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FUNGAL GENOMICS AND PATHOGENESIS (S SHOHAM, SECTION EDITOR)

Emerging Antifungal Drug Resistance in *Aspergillus fumigatus* and Among Other Species of *Aspergillus*

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Abstract

Purpose of Review The purpose of this review is to give an overview of recent findings on antifungal resistance in *Aspergillus fumigatus* (the major causative agent of aspergillosis) and sibling *Aspergillus* species, which can be hidden agents of aspergillosis.

Recent Findings Azole resistance by Cyp51A mutation in *A. fumigatus* is a growing problem worldwide. The resistance can occur in patients or in the environment. The former occurs by drug selection in the host, inducing mutations in Cyp51A. The latter is characterized by a tandem repeat in the promoter region of *cyp51A* gene and mutation(s) in Cyp51A. Environmental resistant strains are prevailing rapidly and globally. Moreover, efflux pump and biofilm formation are closely related with antifungal resistance of *A. fumigatus*. Finally, sibling species of *Aspergillus* are described with regard to antifungal resistance.

Summary Environmental azole-resistant strains have newly emerged and been dispersed globally, and continuous survey and countermeasures are urgently needed against these strains. Although the contributions of Cyp51A and efflux pumps to antifungal resistance are becoming clear, other resistance mechanisms remain unclear. Further investigations including genome comparisons will help to clarify the novel resistant mechanisms and to develop countermeasures or novel antifungal drugs against resistant strains of *A. fumigatus* and other *Aspergillus* species that have low susceptibility to antifungal therapeutics.

Keywords Antifungal drug resistance · Genomic alteration · *Aspergillus fumigatus* · Sibling species · Biofilm formation

Introduction

Aspergillosis and candidiasis are the most common invasive fungal infections found worldwide [1–4, 5•]. The prognosis of aspergillosis continues to be sub-optimal and in chronic pulmonary aspergillosis, which is fairly common in Japan, the 5-year survival rate is approximately 50–60%. The causative agents are *Aspergillus* spp., which are commonly found in the environment and grow there saprophytically. The most common causative agent of aspergillosis is *Aspergillus fumigatus* [2, 3, 6–8]. Although *A. fumigatus* is not prevalent in the air, its characteristics, for example, spore size and easy dispersion of spores, confer advantage in infection. Secondary causative agents of aspergillosis are *A. flavus*, *A. niger*, and *A. terreus* [2, 3, 7]. Under macroscopic and microscopic observation, these species can be differentiated from *A. fumigatus*. Recently, sibling *Aspergillus* species have been recognized as causative agents of aspergillosis by sequencing-based identification methods [9–14]. For instance, in *Aspergillus* section *Fumigati*, several cryptic species such as *A. lentulus*, *A. felis*, and *A. udagawae*, which resemble *A. fumigatus*, have been often misidentified as *A. fumigatus* in the past, and their clinical significance overlooked [13, 15].

Antifungals have been developed for the treatment of aspergillosis and are currently available in three antifungal classes: polyene (amphotericin B), azoles (voriconazole (VRCZ), itraconazole (ITCZ), isavuconazole, and posaconazole), and

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echinocandins (micafungin, caspofungin, and anidulafungin). Among these, azoles are the most commonly used to treat aspergillosis. Some azoles, not medical azoles, have been used as agricultural fungicides.

Antifungal resistance is a growing problem worldwide. Drug resistance in *Candida* spp. and *Aspergillus* spp. has been intensively analyzed [16]. Major antimicrobial resistance mechanisms are roughly classified into four types [17]: (1) modifications of the antimicrobial molecule, (2) decreasing penetration or active exportation of the antimicrobial compounds, (3) modification of drug target protein/enzyme, and (4) global cell adaptation. It is thought that biofilm is a global cell adaptation, resulting in antimicrobial resistance. For antifungal resistance, efflux pumps and target alteration have been characterized among fungi although modification of antifungal compounds by fungi has never been reported. *A. fumigatus* is recognized as a pathogenic fungus that forms biofilms.

As genomic and transcriptomic analyses forward our understanding of the characteristics of *Aspergillus* species, some molecular mechanisms of antifungal resistance, as well as other phenotypes, have been disclosed [18, 19]. The genome sequence of *A. fumigatus* was determined in 2005 [20], as well as that of *A. oryzae*, which is an important microbe for fermentation [21] and *A. nidulans*, an important model organism. The genome sequences of *A. flavus*, *A. niger*, and *A. terreus* have also been determined [22–24]. Genome sequences of several sibling species, such as *A. lentulus* [25] and *A. udagawae* [26], have also recently been determined.

In this review, we describe recent findings on antifungal resistance in causative agents of aspergillosis: *A. fumigatus* and non-*fumigatus Aspergillus*; especially azole resistance by Cyp51A alteration, drug resistance related with efflux pumps, biofilm formation of *A. fumigatus*, and low susceptibility of non-*fumigatus Aspergillus*, including sibling species.

Azole Resistance in *Aspergillus fumigatus* by the Mutation and Increased Expression of Cyp51A

Azole resistance of *A. fumigatus*, a growing problem worldwide, occurs after long azole exposure in a patient (patient route) or in the environment (environmental route) [27–30]. In the patient route, the causative *A. fumigatus* strain is susceptible before entering the host. During treatment with azoles, the exposure in a host induces mutation(s) in the genome, resulting in azole resistance [31–34]. For instance, amino acid substitution(s) in lanosterol-14-α-demethylase Cyp51A, which is the major target of azole antifungals, appears during azole treatment. Hagiwara et al. reported the acquisition of ITCZ resistance in a patient [32], who presented with an aspergilloma and treated with ITCZ for 449 days. Isolates before ITCZ treatment were not resistant to azoles. However, after ITCZ treatment, the isolate possessed a P216L mutation in Cyp51A and was resistant to ITCZ (minimum inhibitory concentration (MIC) 4 μg/mL). In other studies, the acquisition of a G448S mutation after VRCZ treatment and the acquisition of G54E or G54W substitution after ITCZ in each patient have been reported in Cyp51A of *A. fumigatus* [33, 34].

Via the environmental route, *A. fumigatus* acquire azole resistance in the environment. Although it remains unclear how the environmental-resistant strain appeared, it is widely accepted that the selection with agricultural fungicides might induce azole resistance. In contrast to the patient route, the resistant strains possess limited sets of amino acid substitution(s) in Cyp51A and a tandem repeat (TR) in the *cyp51A* promoter region, mainly TR_{34}/L98H and TR_{46}/Y121F/T289A. The TR_{34}/L98H type of resistant strains has been initially reported as isolates from across the Netherlands between 2002 and 2006. Thereafter, isolation cases from Italy and the Netherlands in 1998 were reported [35•, 36]. TR-type strains have been reported not only in European countries but also in the Middle East, South Asia, and East Asia. Recently, in Japan, a TR_{46}/Y121F/T289A strain [37] and a TR_{34}/L98H strain [38] have been isolated from patients. Moreover, a TR_{34}/L98H strain has been isolated from the environment in Japan [39]. The short tandem repeat pattern of the TR_{34}/L98H isolate was close to the patterns of overseas isolates harboring TR_{34}/L98H, but not Japanese azole-susceptible isolates [38, 39], indicating that the isolate was introduced from overseas into Japan. The route how TR-type strain was brought to Japan remains unknown. Spores might adhere to individuals or imported items because *A. fumigatus* could be found in soil worldwide or it may be airborne, because spores of *A. fumigatus* are easily dispersed. Dunne et al. isolated azole-resistant *A. fumigatus* from imported plant bulbs [40••], suggesting that transportation of items associated with soil is a route for intercountry transfer of the resistant strains.

It is thought that most of the mutations in the coding sequence directly affect the structure and binding of azoles to Cyp51A. For example, 54th and 220th residues are located at the mouth of the binding pocket, suggesting that those substitutions affect the drug entering in and binding to the pocket. The 448th residue is located behind heme cofactor, suggesting that the substitutions have the potential to distort the position of heme [41]. Although the 98th residue of Cyp51A is far from the catalytic pocket, by in silico analysis, the binding stability to azoles was lowered by the substitution from leucine to histidine [41].

The tandem or triple repeat found in the environmental strains increased the expression of Cyp51A [42, 43••] and was included in the binding region of a positive regulator, SrbA [44••], suggesting that the TR contributes to azole resistance by the increase of its expression. Zhang et al. [43••] report a possible role of sexual reproduction in the emergence of triple repeat.

As an azole resistance mechanism, Cyp51A upregulation is also reported [45, 46]. As shown in Fig. 1, *cyp51A* regulation is becoming clear. As well as acquisition of tandem or triple repeat in the *cyp51A* promoter region, a transposon insertion in the promoter was reported in a clinical isolate, suggesting that the insertion might be responsible for increased expression of *cyp51A* [45]. Camps et al. identified HapE mutation P88L as the mutation responsible for azole resistance [46]. HapE is a subunit of the CCAAT-binding transcription factor complex, which is important for the repression of *cyp51A* expression [44••]. P88L mutation in HapE could not interact with the *cyp51A* promoter region, resulting in increased expression of *cyp51A* [44••]. Another important transcription factor, SrbA, interacted with the *cyp51A* promoter and induced *cyp51A* expression [44••, 47•]. An *srbA* disruptant decreased *cyp51A* expression, resulting in azole hypersensitivity [44••, 47•]. Notably, the hypersensitivity resulting from the deletion of the *srbA* gene was shown even in the TR_{46}/Y121F/T289A strain [47•]. AtrR, a Zn_2-Cys_6 type transcription factor, also interacts with *cyp51A* promoter and regulates *cyp51A* expression [48••]. High-level expression induced by azole or constitutive expression of *cyp51B*, a paralogue of *cyp51A*, has been reported in azole-resistant clinical isolates of *A. fumigatus* [49].

Contribution of Efflux Pumps to Drug Resistance in *A. fumigatus*

Efflux pumps contribute to drug resistance in microbes, including fungi, especially by overexpression. In *Candida albicans*, ATP-binding cassette (ABC) transporters, CDR1 [50] and CDR2 [51], and a major facilitator superfamily (MFS) protein MDR1 (BEN^r) [52] are well characterized in the contribution to azole resistance. The contribution of some efflux pumps to azole resistance in *A. fumigatus* has also been described. Fraczek et al. [53] and Paul et al. [54] show that an ABC transporter, Cdr1B, is associated with azole resistance in *A. fumigatus*. MdrA, an MFS protein in *A. fumigatus*, is described as a potential protein conferring azole resistance [55]. Recently, Hagiwara et al. elucidated the regulation of *cdr1B* expression as well as *cyp51A* by AtrR [48••]. AtrR is a Zn_2-Cys_6 type transcription factor in *Aspergillus* spp. *atrR* disruptant showed hypersusceptibility to azoles and decreased expression of *cdr1B* and *cyp51A*. AtrR bound to *cdr1B* and *cyp51A* promoters, indicating the direct regulation of their expression.

Biofilm Formation and Antifungal Resistance in *A. fumigatus*

Biofilm formation is a major mechanism in fungal resistance to antimicrobial agents. *C. albicans* is a well-known biofilm former among fungi (in a recent study reviewed by Cavalheiro and Teixeira [56]). The molecular mechanism underlying biofilm formation and the relationship between biofilm formation and antifungal resistance have been extensively investigated in *C. albicans* [56–58].

A. fumigatus, although less well known, is also recognized as a biofilm former [59–64]. *A. fumigatus* forms a biofilm with an extracellular matrix (ECM) in vitro [59, 63–66] and in vivo [67]. Mowat et al. [59] and Seidler et al. [62] developed simple biofilm models of *A. fumigatus* in which antifungals against the biofilm were less effective than against planktonic cells. Fatal bovine serum and fetuin A (a serum glycoprotein) found

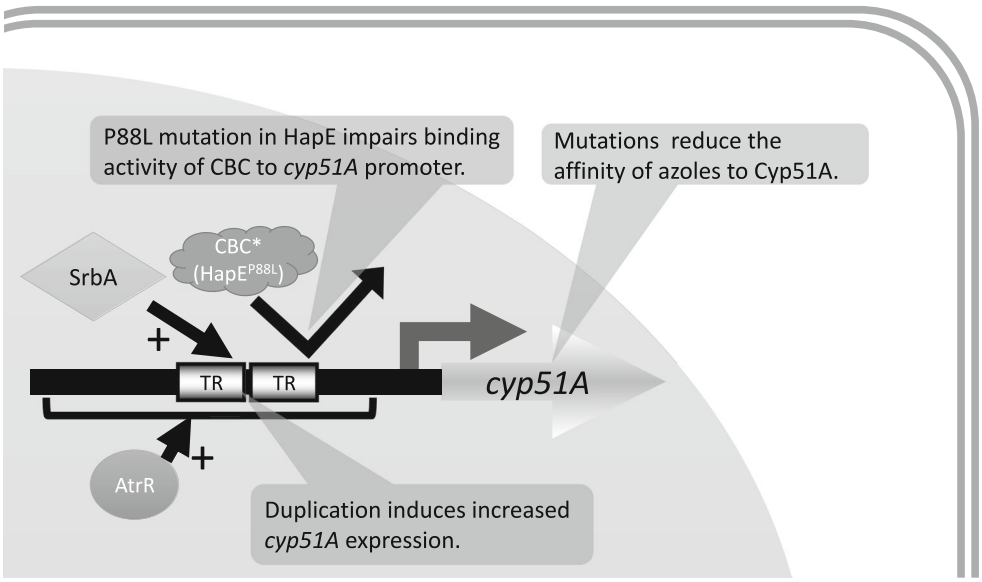

Fig. 1 The regulation of *cyp51A* expression.CBC,CCAAT-binding transcription factor complex;TR,tandem repeat.

in a patient's fungus ball was found to promote biofilm formation by *A. fumigatus* [64]. Biofilms formed in vitro also conferred resistance to antifungals [68].

Several mechanisms, adherence [69], ECM formation [70], and efflux pump expression [71], have been suggested to reduce the efficacy of antifungals in *Candida* biofilm. In *A. fumigatus* biofilm, efflux pump expression has been relatively well-characterized compared with other mechanisms [65, 72, 73]. Transcriptome analyses show that ABC and MFS transporters are upregulated in *A. fumigatus* biofilms [65, 72]. Rajendran showed that MIC of VRCZ was reduced by treatment with an efflux pump inhibitor and the expression of an MFS protein was induced by the exposure of VRCZ [73], strongly suggesting that transporters have a pivotal role in antifungal resistance of *A. fumigatus* biofilm. The ECM produced by *A. fumigatus* has a putative role in antifungal resistance in the biofilm. In *C. albicans* biofilms, β-1,3-glucans have a putative role in antifungal resistance, because planktonic *C. albicans*' susceptibility to fluconazole was significantly reduced by the addition of laminarin and soluble β-1,3-glucan, as well as adding ECMs to *C. albicans* biofilms [70]. The ECM in *A. fumigatus* biofilms is mainly composed of galactosaminogalactan, galactomannan, and α-1,3-glucans [63, 67]. β-1,3-Glucan, chitin, and polygalactosamine were not detected in a model of *A. fumigatus* ECM in vivo [63]. Although soluble β-1,3-glucan was detected at tens of nanograms per milliliter in a supernatant of *A. fumigatus* biofilm [64], the role in antifungal resistance remains unclear.

Antifungal Resistance in *Aspergillus* Species Including Sibling Species

As described above, *A. fumigatus* is the major causative agent of aspergillosis. Other *Aspergillus* species, such as *A. flavus*, *A. niger*, and *A. terreus*, are also recognized as causative agents of aspergillosis. Recently, sibling species, for example, *A. lentulus*, *A. udagawae*, *A. felis* (resembling *A. fumigatus*) and *A. tubingensis* (resembling *A. niger*), have been newly recognized as causative agents of aspergillosis. It is noteworthy that MIC distributions of some species are higher than those of *A. fumigatus*. As shown by FILPOP and TRANSNET studies, strains resistant to antifungals among non-*fumigatus Aspergillus* species are more frequently found among *A. fumigatus* [13, 15, 74]. A case report of aspergillosis due to *A. lentulus* has been published, which showed low sensitivity of the isolate to antifungals (2 mg/L for VRCZ, 4 mg/L for amphotericin B) [75]. *A. felis*, an emerging agent of aspergillosis in humans and animals, is a novel species in *Aspergillus* section *Fumigati* [10]. In a report by Barrs et al., MIC of VRCZ against 3 of 13 *A. felis* strains was 4 mg/L [10]. *A. tubingensis* is recognized as a major causative agent among *Aspergillus* section *Nigri* [13, 15]. Hashimoto and colleagues reported that environmental strains and clinical isolates of *Aspergillus* section *Nigri* showed low susceptibility to azoles [76]: 79.5 and 89.7% of *A. tubingensis* strains showed ITCZ and VRCZ MICs above 2 mg/L, respectively [76]. In our study, five of the eight clinical isolates of *A. tubingensis* showed ITCZ and/or VRCZ MICs ≥ 2 mg/L (unpublished data). In contrast, all nine of our isolates of *A. niger* showed ITCZ and VRCZ MICs < 2 mg/L, suggesting that most *A. tubingensis* isolates are intrinsically resistant or less sensitive to azoles.

The natural resistant mechanisms among sibling species of *Aspergillus* remain mostly unknown. Some of the resistant strains showed the increase of *cyp51A* gene expression [76]; other mechanisms, however, might be hidden. Next-generation sequencing and genome comparison analyses will help to disclose new intrinsic resistant mechanisms in sibling species.

Conclusions

Perspectives

The World Health Organization released a Global Action Plan on antimicrobial resistance in 2015. The importance of understanding antimicrobial resistance and countermeasures has been increasing because new resistance mechanisms are emerging and spreading globally. Antifungal resistance in pathogenic yeasts and fungi is also emerging and spreading. *C. auris* [77], a non-*albicans Candida* not described in this review, is also recognized as an emerging pathogen [78], and the type strain is susceptible to azoles and 5-flucytosine [77]. The species, however, is now recognized as a multidrug-resistant yeast [79]. Along with antifungal stewardship, continuous drug susceptibility testing of clinical and environmental isolates is needed to detect, track, and prevent the emergence and spread of resistant lineages. Besides Cyp51A and efflux pumps, other players such as Hsp90 [80] contribute to antifungal resistance. However, many factors involved in azole resistance remain to be identified. Further analysis, including genome analysis, and deeper understanding of antifungal resistance mechanisms will facilitate the development of new antifungals against less susceptible and resistant strains.

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Compliance with Ethical Standards

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Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major Importance

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