P300: A Novel Mechanism of Voriconazole-Resistance in Aspergillus flavus

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Objectives: *Aspergillus flavus* is the second most common pathogen causing invasive aspergillosis after *A. fumigatus*. In the tropical and sub-tropical regions of Asia, sino-orbital or cerebral aspergillosis due to invasive *Aspergillus* sinusitis are emerging diseases and *A. flavus* is the predominant pathogen in these infections. In invasive aspergillosis, long-term treatment with azole drugs may cause the emergence of azole-resistant *A. fumigatus* and acquired azole-resistant mechanisms in *A. fumigatus* have been well studied. On the other hand, most of azole-resistant risks and mechanisms in *A. flavus* remain to be elucidated. Here, we investigate the risk of acquiring azole-resistance in *A. flavus* due to long-term exposure to azole drugs *in vitro*, and the mechanism of azole-resistance in an isolated azole-resistant mutant. **Methods:** Serial passage cultures (7 days per culture) of *A. flavus* ATCC204304 conidia were performed under exposure to itraconazole or voriconazole in RPMI-agar medium. The MIC was determined by the broth microdilution method according to the CLSI M38-A2 standard. Whole genome sequencing analysis was performed on the Illumina MiSeq platform and CLC Genomics Workbench. Homologous gene replacement studies were performed with *A. flavus* A1421. Gene expression levels were determined using RT-qPCR. **Results:** A mutant with reduced susceptibility to voriconazole with MIC of 16 µg/mL was isolated from the fifth passage culture of *A. flavus* ATCC204304 in the presence of voriconazole whereas the MIC of itraconazole was 0.5 µg/mL. This voriconazole-resistant mutant did not have any mutation in the *cyp51A* gene and its promoter region. Interestingly, we found a mutation in *yap1*, encoding a bZIP transcription factor well-known as the master regulator of oxidative stress responses, which caused Leu558Trp substitution in the carboxy-terminal cysteine-rich domain of Yap1. This mutation was confirmed to be responsible for the voriconazole-resistant phenotype but not for itraconazole by homologous gene replacement studies. Furthermore, we evaluated transcriptional levels of genes which have putative Yap1 response elements in promoter regions, and the expression levels of *atrF*, *dsba*, *gst* and *cyp51A* in the *yap1* mutant were 62-, 27-, 15- and 4.7-fold higher compared to the parental strain, respectively. Notably, we focused on the marked upregulation of the *atrF*, encoding an ABC efflux transporter, and then the deletion of *atrF* in the *yap1* mutant almost restored the susceptibility to voriconazole. **Conclusions:** To the best of our knowledge, this is the first report about a point mutation in *yap1* and subsequent marked *atrF*-upregulation causing voriconazole-resistance in *A. flavus*. These findings provide new insights into azole-resistant mechanisms independent of the *cyp51A* gene mutation in *A. flavus*.

Serial passage culture of A. fumigatus and A. flavus in the presence of azole drugs

Azole MICs and Cyp51A substitution of fifth passage cultures

Strain		MIC (µg/mL)		Substitution
		ITC	VRC	
<i>A. fumigatus</i> ATCC204305	parent	0.5	0.5	-
	5th ITC-passage	>16	4	Gly432Ser
	5th VRC-passage	1	1	none
<i>A. flavus</i> ATCC204304	parent	0.5	0.5	-
	5th ITC-passage	1	2	none
	5th VRC-passage	0.5	16	none

Whole genome sequencing analysis

Locus_Tag	Description	Nucleotide change	Amino acid change	Reference genome
AFLA_129340/	fungal AP-1-like factor (Yap1)	T1673G	L558W	NRRL3357 /AF70
AFLA70_107g002681				

Yap1 substitution in the voriconazole resistant A. flavus

The possible mechanism of VRC resistance via the Yap1 mutant in A. flavus

Homologous gene replacement of the mutated yap1 gene in A. flavus

Strain	Background	Relevant characteristic	MIC (µg/mL)	
			ITC	VRC
SR-A		uracil auxotrophy (+uracil)	0.5	8
SR-B	Yap1 ^{L558W}	pyrG complementation	0.5	8
SR-C	(VRC selection)	Revertant to Yap1 ^{wt} ::pyrG	0.5	0.5
SR-D		ΔatrF::pyrG	0.5	1

Gene expression levels of efflux transporters and possible Yap1 regulating genes

Gene ID	Gene Name	Yap1 response element (YRE)	KEGG description
AFLA_129340	yap1	none	AP-1-like transcription factor
AFLA_036130	cyp51A	TTAGTAA@-165bp	14-alpha sterol demethylase Cyp51A
AFLA_042060	cdr1B	none	ABC transporter, putative
AFLA_048540	mdr1	none	ABC multidrug transporter Mdr1
AFLA_025970	mdr2	none	ABC multidrug transporter Mdr2
AFLA_019740	mdr4	none	multidrug resistance protein 1, 2, putative
AFLA_055690	atrF	TTAGTAA@-468bp	ABC drug exporter AtrF
AFLA_008410	mfs1	none	MFS multidrug transporter, putative
AFLA_022230	dsba	TTATTAA@-114bp	DSBA-like thioredoxin domain protein
AFLA_023740	gst	TTATTAA@-161bp	glutathione S-transferase, putative
AFLA_090690	cat1	TTATTAA@-779bp	mycelial catalase Cat1
AFLA_083370	glr1	TTACTAA@-151bp	glutathione oxidoreductase Glr1, putative