmdA8 (ATTTCGTTCAGAATGCCAGG) (17, 18). Antifungal susceptibility testing was done as per CLSI and EUCAST guidelines (19-22). Coding sequences of the close homologues of *CYP51A* of *A. fumigatus* in *A. flavus* namely *CYP51A* (XM_002375082.1*), CYP51B (*XM_002379089.1) *and CYP51C* (XM_002383890.1) were downloaded from Genbank (http://www.ncbi .nlm.nih.gov/genbank) as mentioned by Liu *et al* (15). Overlapping primer sets were designed for each homologue and PCR amplification of the each open reading frame and upstream and downstream regions of each homologue was performed (Table 2). To reduce errors during amplification, two different high fidelity DNA polymerases (Platinum Taq, Life technologies, Carlsbad,CA and KOD+ Toyobo, Life Science Department, Osaka Japan) were used in different sets of experiment (twice). Sequence amplification and analysis was performed using Big dye terminator ready reaction kit and Genetic Analyzer (Applied Biosystems, Foster city, CA). Consensus of forward and reverse sequences and contig assembly of each product from the overlapping fragments was done in Bionumerics software (Applied Maths, Ghent, Belgium). Sequences were aligned in Clustal-X2 and amino acid sequences were deduced from ExPasy online tool (http://www.expasy.org/translate). To assess the impact of Y319H SNP on the general structure of the *A. flavus CYP51C*, homology modelling and molecular dynamic simulations

were performed for the WT and the Y319H mutant. The amino acid sequence of the query protein was downloaded from Uniprot protein sequence database (Uniprot Id: I8TEB1). The 3D homology models of WT and Y319H mutant were generated using Swiss model (http://swissmodel.expasy.org/interactive #sequence) workspace. LDM (PDBID: 4K0F) structure sharing sequence identity of 50.51% was used as a template for model building. Models were validated using qmean4 score. Production dynamic simulation run was performed using GROMACS 4.6.5 with GROMOS96 43a1 force field. Molecular dynamics (MD) trajectory analysis was performed using Gromacs utilities and all the graphs were plotted using Grace. To study the structural and functional effects of Y319H mutation, the WT and non-WT CYP51C were also analysed on HOPE (23)

Comparison of nucleotide and amino acid sequences of *CYP51A* homologs of non-WT (NCCPF 761157 and NCCPF 760815) and WT strains of *A. flavus (*760816, 760690, 760425 and 760379*)* with reference sequence (NRRL3357) showed G680A transition in *CYP51A* of NCCPF 761157 strain only resulting in amino acid change A205T. The upstream (-1000bp) and downstream (+1000) regulatory regions were intact in all strains. In addition, there was no change in nucleotide or amino acid sequences in *CYP51B.* However, *CYP51C* was most polymorphic in nature (Table 3). Six missense nucleotide changes and resulting amino acid replacements were detected in *CYP51A and CYP51C*. However, 5 of these mutations (A205T, M54T, S240A, D254N, and I285V) did not appear to affect the azole susceptibility of the organism, as these changes were also found in WT isolates. Only one non synonymous mutation T1025C translating to Y319H was found specific to a non- WT isolate (NCCPF 761157) .To confirm these findings, we used 45 wild type isolates to screen for the SNPs and Indels coding for these phenotypes of *CYP51C* in azole sensitive strains. Tandem duplication of promoter sequence, TR 34 along with non-synonymous point mutation L98H was reported for azole resistance in clinical and environmental isolates of

Aspergillus fumigatus. However, mutation of this characteristic was not found in our azole resistant *A. flavus*. Nonetheless, a 4bp deletion was found in the AT- rich intergenic region downstream at position 2734 of *CYP51C* which on screening in WT collection showed that it was not related to the resistant phenotype. Instead, a compensatory 4bp insertion mutation was found in the nearby region in those isolates which harboured this deletion (data not shown). Indel mutations usually arise in intergenic regions which act as mutational hotspots for indels and play a role in purifying selection (24).-The present study also contradicts the finding of Liu *et al* (15) in which the T788G mutation was implicated in mediating voriconazole resistance in *A. flavus*. This mutation was not related to voriconazole resistance in our strains as this SNP was found in all 47 strains tested, irrespective of their susceptibility. Possibly T788G mutation is simply a geographical strain variation as the investigators have compared *CYP51C* sequence of their resistant strain with *A. flavus* NRRL3357 reference sequence only. Alignment of orthologues of Cytochrome P450 of different fungal species and that of human showed mutations including A205T, M54T, S240A, D254N, and I285V were not present in the conserved motifs. (Table 3). Location of Y319H mutation in a highly conserved position of *CYP51C* suggests that this could be one of the possible reasons for azole resistance in our resistant isolate

As the Y319H mutation is located far away from the iron-porhyrin complex, it appears that the mutation affect indirectly on drug binding instead of direct effect on the docking of azoles at the binding site (figure 1). MD simulations revealed that this mutation increases conformational flexibility, as indicated by increased root mean square deviation values (figure 2A) and root mean square fluctuation (RMSF) (figure 2B); there was simultaneous decrease in globularity as depicted by increase in radius of gyration of the mutant protein (figure 2C). Differences in radius of gyration between the WT and non-WT *CYP51C* may be due to loss of non-covalent interactions, which was caused by the

substitution of tyrosine with histidine in the mutant strain. The WT residue tyrosine forms a hydrogen bond with the valine on position 329, and salt bridges with the valine on position 329 and glutamic acid on position 328. Increased flexibility in the non-WT *CYP51C* may be due to the polar nature of histidine causing interatomic repulsions. On the other hand, tyrosine present in wild type can form hydrophobic interactions accounting for lower RMSF. The structural data for the *CYP51C* protein of *A. flavus* is not available to infer the effect of point mutations on the conformations of drug entry channels of orthologous proteins. However, a similar strategy has been applied in earlier studies (25-28). The results from our study provide clues that increased conformational flexibility in the Y319H mutant may be the reason for its reduced drug binding affinity.

However, the Y319H mutation was not found in the other resistant isolate (NCCPF 760815). Absence of the Y319H mutation in NCCPF 760815 may be due to other mechanisms responsible for elevated MICs in this isolate. Nonetheless, our findings need to be evaluated in more non-WT *A. flavus* isolates and by production of a Y319H mutant in a WT background and confirming its azole resistance.

The nucleotide sequences of *CYP51C* of NCCPF 761157 and NCCPF 760815 have been submitted to GenBank with the nucleotide accession numbers KR822399 and KR822400 respectively.

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Table 1: Antifungal susceptibility profile of *A. flavus* isolates for amphotericin B (AMB), voriconazole (VOR), itraconazole (ITR), posaconazole (POS), caspofungin (CSP), micafungin (MCF) and anidulafungin (ANI) performed by CLSI M38-A2..

Strain	MIC/MEC* (µg/mL)						
	AMB	VOR**	ITR	POS	CSP	MCF	ANI
NCCPF 761157	2	4(8)	16	0.25	0.03	0.015	0.0075
NCCPF 760815	4	2(2)	1	0.5	4	0.12	0.25
NCCPF 760816	2	0.5(1)	0.12	0.12	0.06	0.015	0.06
NCCPF 760690	4	0.125 (0.25)	0.06	0.03	0.03	0.015	0.06
NCCPF 761379	1	0.5	0.12	0.06	0.06	0.015	0.06
NCCPF 761425	4	0.5	0.25	0.12	0.03	0.015	0.06

*MEC- minimum effective concentration of ehinocandins.

**The values given in brackets for voriconazole are MICs determined by EUCAST method, E.DEF 9.1.

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248 **Table 2:** Primers used in the study for amplification of homologs of *CYP51*

CYP51 Homologue	Primer Name	Primer sequence (5'……3')	Position on co-ordinate (bases)
CYP51A	AflaCYP51A F1	CAAGAACAGCCTGCACAGAG	324
	AflaCYP51AR1	GGGTGGATCAGTCTTATTA	1126
	AflaCYP51AF2	GCAATCATCGTCCTAAATC	1066
	AflaCYP51AR2	CTGTCCATTCTTGTAGGTA	1899
	AflaCYP51AF3	GCATGAGGGAGATCTATATG	1791
	AflaCYP51AR3	CCTATAATTGCTGGTTTCG	2649
	AflaCYP51AF4	TGAAGCTATTCAATGTAGAC	2480
	AflaCYP51AR4	ACTGCTGATGGTGTGCTAAG	3358
	A205T-F	GGAGTCGCATGTACCATTGA	1510
	A205T-R	TGAAGTTGATCGGAGTGAACC	1716
CYP51B	AflaCYP51B F1	AACACGACTAGGAGCTACAC	4182
	AflaCYP51BR1	CACCAATCCACTCTATC	5082
	AflaCYP51BF2	GATCAGGGAAATGTTCTTC	4948
	AflaCYP51BR2	ACGATCGCTGAGATTAC	5620
	AflaCYP51BF3	GTTCAGCAAATGTCGAG	5550
	AflaCYP51BR3	CCTTTCGTCTACCTGTT	6344
	AflaCYP51BF4	AGTGGAGAGCATCCATAGTGA	6231
	AflaCYP51BR4	ACAACCCGTTCAAGATATCGG	7339
CYP51C	AflaCYP51CF1	CTGTTGCAGAGCCGTTGATG	33
	AflaCYP51CR1	CAAAGAGCGACACATAAG	860
	AflaCYP51CF2	GGTAATGTCTGGTCATAGG	751
	AflaCYP51CR2	ATGAGCTTGGAATTGGG	1453

AflaCYP51CF3	CGAATTCATCCTCAATGG	1336
AflaCYP51CR3	GTCTCTCGGATCACATT	2137
AflaCYP51CF4	GGAACTCTACCAAGAGCA	2018
AflaCYP51CR4	CCTAGATACAGCTAGATACCC	2819
AflaCYP51Cdel-F	CCAGCGCTCATAGGTGTATT	2634
AflaCYP51Cdel-R	CGTGGTCAGTCAATTGGGTA	3102
SNP-F	GCGGTTCTCTACCACGATTTG	677
SNP-R	AGGGTCTCTCGGATCACATTT	1120

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Table 3: Mutational analysis of *CYP51A*, *CYP51B*, *CYP51C* and the corresponding amino acid changes in lanosterol 14 α demethylase (LDM) in resistant and sensitive isolates

Strain	**Mutations in *CYP51***			**Amino acid change in LDM**			**Regulatory region of *CYP51C***
	CYP51A	*CYP51B*	*CYP51C*	*CYP51A*	*CYP51B*	*CYP51C*	
NCCPF 761157	G680A	None	T161C T788G G830A G923A T1025C	A205T	None	M54T S240A D254N I285V **Y319H**	4 bp deletion at 2734 bp
NCCPF 760815	None	None	T161C T788G G830A G923A	None	None	M54T S240A D254N I285V	4 bp deletion at 2734 bp
NCCPF 760816	None	None	T161C T788G	None	None	M54T S240A	None
NCCPF 760690	None	None	T161C T788G	None	None	M54T S240A	None
NCCPF 761379	None	None	T161C	None	None	M54T S240A	None
NCCPF 761425	None	None	T788G	None	None	M54T S240A	None

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Figure legends

Figure 1: Modelled structure of *CYP51C* of *A. flavus* shown in cartoon representation. The porphyrin ring is shown in stick representation in black. Tyrosine residue present in wild type and histidine in mutant are shown in hot pink and green respectively

Figure 2: **A**. Root mean square deviations of Cα backbones of WT and mutant *CYP51C* proteins as a function of time (20 ns); **B.** The graph shows the average fluctuation of Cα atoms for each residue around the average structure of the protein. The black line stands for the WT molecular dynamics trajectory and the red line for the mutant Y319H dynamics trajectory; **C.** Radius of gyration of Cα of WT and mutant *CYP51C* protein as a function of time at 20 nano seconds.