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Diagnostic significance of *Aspergillus* species isolated from respiratory samples in an adult pneumology ward

TAKAYOSHI TASHIRO*, KOICHI IZUMIKAWA†, MASATO TASHIRO†, TAKAHIRO TAKAZONO†, YOSHITOMO MORINAGA†, KAZUKO YAMAMOTO†, YOSHIFUMI IMAMURA†, TAIGA MIYAZAKI†, MASAFUMI SEKI†, HIROSHI KAKEYA†, YOSHIHIRO YAMAMOTO†, KATSUNORI YANAGIHARA‡, AKIRA YASUOKA§ & SHIGERU KOHNO†

*Departments of *Health Sciences and †Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, ‡Department of Laboratory Medicine, and §Nagasaki University Infection Control and Education Center, Nagasaki University Hospital, Nagasaki, Japan*

Although the diagnostic significance of isolating *Aspergillus* spp. from respiratory cultures has been studied in immunocompromised hosts with invasive pulmonary aspergillosis (IPA), little is known of such infections in immunocompetent patients with other forms of aspergillosis. In this study of adult pneumology ward patients, we examined the association between *Aspergillus* spp. and disease prevalence. Laboratory records from April 1998 to March 2009 were reviewed to identify patients with *Aspergillus* spp. in respiratory samples. Correlations between the isolated species and clinical characteristics of patients were evaluated. During the study period, 165 *Aspergillus* spp. isolates were detected in the respiratory cultures of 139 patients. Of these patients, 62 (45%) were colonized with *Aspergillus* spp. and displayed no clinical symptoms of aspergillosis, while 77 (55%) had a form of pulmonary aspergillosis, characterized as either chronic necrotizing pulmonary aspergillosis (CNPA) (48%), aspergilloma (29%), IPA (13%), or allergic bronchopulmonary aspergillosis (ABPA) (10%). The dominant species were *Aspergillus fumigatus* (41%), *A. niger* (32%), and *A. versicolor* (12%). *A. fumigatus* was most commonly isolated in patients with IPA, aspergilloma, and CNPA, whereas *A. niger* was the dominant species in colonized patients and those with ABPA. Isolation of an *Aspergillus* spp. from respiratory samples does not confirm it as the etiologic pathogen because airway colonization by *Aspergillus* spp. is a common feature in several chronic lung diseases. Repeated isolation of the identical *Aspergillus* species and detection of anti-*Aspergillus* antibodies and/or *Aspergillus* antigens in sera are needed to determine the isolate represents the etiologic agent of disease.

Keywords CNPA, CCPA, Aspergilloma, IPA, colonization

Introduction

Members of the genus *Aspergillus* are ubiquitous saprophytic fungi, and due to their widespread presence in the environment, the average person may inhale hundreds of *Aspergillus* conidia per day [1]. Conidia are typically removed from the respiratory tract by mucociliary clearance and phagocytosis by alveolar macrophages, while germinating spores and hyphae are attacked by polymorphonuclear neutrocytes through degranulation and the release of oxidants [2]. Despite these effective clearance mechanisms for the elimination of inhaled conidia from the respiratory tracts of healthy individuals, *Aspergillus* conidia are capable of colonizing injured lung tissue and epithelia. Although such colonization often has no clinical consequences, *Aspergillus* conidia can cause a variety of clinical manifestations depending on the immune status of the host [3,4]. For example, allergic bronchopulmonary

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Correspondence: Koichi Izumikawa, Nagasaki University Graduate School of Biomedical Sciences, Department of Molecular Microbiology and Immunology 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: + 81 95 819 7273; fax: + 81 95 849 7285; E-mail: koizumik@nagasaki-u.ac.jp

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aspergillosis (ABPA), which encompasses hypersensitivity reactions to *Aspergillus* antigens, can develop in atopic patients [5,6], while aspergilloma is mainly observed in patients with chronic cavitary lung disease. Other forms of aspergillosis include chronic necrotizing pulmonary aspergillosis (CNPA), which is seen in mildly immunocompromised patients and those individuals with chronic lung diseases [7], and invasive pulmonary aspergillosis (IPA), which is the most serious lung infection due to *Aspergillus* spp. and typically occurs only in severely immunocompromised patients [8].

Isolation of *Aspergillus* spp. from respiratory samples of a patient with suspected aspergillosis does not necessarily indicate that the identified species is the etiologic agents of disease. Although the diagnostic significance of the isolation of *Aspergillus* spp. from respiratory cultures has been extensively examined in immunocompromised hosts who develop IPA [9–13], little is known in respect to the presence of *Aspergillus* spp. in the respiratory tracts of immunocompetent or mildly immunocompromised patients with other forms of aspergillosis [14–16]. Here, we conducted a retrospective study to evaluate the clinical significance of *Aspergillus*-positive culture results of respiratory samples from adult patients in a pneumology ward.

Materials and methods

The microbiology laboratory records of the Pneumology Department of Nagasaki University Hospital, Japan, from April 1998 to March 2009 were reviewed to identify all patients who had *Aspergillus* spp. isolated in culture from respiratory samples. A total of 350–500 patients including 150–200 new patients per year are admitted to this department, with lung cancer being the most common pulmonary disease. The respiratory samples, which included sputum, endotracheal aspirates, and bronchoalveolar lavage fluid, were obtained as a part of a diagnostic work-up for respiratory disease. Samples were immediately inoculated onto Sabouraud dextrose agar and incubated for 10 days at 30°C. Filamentous fungi recovered in culture were transferred to potato dextrose agar and incubated for 2–3 days at 30°C. The isolates were identified based upon macroscopic and microscopic morphological characteristics following standard mycological procedures. For each patient with a positive sample for *Aspergillus*, the species and clinical characteristics of the patient were recorded. When the same *Aspergillus* species was isolated on more than one occasion from the same patient, it was counted only once.

Aspergilloma was diagnosed when a characteristic fungus ball was detected within a lung cavity by chest radiograph or computed tomography and a positive serological test (antibody detected) was obtained or when an *Aspergillus* species was isolated from respiratory samples. CNPA was diagnosed when chest radiographs showed a cavitary pulmonary lesion with evidence of paracavity infiltrates or cavitary change developing over a time-frame of several weeks to several months in patients with chronic pulmonary symptoms, elevated inflammatory markers, and positive serological tests (antigen/antibody detected) or isolation of *Aspergillus* spp. from respiratory samples [17]. IPA was recorded when chest radiographs showed acute and extensive pneumonia or nodular pneumonia resistant to broad-spectrum antibiotics in immunocompromised patients, and respiratory samples yielded *Aspergillus* spp. without pathogenic bacteria. ABPA was diagnosed according to the criteria of Rosenberg [5]. Serological studies including *Aspergillus* antibody and antigen tests were performed when the patients were suspected of having pulmonary aspergillosis. Patients were diagnosed as having airway colonization when *Aspergillus* spp., were isolated from respiratory samples without clinical or histological evidence of aspergillosis. All of the charts were reviewed by TT, KI, MT, TT, TM, and HK as review committee. All members of the chart review committee discussed each case if there was an incorrect or unclear diagnosis of aspergillosis. All data was reviewed by all authors.

Results

During the 11-year study period, 165 isolates of *Aspergillus* spp. were detected in cultures inoculated with the respiratory samples of 139 patients. Of these patients, 62 (45%) were colonized with *Aspergillus* spp. but displayed no clinical symptoms of aspergillosis, while the remaining 77 (55%) had a form of pulmonary aspergillosis, which could be classified as CNPA (48%), aspergilloma (29%), IPA (13%), or ABPA (10%). The clinical characteristics of the patients are summarized in Table 1.

The patients with CNPA or aspergilloma displayed a high occurrence of associated chronic lung disease, including chronic obstructive pulmonary disease (COPD), nontuberculous mycobacteriosis (NTM), pulmonary tuberculosis, lung cancer, and bullae or a history of pneumothorax, interstitial pneumonia, and pneumoconiosis. In addition, the majority of patients with CNPA suffered from a mild systemic immunosuppressive condition, such as diabetes mellitus, solid-organ cancer, chronic liver disease, and corticosteroid or cytotoxic drug use. In patients with IPA, systemic immunosuppression due to hematologic malignancy, corticosteroid therapy, or chemotherapy was the major predisposing factor, while patients with ABPA often displayed bronchial asthma (88%) and/or other atopic diseases (63%). Of the 62 patients with *Aspergillus* colonization, nearly all had an associated chronic lung disease, such as tuberculosis, COPD, lung cancer, NTM, interstitial

Table 1 Clinical characteristics of patients with *Aspergillus*-positive cultures from respiratory samples.

Characteristic[a]	No. (%) of patients, according to clinical condition				
	CNPA (n = 37)	Aspergilloma (n = 22)	IPA (n = 10)	ABPA (n = 8)	Colonization (n = 62)
Age in years, range (mean)	19–83 (64.7)	46–84 (68.7)	18–83 (58.8)	27–80 (51.6)	20–85 (61.8)
Sex (male/female)	29/8	15/7	7/3	4/4	26/36
Underlying lung disease					
COPD	14 (38)	9 (41)	1 (10)		11 (18)
Non-tuberculous mycobacteriosis	13 (35)	1 (5)		1 (13)	10 (16)
Tuberculosis	9 (24)	9 (41)		1 (13)	14 (23)
Lung cancer survivor	5 (14)	2 (9)		1 (13)	11 (18)
Bullae or pneumothorax	4 (11)	3 (14)			4 (6)
Interstitial pneumonia	2 (5)	1 (5)	1 (10)		10 (16)
Pneumoconiosis	2 (5)	1 (5)			2 (3)
Bronchial asthma	1 (3)			7 (88)	1 (2)
Other lung diseases		2 (9)	2 (20)		8 (13)
Immunosuppressive condition					
Solid organ cancer	6 (16)		1 (10)		4 (6)
Hematologic malignancy	1 (3)		5 (50)		4 (6)
Diabetes mellitus	11 (30)		2 (20)		2 (3)
Chronic liver disease	3 (8)		2 (20)		2 (3)
Chronic kidney disease	1 (3)				3 (5)
Collagen vascular disease					10 (16)
Atopic disease				5 (63)	
Other diseases	2 (5)				
Corticosteroid use	3 (8)		5 (50)		12 (19)
Cytotoxic drug use			5 (50)		2 (3)
Bone marrow transplant			1 (10)		
In-hospital mortality	6 (16)	1 (5)	8 (80)	0 (0)	1 (2)

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis; COPD, chronic obstructive pulmonary disease.

[a]A patient may have more than one characteristic.

pneumonia, and/or an immunosuppressive condition induced by corticosteroid use, collagen vascular disease, solid-organ cancer other than lung, hematologic malignancy, or diabetes mellitus. In-hospital mortality was highest in patients with IPA (80%), whereas it was relatively low in patients with CNPA (16%), aspergilloma (5%), and *Aspergillus* colonization (2%). None of the patients with ABPA died during their hospitalization period.

We determined the distribution of *Aspergillus* spp. among the 165 isolates from the respiratory cultures of 139 patients (Table 2). The two dominant species of *Aspergillus* were *A. fumigatus* (41%) and *A. niger* (32%), with *A. versicolor* (12%), *A. terreus* (6%), *A. flavus* (5%), *A. nidulans* (2%), *A. sydowii* (1%), and unidentifiable *Aspergillus* spp. (0.6%) accounting for the remaining isolates. When the patient data between 1998 and 2004 were compiled, *A. fumigatus* was the most commonly isolated species (58.2%), followed by *A. niger* (20.9%), *A. flavus* (10.4%), *A. versicolor* (6.0%), and *A. terreus* (4.5%). However, between 2005 and 2009, the incidence

Table 2 *Aspergillus* species associated with disease based on positive culture results.

Aspergillus species	No. (%) of positive culture results, according to clinical condition					
	CNPA (n = 50)	Aspergilloma (n = 25)	IPA (n = 11)	ABPA (n = 10)	Colonization (n = 69)	Total (n = 165)
A. fumigatus	27 (54)	17 (68)	9 (82)	3 (30)	11 (16)	67 (41)
A. niger	12 (24)	3 (12)	1 (9)	4 (40)	33 (48)	53 (32)
A. versicolor	3 (6)	1 (4)		2 (20)	14 (20)	20 (12)
A. terreus	5 (10)			1 (10)	4 (6)	10 (6)
A. flavus	2 (4)	3 (12)	1 (9)		3 (4)	9 (5)
A. nidulans	1 (2)	1 (4)			1 (1)	3 (2)
A. sydowii					2 (3)	2 (1)
Not identified					1 (1)	1 (0.6)

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.

of *A. fumigatus* isolation decreased by approximately half (28.6%), whereas that of *A. niger* isolation increased approximately two-fold (39.8%), with *A. versicolor* (16.3%) and *A. terreus* (7.1%) again being the next most common isolates (Fig. 1).

To gain a better understanding of the diagnostic significance of the presence of *Aspergillus* spp. in respiratory samples, we examined the correlation between the identified species and the clinical characteristics of patients with regard to disease. It was revealed that *A. fumigatus* was the most commonly isolated species in patients with IPA (82%), aspergilloma (68%), and CNPA (54%), while *A. niger* was the second most prevalent isolate in patients with these diseases. In patients with ABPA, *A. niger* was the most common isolate (40%), followed by *A. fumigatus* (30%). *A. niger* was also the most common isolate (48%) in patients who were asymptomatic for aspergillosis, followed by *A. versicolor* (20%) and *A. fumigatus* (16%). Interestingly, with regard to colonization by *Aspergillus* species, 70% (14/20) and 62% (33/53) of cases involving isolation of *A. versicolor* and *A. niger* isolation, respectively, were considered as asymptomatic, whereas only 16% (11/67) of cases of *A. fumigatus* isolation were not associated with disease.

Finally, we examined the respiratory tract colonization profiles and patterns among the 139 patients positive for *Aspergillus* spp. In most patients (83%), only a single *Aspergillus* spp. was isolated from respiratory tract samples, whereas two or more species were isolated in the remaining patients (17%). In 17 of these 23 patients, a synchronous isolation pattern was observed, which was characterized by isolation of two or more species in the same sample or different samples obtained within a one-month period (Table 3). The remaining six patients displayed a metachronous isolation pattern, in which different samples obtained with an interval of over one month was revealed (Table 4). The combination of *A. fumigatus* and *A. niger* was the most common profile for both the synchronous and metachronous isolation patterns of the observed species combinations.

Discussion

Although the genus *Aspergillus* consists of approximately 200 species [2,18], only a few are considered human pathogens and generally require immunocompromised hosts to cause disease. Among *Aspergillus* spp., *A. fumigatus* is the primary causative agent of human infections, followed by *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* [8,19–26]. In the present retrospective study, *A. fumigatus* was the most commonly isolated species from respiratory samples, followed by *A. niger*, *A. versicolor*, *A. terreus*, *A. flavus*, *A. nidulans*, and *A. sydowii*. However, our analysis of laboratory records and clinical disease revealed that the isolation of a particular *Aspergillus* spp. does not confirm it is the etiologic pathogen of the patient. The diagnostic value of *Aspergillus* spp. in respiratory samples is not straightforward, principally because of difficulties distinguishing colonization from disease. In our series, 42% (69 of 165) of the isolated *Aspergillus* spp. were found in cases representing colonization, and the most common colonizing species was *A. niger*, followed by *A. versicolor*, *A. fumigatus*, *A. terreus*, *A. flavus*, *A. sydowii*, and *A. nidulans*. Although *A. niger* is less virulent than *A. fumigatus* and *A. flavus*, it can occasionally cause IPA, CNPA, or ABPA, and may colonize respiratory tracts of patients with chronic lung diseases [8,23,24]. From our analyses, *A. niger* was most frequently associated with patients diagnosed with ABPA (40%), but for all other forms of pulmonary aspergillosis, *A. fumigatus* was the dominant isolate. Although *A. fumigatus* is the most pathogenic *Aspergillus* spp., it may also colonize respiratory tracts without leading

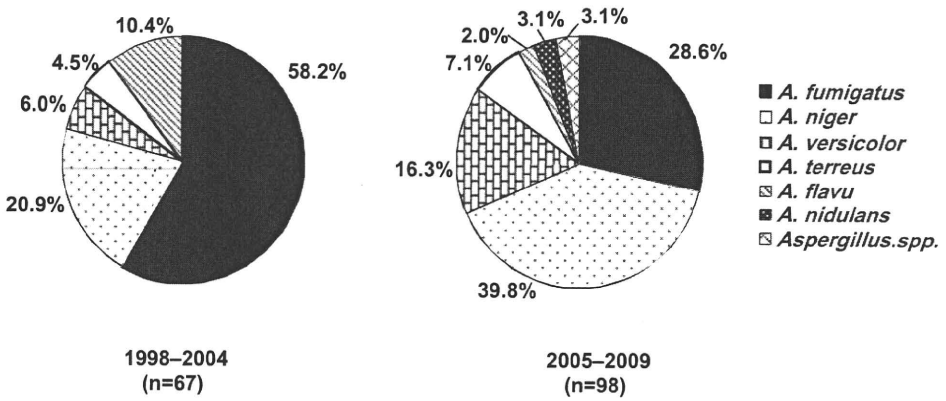

Fig. 1 Comparison of *Aspergillus* spp. isolated during 1998–2004 and 2005–2009 from adults in a pneumology ward. *A. fumigatus* was the most commonly isolated species between 1998 and 2004; however, between 2005 and 2009 its frequency of isolation had decreased by approximately half, while *A. niger* increased approximately two-fold to become the most commonly isolated species.

Table 3 Synchronously isolated *Aspergillus* species according to clinical condition.

Aspergillus species	No. of patients, according to clinical condition					
	CNPA (n = 7)	Aspergilloma (n = 1)	IPA (n = 1)	ABPA (n = 2)	Colonization (n = 6)	Total (n = 17)
A. fumigatus + *A. niger*	3	1		1	2	7
A. niger + *A. versicolor*	1				2	3
A. fumigatus + *A. terreus*	1					1
A. fumigatus + *A. flavus*			1			1
A. niger + *A. flavus*					1	1
A. niger + *A. terreus*				1		1
A. niger + *A. sydowii*					1	1
A. terreus + *A. nidulans*	1					1
A. fumigatus + *A. niger* + *A. versicolor*	1					1

CNPA, chronic necrotizing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.

to clinical symptoms of aspergillosis, as was observed in 16% of cases.

The reported frequency of *Aspergillus* spp. colonization ranges from 36–91% of clinical cases, depending on the patient population studied [9–16]. Perfect *et al.* [11] reported that approximately 50% of *Aspergillus*-positive cultures represented colonization, with *A. fumigatus* (63%) the dominant colonizing species, followed by *A. niger* (14%), *A. flavus* (9%), *A. nidulans* (1%), *A. terreus* (1%), and other species (4%). Although the general trend for the colonizing species in our data was similar, the prevalence of *A. fumigatus* (41%) and *A. niger* (32%) was approximately equal. The difference in the incidence of colonizing species between the two studies is considered to be mainly a result of the study periods and patients. Our data were obtained between 1998 and 2009, whereas Perfect *et al.* [11] conducted their surveillance in 1995. As our data for 1998 and 2004 more closely resembled that of Perfect *et al.* [11] with regard to the distribution of *A. fumigatus* (58.2%) and *A. niger* (20.9%). It appears that *A. niger* is becoming more prevalent in respiratory tract samples, a speculation that is consistent with a reported increase in the frequency of *A. niger* isolation in recent years [27–29]. Colonization may represent transient passage in the airway, as long-term benign carriage is typically observed in patients with localized structural or functional pulmonary deficits. However, it may be a warning sign preceding overt *Aspergillus* infection [14,15].

Table 4 Metachronously isolated *Aspergillus* species according to clinical condition.

Aspergillus species	Clinical condition (No. of patients)
A. fumigatus → *A. niger*	CNPA (n = 2)
A. niger → *A. fumigatus*	CNPA (n = 1)
A. niger → *A. fumigatus* → *A. terreus* → *A. versicolor*	CNPA (n = 1)
A. niger → *A. fumigatus* → *A. nidulans*	Aspergilloma (n = 1)
A. terreus → *A. fumigatus*	Colonization (n = 1)

CNPA, chronic necrotizing pulmonary aspergillosis.

CNPA, which was originally reported by Binder *et al.* [7], has been widely used to characterize a syndrome complex consisting of slowly progressive cavitary lung disease, chronic respiratory symptoms, and the presence of precipitating antibodies to *Aspergillus* spp. A few reports related to CNPA have described direct invasion of pulmonary parenchyma by hyphal elements [7,23,30]. However, the majority of reports have not found clear evidence of parenchymal invasion despite progressive tissue damage. Denning *et al.* [31] proposed that chronic cavitary pulmonary aspergillosis (CCPA) accounts for cases in which there is formation and expansion of multiple cavities over time. Furthermore, they proposed that the term CNPA be reserved for cases in which hyphal invasion of tissue is demonstrated, such as the subacute form of IPA. The majority of our patients with CNPA may have been diagnosed with CCPA, because the pathological appearance of hyphal invasion had not been examined. In our patients with CNPA, *A. fumigatus* was the most commonly isolated species (54%), followed by *A. niger* (24%), *A. terreus* (10%), *A. versicolor* (6%), *A. flavus* (4%), and *A. nidulans* (2%). This result is consistent with that of Perfect *et al.* [11] who reported that the most common *Aspergillus* species identified in patients with CNPA was *A. fumigatus* (80%), followed by *A. niger* (10%), *A. flavus* (2%), and other species (8%).

The profile of *Aspergillus* spp. isolated from patients with aspergilloma was similar to that from patients with CNPA, and consisted of *A. fumigatus* (68%), *A. niger* (12%), *A. terreus* (12%), *A. versicolor* (4%), and *A. nidulans* (4%). Perfect *et al.* [11] reported that the most common *Aspergillus* species identified in patients with aspergilloma was *A. fumigatus* (69%), followed by *A. niger* (13%), *A. flavus* (2%), and other species (5%).

The majority of IPA is caused by *A. fumigatus*, with the second most frequent pathogenic species being *A. flavus* and, to a lesser extent, *A. niger* and *A. terreus* [8,32]. In our retrospective study, the most common *Aspergillus* species identified in patients with IPA was *A. fumigatus* (80%), followed by *A. niger* (9%) and *A. flavus* (9%). Perfect *et al.* [11] reported that *A. fumigatus* (67%) was most commonly isolated, followed by *A. flavus* (16%), *A. niger* (5%), *A. terreus* (3%), and *A. nidulans* (1%).

ABPA is an allergic pulmonary disorder caused by hypersensitivity to *Aspergillus* spp., and *A. fumigatus* is the primary causal organism. In our series, the isolated *Aspergillus* spp. from patients with ABPA were *A. niger* (40%), *A. fumigatus* (30%), *A. versicolor* (20%), and *A. terreus* (10%). Since we did not test allergic reactions to non-*A. fumigatus Aspergillus* spp., isolates other than *A. fumigatus* could not be confirmed as the etiologic antigen to ABPA. However, ABPA due to *A. flavus*, *A. nidulans*, *A. terreus* or *A. niger* has been reported [33–35]. Recently, Benndorf *et al.* [36] identified IgE antibodies that exclusively recognize spore extracts from *A. versicolor* in sera from patients, and also found a relationship between increased spore concentration in indoor air or visible mould affection and a positive reaction of sera to *A. versicolor*. As the isolation of *A. versicolor* in respiratory samples is generally considered as colonization [37–40], the possibility that it is an etiologic agent for aspergillosis, including ABPA, cannot be denied.

Two limitations of this study warrant mentioning. First, as histopathological observations were not available for most patients, we could not differentiate between CNPA and other forms of chronic pulmonary aspergillosis, including CCPA. Second, since immunological tests, such as IgG or IgE antibody titer and antigen tests for non- *fumigatus Aspergillus* spp. were not available, isolates other than *A. fumigatus* could not be confirmed as the etiologic agent of disease in chronic pulmonary aspergillosis including ABPA.

In conclusion, the results of our study show that isolation of *Aspergillus* spp. from respiratory samples does not confirm that they represent the etiologic pathogen, because airway colonization by *Aspergillus* spp. is a common feature in patients with chronic lung disease. Even if an *Aspergillus* is isolated in a patient with clinically diagnosed aspergillosis, the colonizing species may not be associated with mycotic disease. The examined clinical, radiological, and microbiological data from 139 patients positive for *Aspergillus* spp. isolates did not allow differentiation between infection and colonization in several cases. It is therefore considered that repeated isolation of the identical *Aspergillus* species and detection of anti-*Aspergillus* antibodies and/or *Aspergillus* circulating antigens in sera are needed to conclude that an isolated species represents the etiological organism in immunocompetent or mildly immunocompromised individuals.

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Genes to Cells

Transcription factors *CgUPC2A* and *CgUPC2B* regulate ergosterol biosynthetic genes in *Candida glabrata*

Minoru Nagi[1,2†], Hironobu Nakayama[3,a†], Koichi Tanabe[1]*, Martin Bard[4], Toshihiro Aoyama[5], Makoto Okano[3], Satoru Higashi[3], Keigo Ueno[6], Hiroji Chibana[6], Masakazu Niimi[1,b], Satoshi Yamagoe[1], Takashi Umeyama[1], Susumu Kajiwara[2], Hideaki Ohno[1] and Yoshitsugu Miyazaki[1]

[1]*Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-Ku, Tokyo 162-8640, Japan*

[2]*Kajiwara Laboratory, Department of Lifescience, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-Cho, Midori-Ku, Yokohama, Kanagawa 226-8503, Japan*

[3]*Department of Chemistry and Biochemistry, Suzuka National College of Technology, Shiroko, Suzuka, Mie 510-0294, Japan*

[4]*Department of Biology, Indiana University-Purdue University Indianapolis, 723 W. Michigan St., Indianapolis, IN 46202, USA*

[5]*Department of Electronic and Information Engineering, Suzuka National College of Technology, Shiroko, Suzuka, Mie 510-0294, Japan*

[6]*Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chuo-Ku, Chiba 260-8673, Japan*

Zn[2]-Cys[6] binuclear transcription factors Upc2p and Ecm22p regulate the expression of genes involved in ergosterol biosynthesis and exogenous sterol uptake in *Saccharomyces cerevisiae*. We identified two *UPC2/ECM22* homologues in the pathogenic fungus *Candida glabrata* which we designated *CgUPC2A* and *CgUPC2B*. The contribution of these two genes to sterol homeostasis was investigated. Cells that lack *CgUPC2A* (*upc2AΔ*) exhibited enhanced susceptibility to the sterol biosynthesis inhibitors, fluconazole and lovastatin, whereas *upc2BΔ*-mutant cells were as susceptible to the drugs as wild-type cells. The growth of *upc2AΔ* cells was also severely attenuated under anaerobic conditions. Lovastatin treatment enhanced the expression of ergosterol biosynthetic genes, *ERG2* and *ERG3* in wild-type and *upc2BΔ* but not in *upc2AΔ* cells. Similarly, serum-induced expression of *ERG2* and *ERG3* was completely impaired in *upc2AΔ* cells but was unaffected in *upc2BΔ* cells, whereas serum-induced expression of the sterol transporter gene *CgAUS1* was impaired in both *upc2AΔ* and *upc2BΔ* cells. These results suggest that in *C. glabrata CgUPC2A* but not in *CgUPC2B* is the main transcriptional regulator of the genes responsible for maintaining sterol homeostasis as well as susceptibility to sterol inhibitors.

Introduction

Sterols are essential membrane lipid components of most eukaryotic cells including fungi. Ergosterol is the major sterol that mainly resides in the plasma membrane of fungal cells. Although many antifungal drugs target enzymes responsible for ergosterol biosynthesis (Ghannoum & Rice 1999; Akins 2005), transcriptional regulation of genes in the ergosterol biosynthetic pathway is not fully elucidated. In *Saccharomyces cerevisiae*, the Zn[2]-Cys[6] binuclear transcription factors, Upc2p and Ecm22p, bind to a DNA sequence motif reported as a sterol regulatory element (SRE) and regulate the expression of several ergosterol biosynthetic genes (Vik & Rine 2001; Davies *et al.* 2005). Upc2p was also implicated in the expression of genes responsible for anaerobic sterol uptake including *DAN/TIR* and *AUS1* encoding mannoproteins and the sterol transporter, respectively (Abramova *et al.* 2001; Wilcox *et al.* 2002). In

Communicated by: Hiroji Aiba
**Correspondence:* ktanabe@nih.go.jp
[a]*Present address*: Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Suzuka, Japan.
[b]*Present address*: Department of Oral Sciences, School of Dentistry, University of Otago, 310 Great King St, Dunedin 9054, New Zealand.

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Candida albicans, the *UPC2* orthologue, *CaUPC2*, was identified as a transcription factor regulating the expression of genes responsible for ergosterol biosynthesis, and its expression was increased by sterol inhibitors as also observed in *S. cerevisiae* (Silver *et al.* 2004; Hoot *et al.* 2008). These observations suggested that the Upc2p/Ecm22p in *S. cerevisiae* and CaUpc2p in *C. albicans* played crucial roles in the regulation of synthesis and uptake of sterols to maintain cellular sterol homeostasis.

The pathogenic fungus *Candida glabrata* is the second most common causative agent of candidiasis, and systemic infections have been linked to the death of immunocompromised patients (Fortun *et al.* 1997; Fidel *et al.* 1999). Sequencing data have showed that *C. glabrata* is more closely related to *S. cerevisiae* than to *C. albicans* (Barns *et al.* 1991), and some genes are functionally exchangeable between *C. glabrata* and *S. cerevisiae* (Kitada *et al.* 1995). Despite the biologic similarity of these two yeast strains, many *C. glabrata* strains are markedly resistant to azole antifungals that inhibit ergosterol biosynthesis in contrast to *S. cerevisiae* and *C. albicans*. Moreover, unlike *S. cerevisiae* cells, the addition of serum or bile *in vitro* resulted in growth recovery of *C. glabrata* even when cells were treated with high concentration of azoles presumably because of exogenous sterol uptake from serum or bile (Nakayama *et al.* 2007). These observations suggested that *C. glabrata* might have evolved its own molecular machinery to maintain sterol homeostasis distinct from that of *S. cerevisiae*.

In this study, the contribution of two *UPC2/ECM22* homologues (*CgUPC2A* and *CgUPC2B*) to the regulation of sterol homeostasis in *C. glabrata* was investigated. Using mutant strains deleted in each gene, the susceptibilities to sterol biosynthetic inhibitors were determined and anaerobic growth tests were carried out. The expression of genes that participate in sterol biosynthesis (*ERG2* or *ERG3*) and sterol uptake (*CgAUS1*) was also evaluated when cells were cultured in the presence of a sterol biosynthetic inhibitor or serum.

Results

Identification of *C. glabrata* genes homologous to *UPC2*

In *S. cerevisiae*, ergosterol biosynthesis and exogenous sterol uptake are regulated by two transcription factors, Upc2p and Ecm22p harboring a Zn-finger motif. To identify *UPC2/ECM22* orthologues in *C. glabrata*, we selected the *S. cerevisiae UPC2* and *ECM22* genes as queries to perform homology searches. A BLAST search against the *C. glabrata* genome (www.genolevures.org/elt/CAG/CAGL) predicted CAGL0C01199g (*CgUPC2A*) and CAGL0F07865g (*CgUPC2B*) to be possible orthologues of *UPC2* or *ECM22*. The amino acid sequences encoded by these two genes are particularly highly homologous to Upc2p and Ecm22p at separate regions, the amino-terminal transcription factor DNA-binding domain and the carboxyl-terminal transmembrane domain (Fig. 1). Synteny analysis of *CgUPC2A* and *CgUPC2B* against the *S. cerevisiae UPC2* and *ECM22* loci indicated that the genes adjacent to *CgUPC2A* or *CgUPC2B* were not highly conserved between *C. glabrata* and *S. cerevisiae*, suggesting that significant genome rearrangements of these regions occurred since separation from a common ancestor (Fig. S1A–D in Supporting Information).

Susceptibility of *CgUPC2*-mutant cells to antifungal drugs

Strains lacking either *CgUPC2A* or *CgUPC2B* were created (*upc2AΔ* or *upc2BΔ*, respectively) as described in Experimental procedures and listed in Table 1. The successful disruption of each gene was confirmed

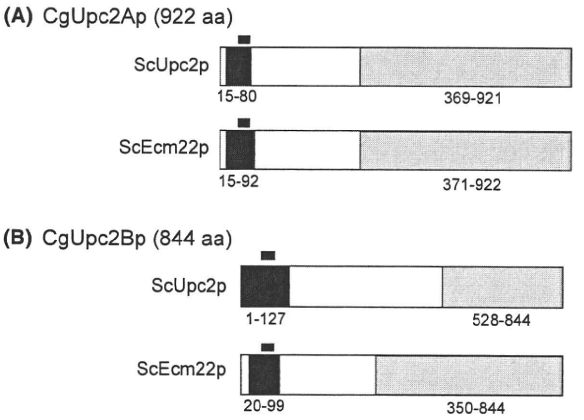

Figure 1 Alignment of *Candida glabrata* and *Saccharomyces cerevisiae* Upc2p/Ecm22p. *S. cerevisiae* Upc2p and Ecm22p (ScUpc2p and ScEcm22p) were aligned with (A) *C. glabrata* Upc2Ap; CgUpc2Ap and (B) *C. glabrata* Upc2Bp; CgUpc2Bp. Regions with highly homologous sequences are labeled. Black boxes indicate amino-terminal homologous regions that include Zn(2)-Cys(6) transcription factor domains indicated by solid bars earlier. The gray boxes indicate carboxyl-terminal transmembrane domains.

by RT-PCR analysis using RNA extracted from *upc2AΔ* or *upc2BΔ* cells (Fig. 5) and by Southern analysis (Fig. S4 in Supporting Information). Susceptibilities of these mutants to two sterol biosynthetic inhibitors (fluconazole and lovastatin) were determined by a liquid microdilution assay (MIC). Fluconazole is an inhibitor of lanosterol 14-alpha demethylase, and lovastatin is a known HMG-CoA reductase inhibitor. Minimum inhibitory concentrations (MICs) were determined for the polyene, amphotericin B, which does not affect sterol biosynthesis but specifically binds to ergosterol, leading to lethal pore formation of fungal cell membranes. *UPC2* disruptions were expected to affect the susceptibility to antifungal drugs that target ergosterol biosynthesis (Vik & Rine 2001; Marie *et al.* 2008). The susceptibility of *upc2AΔ* to fluconazole was increased fourfold (Table 2). A dramatic 16-fold increase in lovastatin susceptibility was also observed for *upc2AΔ*, whereas the MIC of *upc2BΔ* for lovastatin was the same as the parental KUE200 strain. The complementing wild-type allele of *upc2AΔ* (*upc2AΔ/UPC2A*) was as susceptible to the drugs as the parental strain. In contrast, no significant differences in susceptibilities to amphotericin B were observed between any of the strains tested (Table 2).

Table 2 Drug susceptibilities of *Candida glabrata* strains

Strain	MIC_{80} (μg/mL)		
	Fluconazole	Lovastatin	Amphotericin B
Wild type	128	64	4
upc2AΔ	32	4	2
upc2AΔ/UPC2A	64	64	2
upc2BΔ	128	64	2
upc2BΔ/UPC2B	64	128	2

CgUPC2A is required for anaerobic growth

A *C. albicans*-mutant strain in which both alleles of *UPC2* are deleted shows severely attenuated growth under anaerobic conditions (MacPherson *et al.* 2005). We therefore examined the anaerobic growth of *C. glabrata upc2AΔ*- and *upc2BΔ*-mutant cells on CSM and YPD agar medium as described in Experimental procedures. All tested strains grew at similar rates under aerobic conditions, whereas under anaerobic conditions only the growth of *upc2AΔ* was severely attenuated (Fig. 2).

CgUPC2A but not *CgUPC2B* regulates the lovastatin-induced expression of *ERG2* and *ERG3*

Lovastatin treatment results in *S. cerevisiae* cells becoming depleted of ergosterol (Lorenz & Parks 1990). Several *ERG* genes are induced in the presence of this inhibitor to compensate for sterol depletion, and it was previously showed that *UPC2* and *ECM22* redundantly contributed to the lovastatin-induced expression of *ERG2* and *ERG3* in *S. cerevisiae* (Vik & Rine 2001). The expression of *ERG2* and *ERG3* was quantified by RT-PCR using total RNA from lovastatin-treated *upc2AΔ* and *upc2BΔ* mutants of *C. glabrata*. The expression levels of *ERG2* and *ERG3* were not significantly different among all tested strains in the absence of lovastatin (Fig. 3). In the parental KUE200 cells, the addition of lovastatin induced an approximate threefold and fivefold increase in the expression of *ERG2* and *ERG3* (Fig. 3), respectively, as has been also shown in *S. cerevisiae* (Vik & Rine 2001). In contrast, lovastatin treatment was not able to induce the expression of *ERG2* and *ERG3* in *upc2AΔ*

Table 1 Strains used in the present study

Strain	Genotype	References
Candida glabrata		
CBS138	ATCC type culture	
KUE200	*Δtrp1 ::Scura3 Δhis3::ScURA3 Δura3 FRT-YKU80*	Ueno *et al.* (2007)
upc2AΔ	*Δtrp1 ::Scura3 Δhis3::ScURA3 Δura3 FRT-YKU80 upc2A::HIS3*	This study
upc2BΔ	*Δtrp1 ::Scura3 Δhis3::ScURA3 Δura3 FRT-YKU80 upc2B::HIS3*	This study
upc2AΔ/UPC2A	*Δtrp1 ::Scura3 Δhis3::ScURA3 Δura3 FRT-YKU80 upc2A::HIS3-UPC2A-TRP1*	This study
upc2BΔ/UPC2B	*Δtrp1 ::Scura3 Δhis3::ScURA3 Δura3 FRT-YKU80 upc2B::HIS3-UPC2B-TPR1*	This study
Saccharomyces cerevisiae		
W303-1a	*MATaade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100*	

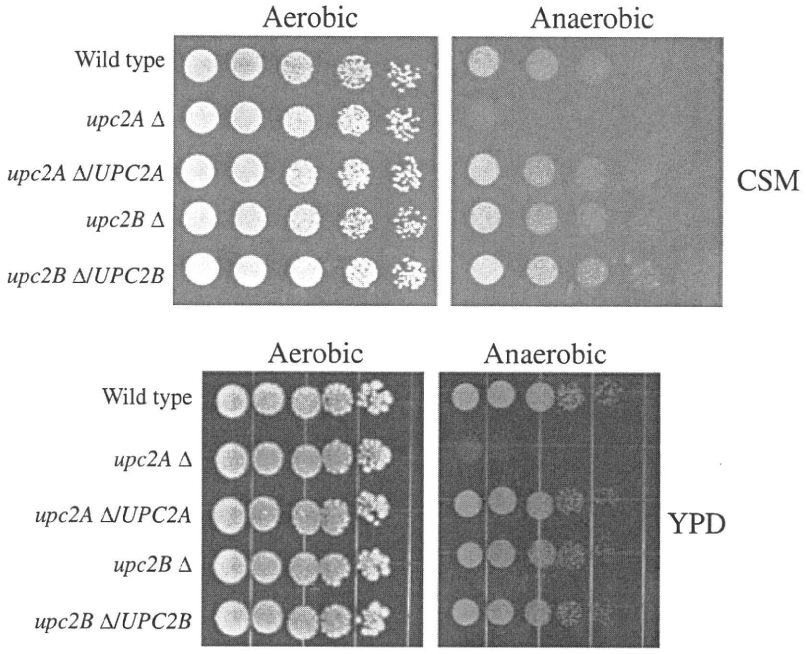

Figure 2 *CgUPC2A* is required for anaerobic growth of *Candida glabrata*. Overnight-grown *upc2*-mutant cells were serially diluted and spotted on CSM or YPD agar plates. The agar plates were incubated at 37 °C for 24 h under aerobic or anaerobic condition.

cells, whereas the induction of these *ERG* genes was unchanged in the *upc2BΔ* strain (Fig. 3). However, the induction of *ERG2* and *ERG3* by lovastatin was restored in the *upc2AΔ/UPC2A* strain.

CgUPC2A contributes to regulated expression of *ERG* genes and *CgAUS1* in the presence of serum

It had been shown that in *S. cerevisiae*, *UPC2* and *ECM22* are regulators of sterol homeostasis including sterol biosynthesis and uptake of exogenous sterol (Vik & Rine 2001; Wilcox *et al.* 2002). Nakayama *et al.* (2007) previously showed that the growth of sterol-depleted *C. glabrata ERG9*-disrupted cells could be rescued by the addition of cholesterol-containing serum. Thus, we hypothesized that serum, which potentially affects the physiology of pathogenic *C. glabrata*, may influence the expression of genes related to sterol homeostasis in *C. glabrata*. The expression of *ERG2*, *ERG3* and *ERG11* was quantified by RT-PCR using total RNA from cells treated with 10% bovine serum. The addition of serum increased the expression of *ERG3* in the parental KUE200 strain (fourfold, Fig. 4B), but only slight increases were observed in the expression levels of *ERG2* and *ERG11* (Fig. 4A,C). The role of *CgUPC2A* and *CgUPC2B* in serum-mediated transcriptional regulation of the *ERG* genes was investigated. A severe repression of these three genes was observed in the *upc2AΔ* strain upon the addition of serum (Fig. 4A–C). The expression of *ERG* genes in the *upc2AΔ/UPC2A* was restored to the levels of KUE200 in the presence of serum.

As *CgAUS1*, which encodes a sterol transporter, was required for the serum-mediated growth of sterol-depleted *C. glabrata* cells (Nakayama *et al.* 2007), the expression of *CgAUS1* was also quantified as described earlier. The addition of serum markedly enhanced the expression of *CgAUS1* to approximately 14-fold in the parental KUE200 strain (Fig. 4D). The serum-induced expression of *CgAUS1* was completely abolished in the *upc2AΔ* strain, and an approximately 70% decrease was observed in the *upc2BΔ* strain. Complementing *CgUPC2A* and *CgUPC2B* of *upc2AΔ* and *upc2BΔ* resulted in the fully restored serum-induced expression of *CgAUS1*, respectively.

Consistently, serum-mediated growth rescue was completely abolished in fluconazole-treated *upc2AΔ* cells. Although the serum-mediated growth rescue of fluconazole-treated *upc2BΔ* cells was also severely impaired, unlike *upc2AΔ*, serum was still able to rescue the growth of fluconazole-treated *upc2BΔ* cells to

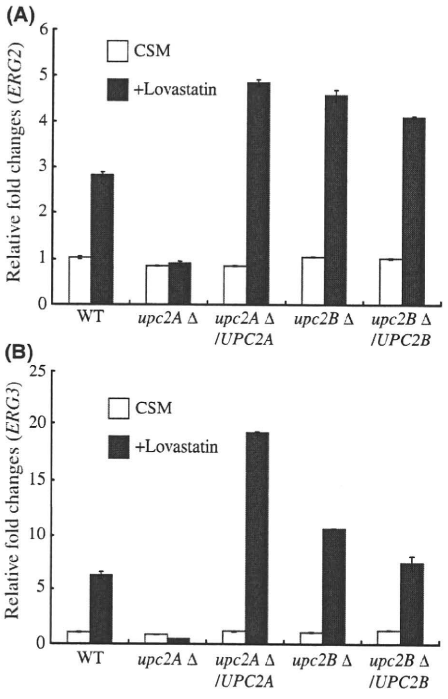

Figure 3 *CgUPC2A* is required for the up-regulation of *ERG2* and *ERG3* in the presence of lovastatin. cDNA was prepared with total RNA samples from 6×10^6 cells incubated with or without 8 μg/mL lovastatin for 4h. Quantitative RT-PCR analysis was performed, and the expression level of each strain was exhibited as relative fold changes compared to wild-type cells incubated without lovastatin. Values are mean and standard deviation of duplicate measurements from a representative experiment. (A) *ERG2*, (B) *ERG3*.

some extent (Fig. 5). These growth defects were restored in *upc2AΔ/UPC2A* and *upc2BΔ/UPC2B*.

CgUPC2A is required for transcriptional regulation of *CgUPC2B* in response to sterol depletion

The expression of *UPC2* and *ECM22* in *S. cerevisiae* was increased and decreased in response to lovastatin, respectively (Davies *et al.* 2005). The expression of *CgUPC2A* and *CgUPC2B* was quantified by RT-PCR using total RNA from *upc2AΔ* and *upc2BΔ* cells in the presence or absence of lovastatin or serum. In wild-type cells, cultivation with lovastatin or serum did not significantly affect the expression level of *CgUPC2A* (Fig. 6A,B), whereas the expression of *CgUPC2B* was enhanced in the presence of lovastatin or serum (Fig. 6C,D). The induction of *CgUPC2B* by lovastatin or serum was abolished in *upc2AΔ* cells (Fig. 6C,D), but the expression level of *CgUPC2A* remained unchanged in *upc2BΔ* cells (Fig. 6A,B).

Discussion

In *S. cerevisiae, UPC2* and *ECM22* were identified as sterol regulatory transcription factors that induce the transcription of genes related to sterol biosynthesis or sterol uptake. In this study, two *C. glabrata* genes (*CgUPC2A* and *CgUPC2B*) homologous to *S. cerevisiae UPC2* were investigated to assess their role in the transcriptional regulation of sterol homeostatic genes.

Drug susceptibilities of the mutants lacking either *CgUPC2A* or *CgUPC2B* showed several lines of evidence that elucidated some of their functional roles. The susceptibility of either *upc2AΔ* or *upc2BΔ* to amphotericin B, which binds to plasma membrane ergosterol, was not different from that of wild-type cells. These results indicate that ergosterol biosynthesis may not be severely impaired in the absence of CgUpc2Ap or CgUpc2Bp under normal growth conditions. Fluconazole and lovastatin deplete yeast cells' ergosterol by inhibiting a late and early step of ergosterol biosynthesis, respectively. The susceptibility of *upc2AΔ* to fluconazole and lovastatin was increased compared to the parental wild-type strain (fourfold and 16-fold, respectively), whereas *upc2BΔ* cells were as susceptible to these drugs as wild-type cells (Table 2). This result suggests that *CgUPC2A* rather than *CgUPC2B* plays a major role in the transcriptional regulation of sterol homeostatic genes under sterol-depleted conditions. Despite their genetic similarity, the disruption of either *UPC2* or *ECM22* in *S. cerevisiae* did not significantly increase its susceptibility to fluconazole or lovastatin, probably because of their functional redundancy. Indeed, a double knockout mutant of both *UPC2* and *ECM22* in *S. cerevisiae* was extremely susceptible to these drugs (Vik & Rine 2001; Marie *et al.* 2008). However, the homozygous deletion of *UPC2* in *C. albicans* resulted in enhanced susceptibility to fluconazole and lovastatin with no change in susceptibility to amphotericin B (Silver *et al.* 2004). Moreover, the *upc2AΔ*-mutant cells alone exhibited severely attenuated growth under anaerobic conditions (Fig. 2) as observed in a homozygous *C. albicans UPC2* (MacPherson *et al.* 2005). Thus, *CgUPC2A* is more functionally similar to *C. albicans UPC2* than either *UPC2 or ECM22* of *S. cerevisiae*.

Lovastatin treatment induced the expression of *ERG2* and *ERG3* in wild-type *C. glabrata* cells, as has been reported in *S. cerevisiae* (Vik & Rine 2001) (Fig. 3). As expected from our drug susceptibility experiments, deletion of *CgUPC2A* in *C. glabrata* attenuated the lovastatin-induced expression of *ERG2* and *ERG3*; however, no significant changes in the

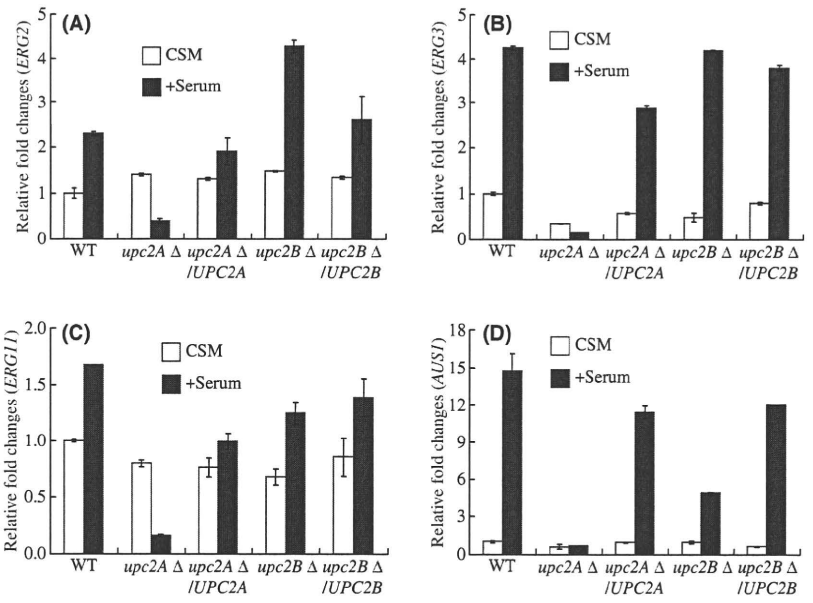

Figure 4 *CgUPC2A* contributes more significantly to the enhanced expression of *ERG* genes or *CgAUS1* in the presence of serum. Total RNA samples were prepared from 6×10^6 cells incubated with or without 10% bovine serum for 4h. Quantitative RT-PCR analysis was performed as shown in Fig. 3. Values are mean and standard deviations of duplicate measurements from a representative experiment. (A) *ERG2*, (B) *ERG3*, (C) *ERG11*, (D) *CgAUS1*.

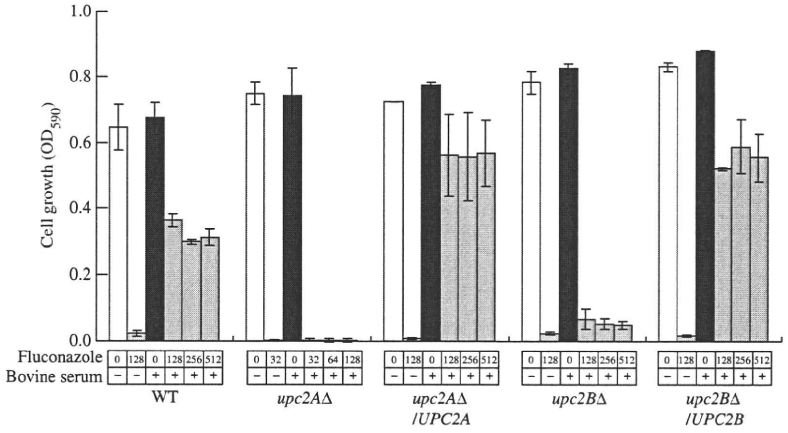

Figure 5 Serum does not promote the growth of either *upc2AΔ* or *upc2BΔ* treated with fluconazole; 6×10^3 cells were inoculated in CSM medium in the presence or absence of serially diluted fluconazole or 10% bovine serum. OD at 590 nm was measured after cultivation at 37 °C for 24 h in the presence of indicated concentrations of drugs. Values are mean and standard deviations of duplicate measurements.

lovastatin-induced *ERG* gene expression could be observed in *upc2BΔ* (Fig. 3). In *S. cerevisiae*, the induction of *ERG2* by lovastatin was severely impaired in *upc2Δ* but not in *ecm22Δ* (Vik & Rine 2001). Our results suggest that *CgUPC2A* in *C. glabrata* as well as *UPC2* in *S. cerevisiae* play major roles in the transcriptional activation of *ERG2* and *ERG3* in response to sterol depletion. It was expected that serum would affect the expression of sterol homeostatic genes, as serum rescued the growth of sterol-

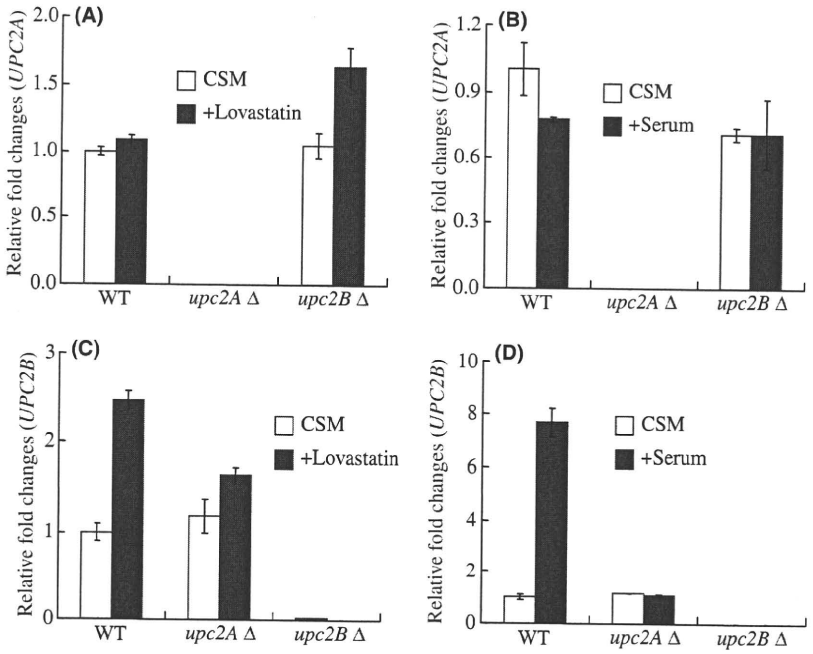

Figure 6 *CgUPC2A* is required for the induction of *CgUPC2B* in response to sterol depletion or serum. *Candida glabrata* cells were incubated in the presence or absence of lovastatin (A, C) or serum (B, D). The expression level of either *CgUPC2A* (A, B) or *CgUPC2B* (C, D) was evaluated by quantitative RT-PCR analysis as shown in Fig. 3. Values are mean and standard deviations of duplicate measurements from a representative experiment.

depleted *C. glabrtata* cells and the amount of cellular sterol was affected by serum (Nakayama *et al.* 2007). The addition of serum promoted the expression of *ERG3* and the sterol transporter *CgAUS1* in *C. glabrata* (Fig. 4B,D), indicating that *C. glabrata* cells up-regulate both ergosterol biosynthesis and sterol uptake in response to serum. One possible explanation is that serum may cause sterol depletion in *C. glabrata* similar to that in lovastatin-treated cells; however, no significant growth defect was observed in the presence of 10% serum, possibly because cholesterol can supplement for the depletion of ergosterol (data not shown). Although most of the genes related to sterol homeostasis are thought to be conserved between *C. glabrata* and *S. cerevisiae*, serum did not induce the expression of *ERG2*, *ERG3* and *AUS1/PDR11* in *S. cerevisiae* (Fig. S2 in Supporting Information), suggesting that serum-induced expression of *ERG* genes and *CgAUS1* may be specific to the pathogenic yeast, *C. glabrata*.

The addition of serum suppressed the expression of *ERG2*, *ERG3* and *ERG11* in *upc2AΔ* (Fig. 4A–C); serum-mediated induction of *CgAUS1* was completely abolished in *upc2AΔ* and markedly decreased in *upc2BΔ* (Fig. 4D). We previously showed that *CgAUS1* is required for serum-mediated growth of sterol-depleted *C. glabrata* cells (Nakayama *et al.* 2007), and we now show that *upc2AΔ* and *upc2BΔ* cells treated with fluconazole in the presence of serum result in no growth or only very modest growth, respectively (Figs 5 and S3 in Supporting Information). Together these results suggest that only the CgUpc2Ap is required for the up-regulation of *ERG* gene expression in response to serum or sterol biosynthesis inhibitors, whereas both CgUpc2Ap and CgUpc2Bp are required for full induction of *CgAUS1* in response to serum and for the growth restoration of fluconazole-inhibited *C. glabrata* cells in response to serum.

The expression levels of *CgUPC2B* but not *CgUPC2A* were up-regulated in the presence of lovastatin or serum (Fig. 6). The induction of *CgUPC2B* was absent in *upc2AΔ*-mutant cells. These results suggest that *CgUPC2A* directly or indirectly regulates the expression of *CgUPC2B* in response to sterol depletion. It was also shown that in *S. cerevisiae* the expression of *UPC2* but not *ECM22* is up-regulated under sterol-depleted conditions (Davies *et al.* 2005).

In conclusion, we present a model in which two putative transcription factors, *CgUPC2A* and

CgUPC2B, have distinct but partly overlapping functions in maintaining sterol homeostasis of *C. glabrata*. *CgUPC2A* regulates the transcription of ergosterol biosynthetic genes under sterol-depleted conditions and plays crucial roles in response to sterol inhibitors. However, both *CgUPC2A* and *CgUPC2B* regulate the genes responsible for exogenous sterol uptake in response to serum. These observations suggest that both *CgUPC2A* and *CgUPC2B* possibly play crucial roles in sterol biosynthesis and exogenous sterol uptake in blood stream infections involving *C. glabrata*.

The sterol regulatory element–binding protein (SREBP), originally found in mammals, is not structurally homologous to *UPC2*, but controls the expression of a wide range of genes related to sterol homeostasis. A pathogenic fungus *Cryptococcus neoformans* containing the *SRE1* gene is homologous to the mammalian SREBP, and deletion of *SRE1* results in enhanced susceptibility to antifungal drugs and decreased pathogenicity (Chang *et al.* 2007). Despite differences in amino acid sequences, similar sterol regulatory transcription factors may be conserved among fungal strains. It will be valuable to analyze and compare sterol regulatory transcription in various fungi to understand how the sterol regulatory gene expression system evolved.

Experimental procedures

Strains and media

Escherichia coli DH5α (F-, φ80, lacZΔM15, Δ(lacZYA-argF) U169, hsdR17(rk− mk+), recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1 λ-) was used for plasmid propagation. Bacterial strains were grown in LB with 50 μg/mL ampicillin. The *C. glabrata* or *S. cerevisiae* strains used in this study are listed in Table 1. The conditional *YKU80* knockout strains, KUE200, were used to generate deletants (Ueno *et al.* 2007). All yeast strains were usually grown in either YPD medium (1% Bacto yeast extract (Difco Laboratories, Detroit, MI, USA) 2% Bacto-peptone (Difco) and 2% glucose) or CSM medium (pH5.8) (0.67% Bacto yeast nitrogen base without amino acids (Difco), and 0.079% CSM complete supplement mixture (Bio101, Vista, CA, USA), 2% glucose and 40 mg/L additional adenine and 60 mg/L additional histidine). Solid media were supplemented with 2% agar (Nacalai Tesque Inc. Kyoto, Japan). Bovine serum was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of *upc2A* and *upc2B* mutants

The DNA fragment used to replace *CgUPC2A* or *CgUPC2B* ORF with *HIS3* was amplified from the plasmid pHIS916 (Ueno *et al.* 2007) using primer sets 1199DF and 1199DR, or 7865DF and 7865DR, respectively. Each amplified fragment (approximately 1 kb) was used to transform KUE200. Disruption of *CgUPC2* genes was confirmed by PCR using primers pTET12F and 1199CHR for *CgUPC2A* or pTET12F and 7865CHR for *CgUPC2B*, respectively. The sequences of all primers are listed in Table S1 (Supporting Information). Integration of DNA fragments into each *UPC2* locus was also confirmed by Southern analysis (Fig. S4 in Supporting Information).

Reintroduction of wild-type alleles into *upc2A* and *upc2B* mutants

DNA fragments harboring *CgUPC2A* or *CgUPC2B* ORF were amplified from the CBS138 genome using primers, 1199compF and 1199compR, or 7865compF and 7865compR, respectively. The amplified fragment containing *CgUPC2A* (approximately 5.0 kb) was digested with *Eco*RI and *Xho*I and yielded a DNA fragment (approximately 3.8 kb) that was cloned into *Eco*RI and *Sal*I sites of pTi-comp (obtained by deleting *CgARS* and *CgCEN* regions from pACT,, Kitada *et al.* 1996). The amplified DNA fragment containing *CgUPC2B* (approximately 4.0 kb) was digested with *Eco*RI and *Sph*I and yielded a fragment (approximately 3.3 kb) that was cloned into the *Eco*RI and *Sph*I sites of pTi-comp. Using these plasmids as the template, insertion fragments were amplified using primers, 1199-Rev-R and 1199-Rev-F, or 7865 Rev-R and 7865 Rev-F, respectively. The resulting DNA fragment (approximately 7 kb) was introduced into the *HIS3* gene-replaced locus on each deletant chromosome by end-in type recombination (Yamana *et al.* 2005). Accurate insertion of each amplified fragment into the correct chromosomal locus was confirmed by PCR using primers 1199KOF and 1199CHF, or 7865DF1 and 7865KOCHR, respectively. The sequences of all primers are listed in Table S1 (Supporting Information). Integration of DNA fragments into the designated locus of each strain was also confirmed by Southern analysis (Fig. S4 in Supporting Information).

Southern blot analysis

Cells grown on 5 mL of YPD medium at 37 °C overnight were collected and resuspended in 400 μL of Lysis buffer (from DNA prep kit) and vigorously shaken with 0.5 g of acid-washed glass bead. Genomic DNA was isolated from the lysate with DNeasy Plant Mini Kit (QIAGEN K.K., Japan) following the manufacturer's instruction, and approximately 4 μg was digested with *Eco*RI. Digests were electrophoresed on a 1% agarose-1× Tris-acetate-EDTA gel and transferred to a nylon membrane (Hybond-N; Amersham). The probe, corresponding to nucleotides −98 to +823 or +180 to +743 relative to the start codon of *HIS3* or *TRP1*, was amplified with the oligonucleotides HIS3f and HIS3r or TRP1f and TRP1r, respectively (Table S1 in Supporting Information).

Sequence analysis

Nucleotide/amino acid sequence analyses and homology searches were performed using the BLAST search, the CLUSTAL W (1.83) multiple/pairwise sequence alignment program and the T-Coffee (3.27) multiple/pairwise sequence alignment program. Synteny was analyzed using our scripts incorporated into Ruby (http://www.ruby-lang.org/) and BioRuby (http://bioruby.org/). The synteny around a targeted gene was analyzed by comparing chromosomal genes near the target gene with adjacent genes close to homologues corresponding to the target gene. The *C. glabrata* homologue was defined as best hit in a BLAST search. The nucleotide sequences were obtained from Genbank (http://www.ncbi.nih.gov/) for *C. glabrata* and *S. cerevisiae*.

Semi-quantitative RT-PCR

Cells grown on YPD medium at 37 °C overnight were inoculated at approximately 1×10^6 cells/mL and cultured for 4 h in CSM medium with and without 8 μg/mL lovastatin or 10% bovine serum. Cells were collected by centrifugation and washed twice with ice-cold water. Crude RNA was extracted as described elsewhere (Hanaoka *et al.* 2008) and digested with DNaseI (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, and total RNA was precipitated with ethanol. cDNA was synthesized with SuperScript III reverse transcriptase (SuperScript III Platinum two-step qRT-PCR kit with SYBR green, Invitrogen). The amount of mRNA was determined by quantitative real-time PCR (qRT-PCR) using ABI Prism 7000 (Applied Biosystems, Carlsbad, CA, USA) and SYBR Premix ExTaq (Takara, Otsu, Japan) and was normalized against 18S rRNA. The PCR conditions consisted of ExTaq HS activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. All primers used for qRT-PCR are listed in Table S1 (Supporting Information). Experiments were performed in triplicate. All experiments were repeated with three independent preparations of RNA and a representative result is exhibited.

Drug susceptibility determination by liquid microdilution assay

Approximately 6×10^3 cells were used to inoculate CSM medium (200 μL) in microtitre plates and then cultured with serially diluted fluconazole (from 0 to 1024 μg/mL; Pfizer Inc., NY, USA). After incubation at 37 °C for 24 h, the OD at 590 nm of the cells was measured. The MIC_{80} is defined as the lowest concentration of drug that inhibited growth yield by at least 80% compared with growth for a no-drug control. Susceptibilities to fluconazole were also examined in the presence of serially diluted bovine serum. Susceptibilities to lovastatin (mevinolin) (from 0 to 128 μg/mL; Sigma-Aldrich) and amphotericin B (from 0 to 32 μg/mL; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were examined as described earlier. CSM medium was chosen for the cultivation of cells, as severe *C. glabrata* growth defects were observed in RPMI1640 media containing serum (human, fetal bovine or bovine). All drug susceptibility tests were repeated twice.

Anaerobic growth test

Cells grown on YPD medium at 37 °C overnight were serially diluted to O.D.s (600 nm) of 0.1, 0.02, 0.004, 0.0008 and 0.000016. Ten microliters of each dilution was spotted on the CSM agar or YPD medium. Anaerobic conditions were obtained with an anaerobic rectangular jar (Mitsubishi Gas Chemical Company, inc.) and Anaero pack-Anaero (Mitsubishi Gas Chemical Company, inc.). Strains were grown at 37 °C for 24 h.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Synteny analysis of *UPC2/ECM22* genes.

Figure S2 Serum does not induce the expression of *ERG2*, *AUS1* and *PDR11* in *S. cerevisiae*.

Figure S3 Checkerboard growth promotion of mutant *C. glabrata* strains by serum.

Figure S4 Southern blot analysis of strains carrying deletion of the *UPC2* genes.

Table S1 Primers used in this study

Additional Supporting Information may be found in the online version of this article.

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Anti-*Candida*-biofilm activity of micafungin is attenuated by voriconazole but restored by pharmacological inhibition of Hsp90-related stress responses

YUKIHIRO KANEKO*, HIDEAKI OHNO*, HIDESUKE FUKAZAWA*, YUKO MURAKAMI*, YOSHIFUMI IMAMURA†, SHIGERU KOHNO† & YOSHITSUGU MIYAZAKI*

**Department of Chemotherapy and Mycosis, National Institute of Infectious Diseases, Tokyo, Japan, and †Department of Molecular Microbiology & Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.*

We have conducted an *in vitro* evaluation of the efficacy of a voriconazole-micafungin combination against *Candida albicans*. When used alone, both micafungin and voriconazole decreased the metabolic activity of planktonic cells, but only micafungin displayed potent anti-biofilm activity. Their combination appeared to have an additive effect against planktonic cells. However, voriconazole significantly antagonized the fungicidal effect of micafungin against *Candida* biofilms. Time-lag experiments showed that pretreatment with voriconazole induced resistance to micafungin in *Candida* biofilms. The micafungin-antagonizing effect of voriconazole persisted even when the biofilm was no longer exposed to voriconazole. In contrast, voriconazole addition after 24 h of micafungin treatment did not alter micafungin sensitivity. To investigate the mechanism of antagonism, we used inhibitors of Hsp90 and its effectors because Hsp90 seems to be implicated in the resistance to micafungin. These molecules reversed the voriconazole-induced resistance to micafungin which suggests that Hsp90-related stress responses are involved in the antagonism. Our results may provide clues as to the mechanism of increased drug resistance in *Candida* biofilms and raises concerns about the use of the voriconazole-micafungin combination in clinical settings.

Keywords *Candida*, biofilm, Hsp90, voriconazole, micafungin

Introduction

The use of long-term implants in clinical practice has been shown to promote colonization and biofilm formation [1]. *Candida albicans* (*C. albicans*) is a pathogen associated with biofilm-related diseases, as well as the primary cause of systemic candidiasis. It is known that the latter has a high mortality rate [2]. Unfortunately, only a limited number of antifungal drugs are available for use in *Candida* infections and the formation of biofilms renders these infections intractable [3]. The common treatment approach in cases of biofilm formation is to remove the implants as soon as possible, but removal is not always possible as is the case with pacemakers or artificial heart valves [4]. Besides, biofilm-related infections may remain unnoticed when they are refractory to intensive antifungal therapy because alternative treatments need to be considered and it is not always possible to confirm the presence of biofilms. Therefore, it is necessary to develop highly effective antifungal drugs against such infections, as well as to utilize existing antifungal agents more effectively.

Combination therapy is a good option in this regard because it is thought to be appropriate for intractable infections [5]. Several new commercially available safe antifungal agents can be considered for combination therapy [6]. As expected, combinations of new azoles and echinocandins have been reported to exert an additive or synergistic effect against most *Candida* species [7]. However, this effect is limited to growth inhibition, and some *in vitro* studies have shown that azoles antagonized the fungicidal effect of echinocandins, especially in biofilms [8–10]. The mechanism underlying this antagonistic effect has never been

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Correspondence: Y. Kaneko, Department of Chemotherapy and Mycosis, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81 (0)3 5285-1111 (2327), fax: +81 (0)3 5285-1272; E-mail: ykaneko@nih.go.jp

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