Review

Promising Therapies for Fungal Infection Based on the Study to Elucidate Mechanisms to Cope with Stress in *Candida* Species

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ABSTRACT

In recent years, the incidence of fungal infections has been increasing, particularly among patients with immune systems compromised by human immunodeficiency virus infection, organ transplantation, and/or chemotherapy for cancer. Current therapies for treating systemic fungal infection have limited effectiveness and have created problems of adverse reactions and drug resistance. These issues therefore motivate us to develop novel antifungals. Elucidation of stress response mechanisms and virulence factors in pathogenic fungi is required in developing an effective antifungal strategy. There are actually numerous studies concerning various stress responses in several important fungal pathogens. Among these responses, we focused on stress response for iron starvation to identify potential targets for novel antifungals because iron starvation occurs in blood, where pathogenic fungi often infect. Here we show recent progress of studies on iron homeostasis in *Candida* species, especially focusing on *Candida glabrata*, and propose novel antifungal targets.

Key words : *Candida*, iron metabolism, iron starvation, molecular targets, stress response

Introduction

Many deep-seated fungal infections can occur as opportunistic infections in immunocompromised patients, but have been steadily increasing due to clinical situation including increase of ageing population and advanced medical treatments. The number of cases where patients have died after contracting these infections is also not insignificant. In general, *Candida* infection is one of the major cases of deep mycoses, *Candida* species is located above the causative agents isolated from patients attacked by septicemia. Invasive aspergillosis, which is caused by the *Aspergillus* genus, has a high mortality rate in advanced immunocompromised patients¹⁾. Cryptococcosis, which is caused by the *Cryptococcus* genus, can also occur in healthy individuals, and can sometimes develop into severe meningitis in immunocompromised patients²⁾. The number of

treatments for fungal infections that are in current use is limited for various reasons including the difficulty of setting a target molecule for eukaryotes (unlike bacteria and the like), and the poor understanding of their infection mechanisms. Furthermore, the use of these therapeutic agents can itself sometimes cause problems including serious side-effects and narrowing of the antifungal spectrum, so the development of therapeutic agents for fungal infections is urgently needed.

Criteria for the selection of a target molecule for an antifungal agent are importance for growth, less homology with humans, and contribution to pathogenicity. In particular, information on the essentiality of fungal growth is the most important among the selection criteria, but since it is necessary to determine the importance in the host body environment, simple *in vitro* culture environments do not provide sufficient information. This should be kept in mind before reaching any conclusions³⁾. Considering the complexity of

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Table 1

Stress from host	Fungi response, etc.
High temperature	
Immune attack	
Oxidative stress	Resistance mechanisms
Low pH	
Protease	
Restricted sources of nutrients	Hyphal formation
	Maintenance of homeostasis (including uptake from external sources)
	Degradation of tissue by hydrolytic enzymes: protease secretion
Drug resistance	Overexpression/mutation of target enzymes
	Expression of drug efflux pumps, etc.

host body environments, it is possible that fungi are subjected to various environmental stresses, including high temperatures, immune attack, and restricted sources of nutrients including ions (Table 1). Therefore, to develop effective treatments, numerous studies are using a variety of fungi to elucidate their response mechanisms under environmental stress⁴⁾. Of the many different stress response mechanisms, the responses to restricted sources of nutrients have a strong association with disseminated infection. In this review, we therefore focused on the stress response mechanism to cope with iron starvation. After showing recent progress of studies in *Candida* species (especially *C. glabrata*) including our results, we propose novel antifungal targets.

Characteristics of *C. glabrata*

C. glabrata is an opportunistic fungal pathogen that causes severe invasive infection, and is the second most frequently species isolated after *C. albicans*. Isolated ratio is 10% for invasive *Candida* and over 20% for blood infection, and patients infected with *C. glabrata* are categorized as poor prognosis⁵⁾. Also, *C. glabrata* is not only a clinically important strain, but it also has highly reliable genomic information^{6, 7)}, resulting in easy genetic manipulation and analysis. Furthermore, infection models including mice and silkworms have been established. With this background, *C. glabrata* can be recognized as one of the most important model organisms for study of pathogenic fungi. Therefore, the studies to clarify the association between stress response mechanisms and infec-

tion mechanisms are in progress, and the reports of various studies on stress responses have already been published. The topics of these studies include the response mechanism to oxidative stress to evade attack from immunocompetent cells⁸⁾, calcineurin signaling pathways associated with various stress responses⁹⁾, and stress response mechanisms of the endoplasmic reticulum¹⁰⁾.

Response to iron starvation in fungi (maintenance of iron homeostasis)

Since iron is used in various reactions including redox reactions in cells, an excess or shortage of iron affects multiple systems including not only the cell metabolism but also the functioning of mitochondria and the integrity of the cell wall. Therefore, maintaining homeostasis of iron concentration is important for the survival of pathogenic fungi as well as other organisms. In particular, the maintenance of iron homeostasis in the stress response to iron starvation is strongly associated with pathogenesis and is therefore receiving a lot of attention¹¹⁾. Although *C. glabrata* is a fungus that infects in the gastrointestinal tract, it can spread from there to the blood vessels in immunocompromised patients (e. g., organ transplant recipients), resulting in disseminated hematogenous infections. Although free iron concentration in the gastrointestinal tract is sufficient for growth, but the concentration in the blood is kept much lower due the action of iron-binding proteins like transferrin and ferritin. Therefore, a response to iron starvation stress is one of the environmental responses it needs to cause disseminated infection. The maintenance of

Fig. 1 Iron acquisition mechanism in *C. albicans*.

iron homeostasis is easier to understand if it is separated into the maintenance of iron uptake and the maintenance of intracellular iron concentration. The following sections present separate discussions of recent trends in the elucidation of molecular maintenance mechanisms, including novel antifungal targets.

Iron uptake in the *Candida* genus

Fungus acquires iron via siderophore transporters (siderophore is a small peptide molecule generated by some bacteria and fungi). Since fungus has hemolytic activity similar that of bacteria, free iron generated by lysis of blood cells was taken by siderophores. It has also been reported the lines of evidence that show the direct iron removal from the host iron binding protein via ferritin, transferrin receptors and hemoglobin receptors¹²⁾. It has been assumed that similar uptake mechanism is conserved in *Candida* species. Therefore, studies to identify the iron uptake mechanism in *C. albicans* are actively carried out¹³⁾ (Fig. 1). However, the factors participating in iron removal from host iron binding proteins have not yet been identified in *C. glabrata*, and only the siderophore transporter the *SIT1* gene has been isolated¹⁴⁾. Also, the ornithine-N5-oxygenase orthologue that plays an essential role in the siderophore biosynthesis pathway has also not been identified¹⁵⁾. Like *C. albicans* and budding yeast *Saccharomyces*

cerevisiae, *C. glabrata* utilizes the siderophores of other fungi (hydroxamate type siderophore) when it takes up iron via Sit1¹⁴⁾. It has been shown that the *sit1*-deleted strain (*sit1* Δ) shows a lower survival rate within macrophages, suggesting that inhibitors of siderophore transporter would be promising antifungal drugs. However, considering *C. glabrata* utilizes siderophore of other fungi, we can not conclude that *C. glabrata* always utilizes a siderophore transporters as a method to acquire iron. Furthermore, other pathogenic fungi including *C. albicans* have multiple iron uptake mechanisms, and study using mouse infection model revealed that siderophore-deleted strain (*sit1* Δ /*sit1* Δ) was not avirulent. On the basis of these results, it is considered that it is not optimal to target only siderophore transporters¹⁶⁾.

Mechanisms to maintain iron concentration in fungal cells

The regulation of iron concentration in fungal cells consists iron uptake/release control, storage in organelles, and changes in metabolism. The studies using *S. cerevisiae* are carried out most actively, showing the most advanced evidence. Therefore, we show here a brief overview of recent studies on iron uptake/release mechanisms in *S. cerevisiae*.

It has been demonstrated that iron uptake is activated and repressed by transcription factors Aft1/Aft2 and Yap5 respectively, resulting in the expression of gene clusters in the corresponding

Fig. 2 Regulation of iron concentration in yeast.

regulons¹⁷. When iron starvation occurs, it is known that Aft1/Aft2 is transported into the nucleus by karyopherin Pse1, which belongs to a family of nuclear transport receptors¹⁸. On the other hand, when there is a high iron concentration in the cell, it has been reported that iron is stored in vacuoles and other organelles via the transporter Ccc1, with Fe-S clusters produced by mitochondria acting as signaling molecules¹⁹. These Fe-S clusters are released from the mitochondria via Atm1, sent to the nucleus after binding with glutathione (GSH) and monothiol glutaredoxin Grx3/Grx4, and bind with Aft1/Aft2 to remove Aft1/Aft2 from the DNA and stop the transcription of gene clusters in the regulon (Fig. 2). It has also been shown that post-transcriptional regulation via mRNA binding protein Cth1/Cth2 works when iron starvation occurs²⁰. The *CTH1* and *CTH2* gene are located in the Aft1/Aft2 regulon, and their transcripts, Cth1 and Cth2, bind mRNAs harboring AU rich mRNA sequence (ARE: AU-rich element) and their binding complexes are digested. The mRNAs harboring ARE encode the proteins related to iron utilization processes that include respiration, the TCA cycle, heme synthesis, amino acid synthesis, sterol and fatty acid synthesis, and the formation of Fe-S clusters by mitochondria. Therefore, Cth1 and Cth2 stop the syntheses of these proteins at the post-transcriptional level, resulting in suppression of

iron utilization. Also, since the mRNAs of the *CTH1* and *CTH2* gene harbor ARE, they also regulate their own transcription, thereby preventing excessive suppression of iron utilization.

Further research is awaited to determine whether or not the above mechanism is conserved in most pathogenic fungi including *Candida*, but according to predictions based on genomic sequences of *C. glabrata*, they have transcription factors similar to *S. cerevisiae* and may participate in metabolic regulation of intracellular iron. Also, as in prokaryotes, since the gene clusters associated with iron metabolism have a regulon structure and are controlled at the transcription level, it is thought that the mechanism is not the same as the post-transcriptional regulation by iron regulatory protein (IRP) in mammals (Fig. 3). From the above consideration, it seems highly likely that inhibitors of Aft1/Aft2 orthologues could be used for the therapy for fungal infections.

Adaptation by metabolic changes due to iron starvation

As described in the former section, iron is utilized in cellular processes including respiration, the TCA cycle, heme synthesis, amino acid synthesis, sterol and fatty acid synthesis, and mitochondrial Fe-S cluster synthesis. Therefore, reducing the concentration of iron in a cell

Fig. 3 Regulation of iron concentration in mammals.

hinders many biological reactions, causing major changes in metabolism. From a transcriptome analysis of *C. glabrata* using a DNA microarray, we focused on the increased expression of *ATG32* in response to iron starvation, and showed that the degradation of mitochondria (mitophagy) is mediated by Atg32²¹. Further study is needed to confirm whether this mitophagy happens because of disfunction of mitochondria due to iron starvation or due to the supply of iron, but since an *atg32*-deleted strains (*atg32* Δ) has lower pathogenicity, it is thought that the inhibition of mitophagy is also one of the targets for antifungal therapy. We also discovered that during iron starvation there are changes in the expression of genes associated with iron metabolism and sterol uptake (Table 2). In fact, iron starvation causes reduce of heme iron, inactivation of the enzyme Erg11 (sterol 14 α demethylase) participating in ergosterol biosynthesis, resulting in ergosterol depletion. As result of this, the cells take in exogenous sterol to rescue sterol depletion (Fig. 4)^{22, 23}. Since serum component such as transferrin functions as iron chelators, this metabolic change is also observed by the addition of serum, and sterol uptake during iron starvation is thought to be related to disseminated infections of *C. glabrata*. It has been confirmed that an ABC transporter-deleted strain, *aus1* Δ (which plays a central role

in the uptake of exogenous sterol), has reduced pathogenicity²³, and sterol uptake during iron starvation has not been so far reported in other *Candida* species²⁴. It is unlikely that sterol uptake is a promising target for antifungal therapy. On the other hand, it is suggested that transcription factor Upc2 (which is activated in response to iron starvation to regulate sterol homeostasis) and its orthologues are associated with pathogenicity and drug resistance even in *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, so we can expect that inhibiting this transcriptional activity could be a target of antifungal therapy. In fact, it has been reported that screening of inhibitors of Upc2 function using *C. albicans* has been in progress^{25, 26}.

Conclusion

In this paper, we showed the stress response to iron starvation, and from the molecular mechanism, as the target molecule of antifungal therapies, we considered transcription factors, proton pump and transporter involved in iron metabolism. Anti-fungal drugs inhibiting function(s) of transcription factors have not developed so far. Therefore, such inhibitors are expected to be novel type of antifungal agents although some issues including their delivery to nucleus or

Table 2

Gene ID	<i>S. cerevisiae</i> homologue	Fold change (-Fe/control) > 3	Putative function
Related to iron homeostasis			
CAGL0H06545g	ATG32	3.18	Mitochondrial-anchored receptor that interacts with the autophagy adaptor protein, Atg11p, and is essential for mitophagy
CAGL0A03905g	HMX1	3.25	ER localized heme oxygenase, involved in heme degradation during iron starvation and in the oxidative stress response
CAGL0M07942g	FRE8	3.34	Protein with sequence similarity to iron/copper reductases, involved in iron homeostasis
CAGL0J07980g	ATX1	3.37	Cytosolic copper metallochaperone that transports copper to the secretory vesicle copper transporter Ccc2p
CAGL0F00187g	FET4	5.05	Low-affinity Fe(II) transporter of the plasma membrane
CAGL0K08756g	YAP5	5.89	Basic leucine zipper (bZIP) transcription factor
CAGL0G06798g	LSO1	8.92	Protein with a potential role in response to iron deprivation
CAGL0H09966g	FMP23	9.85	Putative protein of unknown function; proposed to be involved in iron or copper homeostasis
CAGL0L05258g	SRX1	10.45	Sulfiredoxin, contributes to oxidative stress resistance by reducing cysteine-sulfinic acid groups
CAGL0L11990g	GRX4	12.63	Glutathione-dependent oxidoreductase; hydroperoxide and superoxide-radical responsive
Sterol uptake			
CAGL0F07865g	UPC2/ECM22	3.12	Sterol regulatory element binding protein, induces transcription of sterol biosynthetic genes and of DAN/TIR gene products
CAGL0F01419g	AUS1	3.92	Plasma membrane sterol transporter of the ATP-binding cassette family
CAGL0J03916g	KES1	4.40	Member of the oxysterol binding protein family, which includes seven yeast homologs
CAGL0C03872g	TIR3	6.22	Cell wall mannoprotein of the Srp1p/Tiip1p family of serine-alanine-rich proteins, is required for sterol uptake
CAGL0D00946g	CSR1	6.84	Phosphatidylinositol transfer protein with a potential role in regulating lipid and fatty acid metabolism under heme-depleted condition

Enhanced expression was observed in 34 out of 5,300 genes (ratio of 3 or more in an iron bonding medium/synthesis medium). RNA adjusted from *C. glabrata* ATCC2001 cultured for 4 hours in an iron starved medium (YNB + CSM + Ferrozine) and synthetic medium (YNB + CSM), and subjected to microarray analysis.

Synthetic medium: 0.67% Bacto yeast nitrogen base without amino acids (Difco), 0.079% CSM complete supplement mixture (Bio101, Vista, CA, USA), and 2% glucose.

Iron starved medium: 0.67% Bacto yeast nitrogen base without amino acids (Difco), 0.079% CSM complete supplement mixture (Bio101, Vista, CA, USA), 2% glucose and 60 μ M Ferrozine (23).

Fig. 4 Sterol homeostasis during iron starvation.

cytosol around nuclear should be overcome. In addition, the factors participating in various cellular responses have been conserved between fungi and mammals since fungi are eukaryotes. In fact, drugs such as calcineurin inhibitors (cyclosporine or FK506) and HSP90 inhibitors (geldanamycin) have also been reported to have a certain level of antifungal activity²⁷⁾. Based on these findings, it would be an alternative approach to hunt antifungal activity against the compounds that are developed or have been developed as possible inhibitors of iron starvation response.

Furthermore, in clinical practice, infections are often accompanied by bacterial infections. Responses to iron starvation are also exhibited by bacteria, and in some areas the research into bacteria is more advanced than the research into fungi, including the lines of evidence to show iron acquisition using host factors including catecholamines such as epinephrine or norepinephrine^{28, 29)}. To identify mechanism of fungal iron acquisition using host factor(s) might provide us to uncover conserved mechanism(s) of iron acquisition in "unicellular organisms" including fungi and bacteria. Then, such studies could provide us a chance to develop better therapy for fungal infections. Therefore, it is worth keeping an eye on develop-

ments in the research of bacteria.

Conflict of interest

None.

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