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Outline

Doctoral thesis

Identification and functional characterization of drug:H^+ antiporters of an endemic pathogen *Penicillium marneffei*

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THESIS OUTLINE

INTRODUCTION

Penicillium marneffei (*Talaromyces marneffei*) is the only thermally dimorphic fungus that can switch the cell shape from mycelia at 25°C to yeast at 37°C. The opportunistic infections caused by this species called penicilliosis are common in immunocompromised patients and has been found endemic in tropical Asia. The combination therapy of amphotericin B followed by itraconazole or voriconazole are effective, however, prolonged prophylaxis with azoles could cause the emergence of drug-resistance strains. Various mechanisms are involved in azole resistance. Overexpression of drug efflux pumps that presumably catalyze the efflux of a broad range of cytotoxic substrates play an important role in multidrug resistance (MDR) processes.

1. Identification and classification of major facilitator superfamily (MFS) family of *P. marneffei*

To date, MDR transporters have been identified in three kingdoms (eukaryotes, prokaryotes, and archaea) and have been classified into two types of transport systems, the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS). The role of ABC transporters has been well characterized in the past decades, however much less attention in MFS transporters. According to transporter classification database (TCDB), MFS transporter superfamily is classified into 88 subfamilies in living organisms. *P. marneffei* has 17 subfamilies of those five are considered as major and twelve are minor MFS subfamilies. Five major MFS subfamilies include sugar porter (SP), drug:H^+ antiporter 1 (DHA1), drug:H^+ antiporter 2 (DHA2), monocarboxylate transporter (MCT) and anion cation symporter (ACS). Twelve minor subfamlies include fucose symporter family (FHS), phospate:H^+ symporter (PHS), oxalate:formate antiporter (OFA), siderophore-iron transporter (SIT), two types of vacuolar basic amino acid transporter (VBAAT and V-BAAT), peptide/acetyl-coenzyme A/drug transporter (PAT), feline leukimia virus C receptor (FLVCR), L-amino acid transporter-3 (LAT-3), N-acetylglucosamine transporter (NAG-T), unidentified major-facilitator-12 (UMF12) and -23 (UMF23). Among MFS transporters, drug:H^+ antiporter (DHA) transporter has been proved to have a clinical importance such as *CaMDR1* from *Candida albicans*. It makes this transporter become interesting candidates for the development of new antifungal drugs. Thus, identification and characterization of *P. marneffei* MFS superfamily, specifically related to DHA subfamilies are important.

Based on NCBI databank, *P. marneffei* has 346 proteins, 256 putative (known function) and 90 hypothetical (unknown function) proteins predicted to belong to the MFS superfamily. Out of 256 putative proteins, 177 proteins were predicted to contain the 12 or 14 transmembrane segments (TMSs) that are typically seen in MFS transporters. DHA1 subfamily that possess 12 TMSs has 39 proteins, and DHA2 subfamily that possess 14 TMSs has 16 proteins.

2. Functional characterization of drug:H+ antiporter 1 (DHA1) of *P. marneffei*

Three proteins from DHA1 subfamily, *PmMDR1*, *PmMDR2*, and *PmMDR3*, were selected according to their high similarity to the characterized protein such as *CaMDR1* and *ScTPO* from *Saccharomyces cerevisiae*. The gene expression levels of all proteins were accumulated to higher levels in the presence of 0.001 μg/mL itraconazole (ITC) and expression fell in the presence of a higher concentration of ITC (0.002 μg/mL), except *PmMDR1* transcript accumulated to higher levels, with the mRNA levels exhibiting an apparently dose-dependent increase. It is suggesting that the *PmMDR* proteins may contribute to protecting cells from toxic substrates, such as azoles. All of protein were cloned and subjected to heterologous overexpression in a transporter-deficient strain of *S. cerevisiae* (ADΔ) to analyze potential

contributions to MDR. This strain is a suitable host to express and functionally characterize membrane proteins including transporters. Overexpression of efflux pumps made ADΔ highly resistant, since this strain had seven major ABC transporter genes deleted. The gene inserted into PDR5 locus will be overexpressed due to the gain of function mutation (PDR1-3) which upregulates gene expression through PDR5 promoter. All *Pm*MDR proteins conferred MDR at various levels when produced in *S. cerevisiae* ADΔ. *Pm*MDR1 conferred the broadest resistance to various drugs, including fluconazole (FLC), ketoconazole (KTC), voriconazole (VRC), posaconazole (POS), nystatin (Nys), micafungin (MFG), terbinafine (TRB), nourceothricine (Nour), 5-fluocytocine (5FC), cycloheximide (CHX), G418, and quinidine (Quin). *Pm*MDR2 conferred modest resistance to KTC, POS, R123, 5FC, G418, Quin, and mechopenolic acid (MPA). The drug-resistance phenotype of *Pm*MDR3 was narrower than *Pm*MDR1, including cerulenin (CER), R123, Quin, CHX, 5FC, POS, MFG, G418, MPA, miconazole (MCZ), VRC, and FLC. The substrate specificity of *Pm*MDR3 was similar to that of *Ca*MDR1, aside from minor differences in susceptibility to MFG and CER.

3. Functional characterization of drug:H+ antiporter 2 (DHA2) of *P. marneffei*

One protein from DHA2 subfamily *PmMDR4* were selected according to their high similarity to the characterized protein such as *CaMDR1*. The gene expression levels of this protein were accumulated to higher levels in the presence of 0.001 μg/mL itraconazole (ITC) and expression fell in the presence of a higher concentration of ITC (0.002 μg/mL). This protein was cloned and subjected to heterologous overexpression in a transporter-deficient strain of *S. cerevisiae* (ADΔ) to analyze potential contributions to MDR. The functional characterization of *Pm*MDR4 appeared to be an efficient efflux pump to 5FC and substrate specificity of *Pm*MDR4 was similar to *Ca*MDR1 to recognize MCZ, POS, MFG, G418, Quin, and MPA.

CONCLUSIONS

In this study, *Pm*MDR1, *Pm*MDR2, *Pm*MDR3 of DHA1 subfamily and *Pm*MDR4 of DHA2 subfamily suggested to have a function as multidrug efflux pumps at different degrees and might play an important role in drug resistance of *P. marneffei*. The strains constructed here, in particular *Pm*MDR1, are expected to find use as tools for screening of pump inhibitors. Such inhibitors might by employed in combination therapy with existing antifungal agents such as azoles to prevent DHA transporter-mediated drug resistance.