Construction and analysis of the cDNA subtraction library of yeast and mycelial phases of *Sporothrix globosa* isolated in China: identification of differentially expressed genes*

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Abstract: Species included in the *Sporothrix schenckii* complex are temperature-dependent with dimorphic growth and cause sporotrichosis that is characterized by chronic and fatal lymphocutaneous lesions. The putative species included in the *Sporothrix* complex are *S. brasiliensis*, *S. globosa*, *S. mexicana*, *S. pallida*, *S. schenckii*, and *S. luriei*. *S. globosa* is the causal agent of sporotrichosis in China, and its pathogenicity appears to be closely related to the dimorphic transition, i.e. from the mycelial to the yeast phase, it adapts to changing environmental conditions. To determine the molecular mechanisms of the switching process that mediates the dimorphic transition of *S. globosa*, suppression subtractive hybridization (SSH) was used to prepare a complementary DNA (cDNA) subtraction library from the yeast and mycelial phases. Bioinformatics analysis was performed to profile the relationship between differently expressed genes and the dimorphic transition. Two genes that were expressed at higher levels by the yeast form were selected, and their differential expression levels were verified using a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). It is believed that these differently expressed genes are involved in the pathogenesis of *S. globosa* infection in China.

Key words: *Sporothrix globosa*, Dimorphism, Suppression subtractive hybridization (SSH), Real-time PCR

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1 Introduction

Species included in the *Sporothrix schenckii* complex are the etiological agents of sporotrichosis, which is characterized by acute or chronic lymphocutaneous lesions that can develop into disseminated disease in immunocompromised individuals (Yap, 2011). The *S. schenckii* complex includes *S. brasiliensis*, *S. globosa*, *S. mexicana*, *S. pallida*, *S. schenckii*, and *S. schenckii luriei* (Marimon et al., 2006; 2007; Madrid et al., 2010). Different species of *Sporothrix*

are distributed in various regions of the world (Marimon et al., 2007; Zhou et al., 2013). For example, *S. brasiliensis* is found only in Brazil, and *S. schenckii* is present mainly in the Americas, Africa, and parts of Asia. Different clinical forms of sporotrichosis are related to the condition of the host, route of infection, and virulence of the pathogen, and virulence may be associated with different *Sporothrix* species (Arrillaga-Moncrieff et al., 2009; Fernandes et al., 2013). Previous studies have shown that *S. globosa* is the causal agent of sporotrichosis in China (Tan et al., 2013; Yu et al., 2013; Liu et al., 2014).

Sporothrix includes pathogenic fungi that undergo dimorphic growth from mycelial to yeast phases in response to environmental conditions, such as temperature. In particular, the fungi grow in the

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filamentous form at 25 °C and in the yeast form at 37 °C. Sporotrichosis is caused by the multiplication of the yeast form (Li *et al.*, 2007). Its pathogenicity appears to be closely related to the dimorphic transition from the mycelial to the yeast phase in humans through an unknown mechanism.

Several genes that are differentially expressed during the transition from yeast to mycelial forms have been identified. For example, protein kinase C (PKC) (Aquino-Pinero *et al.*, 1997), Pho85s (de Jesus-Berrios *et al.*, 2002), and calcium/calmodulin (Valle-Aviles *et al.*, 2007) may be involved in the regulation of dimorphism. Further, the cPLA2 homolog (Valentin-Berrios *et al.*, 2009) and a 45-kDa protein (Han-Yaku *et al.*, 1996) are involved in the dimorphic transition of *S. schenckii*. However, the mechanism of dimorphic transition is complicated and unclear. Although signal transduction modulated by factors such as mitogen-activated protein kinases, cyclic adenosine monophosphate (cAMP), and pH-responsive modules may regulate this transition, the mechanism of the dimorphic transition remains undetermined. Further, the species included in the *S. schenckii* complex may harbor different genes that mediate the dimorphic transition. Therefore, studies of the mechanism of the dimorphic transition are required to understand the mechanism of the pathogenesis of *S. globosa*.

To identify differentially expressed genes during the dimorphic transition of *S. globosa*, suppression subtractive hybridization (SSH) was used. This is a powerful method for identifying differentially expressed genes in fungi (Marques *et al.*, 2004), plants (Zeng *et al.*, 2006), and tumors (Ma *et al.*, 2005). In the present study, differentially expressed sequence tags (ESTs) were isolated using SSH and bioinformatics analysis, and two genes were selected and identified using a relative quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). These two genes reside within the mycelial (M)+yeast (Y)contig63 encoding structural genes and the M+Ycontig5 encoding cell-surface molecules.

2 Materials and methods

2.1 Strains and culture conditions

The standard *S. globosa* strain (C26471) was acquired from the Dermatology Department of the

First Affiliated Hospital, Chongqing Medical University (Chongqing, China). The strain was identified as *S. globosa* according to its morphology and internal transcribed spacer (ITS) region (GenBank No. JX997727) (Liu *et al.*, 2014). The cells were cultured in the mycelial phase on a freshly prepared potato dextrose agar (PDA) plating medium at 25 °C, and yeast-phase cells were obtained by culturing mycelial colonies for 7 d at 37 °C on a freshly prepared brain heart infusion (BHI) solid medium supplemented with 5 g/L glucose. Continuous culture for three generations insured stable and pure yeast cells.

2.2 Construction of a complementary DNA (cDNA) subtraction library

Approximately 200 mg of mycelia (M) and yeast (Y) cells were extracted and purified directly using an Easy Spin Total Plant RNA Extraction Kit (Aidlab, China) according to the manufacturer's instructions. The quality and quantity of total RNA preparations were evaluated using 1% (0.01 g/ml) agarose gel electrophoresis. The cDNA was synthesized and amplified using the SMART cDNA Amplification Kit (Clontech, USA) with 1.0 µg total RNA prepared from mycelial and yeast forms. The SSH assay was performed using the PCR-Select cDNA Subtraction Kit (Clontech, USA) following the manufacturer's instructions. Briefly, the mycelial and yeast cDNAs were digested with *RsaI* to generate cDNAs with blunt ends. Adaptors were ligated to tester cDNAs followed by two rounds of subtractive hybridization, two rounds each of PCR amplification, and product purification. Each product designated "M+Y" (cDNAs from yeast as tester and cDNAs from mycelium as driver) and "Y+M" (mycelium as tester and yeast as driver) was isolated using SSH according to the method of Diatchenko *et al.* (1996). The "M+Y" and "Y+M" cDNAs were inserted into the pGEM (Promega, USA) plasmid vector and used to transform *Escherichia coli* TOP10, which was cultured on Luria broth (LB) agar plates with ampicillin, isopropyl-β-D-thiogalactopyranoside, and X-gal (Autolab, China). Recombinant bacteria were selected from white colonies, which were inoculated in 50 µl of LB containing 100 mg/L ampicillin and incubated at 37 °C for 5 h. The DNA of 1 µl of each LB culture was amplified in 25 µl of PCR reaction mixture using nested PCR primers T7 and SP6 (Clontech PCR-Select cDNA Subtraction Kit) in a 96-well plate. PCR was

performed as follows: 75 °C for 7 min, 20 cycles of 91 °C for 30 s, 68 °C for 30 s, and 72 °C for 2.5 min. The PCR product (10 µl) was analyzed using 2% (0.02 g/ml) agarose gel electrophoresis. The subtractive cDNA libraries were constructed and named the “M+Y” and “Y+M” libraries.

2.3 DNA sequence analysis

Positive colonies with inserts >250 bp and a single strip from two subtraction libraries were selected for sequence analysis to identify ESTs using forward primers T7 or SP6 and an ABI-9600 DNA sequencing system. Data processing of ESTs was performed by masking vector and repetitive sequences and by eliminating chimeric, contaminating, and poly(A) sequences. Consensus sequences were obtained by assembling ESTs and performing cluster analysis. We used the longest predicted open reading frame (ORF) of each unique gene. The differentially expressed sequences were used to query the National Center for Biotechnology Information (NCBI) databases using BLASTN (<http://www.ncbi.nlm.nih.gov/BLASTN>), BLASTP (<http://www.ncbi.nlm.nih.gov/BLASTP>), SwissPort (<http://www.expasy.org/sport/BLASTX>), Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/BLASTP>), Clusters of Orthologous of proteins (COG; <http://www.ncbi.nlm.nih.gov/COG/BLASTX>), InterPro (<http://www.ebi.ac.uk/interpro/Interproscan>), and Gene Ontology (GO; <http://www.geneontology.org> or <http://www.ebi.ac.uk/GO/index.html>). Differentially expressed genes were annotated and classified according to the function.

2.4 Relative quantization of mRNA expression using real-time RT-PCR

According to the results of the bioinformatics analyses described above, two genes were selected for further analysis using real-time RT-PCR. The mRNA

encoding β -tubulin was selected as the internal control. Primers for target genes were designed and synthesized by Beijing Genomics Institute (BGI, China) as shown in Table 1, and 700 ng of total RNA from mycelial and yeast phases of *S. globosa* were reverse-transcribed using the Prime Script RT Kit (TaKaRa, Japan). cDNAs (5 µl) were diluted 1:3 (v/v) with sterile water for use as the targets of RT-PCR. Sixteen nanograms of cDNAs were assayed from the mycelial and yeast phases of *S. globosa* in duplicate using SYBR Premix EX Taq II (TaKaRa, Japan). Control reactions without a template were performed in duplicate. Amplification was performed using a C1000 Thermal Cycler Dice Real-time system (Bio-Rad, USA) as follows: 95 °C, 30 s; 40 cycles at 95 °C for 5 s and 60 °C for 60 s. Melting of the PCR products was performed at 60 °C.

3 Results

3.1 Sequence analysis of differentially expressed genes

Colonies with inserts ranging from 400 to 1000 bp were sequenced. We obtained 751 ESTs from the “M+Y” library (average 690.98 bp) and 875 ESTs were obtained from the “Y+M” library (average 575.9 bp). After deleting ESTs, 101 unique genes, including 52 contigs and 49 singlets, were obtained from the “M+Y” library and 249 unique genes, including 109 contigs and 140 singlets, were obtained from the “Y+M” library. There were 35 and 91 gene sequences in the “M+Y” and “Y+M” libraries, respectively, that are present in the NCBI nucleotide sequence database. Their putative identities, nucleotide sequence similarities with other sequences, and insert sizes are shown in Tables 2 and 3. The distribution of differentially expressed genes varied with

Table 1 Real-time RT-PCR primers and probe sequences

Target cDNA	Primer	Primer sequence (5'→3')	T_m (°C)	PCR product size (bp)
β -tubulin	BTI	GGTAACCAAATCGGTGCTGCTTTC	60.40	120
	BTII	ACCCCTCAGTGTAAGTACCCTTGCC	63.82	
M+Ycontig63	63I	CCCATCAAACCTGCTCAGAAA	60.00	150
	63II	GCACTGCTACTGTCCGTTTG	58.00	
M+Ycontig5	5I	AGCCGTCGAAAGTTGATAGG	60.00	143
	5II	CGACTCGGAAGGGATGTAT	60.00	

T_m : annealing temperature used in real-time RT-PCR with each primer pair

the type of dimorphic transition. Upregulated genes were divided into those encoding structural proteins, metabolic enzymes, cell-surface proteins, and molecules with unknown function. Approximately 40% of the EST sequences were similar to the coding regions of proteins involved in protein synthesis, including several ribosomal proteins. The remaining ESTs were related to genes involved in transcription (about 20%), metabolism (20%), and signaling (20%). Two genes were selected and identified using relative quantification real-time RT-PCR. These genes were present

in M+Ycontig63 and M+Ycontig5 and encoded structural and cell-surface molecules, respectively.

3.2 Relative quantification of gene expression using real-time RT-PCR

To enhance the reliability of SSH findings, the expression levels of two genes in two forms were analyzed using real-time RT-PCR. The results are shown in Table 4. Two genes (M+Ycontig5, M+Ycontig63) present in the “M+Y” library were expressed at higher levels in the yeast phase.

Table 2 Partial gene fragments preferentially expressed in the “M+Y” library of *S. globosa*

Clone name	Size (bp)	BLAST database match	Identity (%)	Score	E-value
M+Ycontig5	569	CHK1 checkpoint-like protein (<i>Helicoverpa armigera</i>)	98	959	0
M+Ycontig12	431	<i>Sporothrix schenckii</i> isolate CDM 18 28S ribosomal RNA gene	100	272	10 ⁻⁶⁹
M+Ycontig44	855	<i>Leptographium brachiatum</i> internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	99	422	10 ⁻¹¹⁴
M+Ycontig46	609	<i>Paenibacillus polymyxa</i> strain PKB1 glutamine amidotransferase	97	1019	0
M+Ycontig63	905	<i>Ophiostoma stenoceras</i> 18S small subunit ribosomal RNA gene	100	1130	0
M+Ycontig68	638	<i>O. stenoceras</i> 12S small subunit ribosomal RNA gene	98	153	10 ⁻³³

Table 3 Partial gene fragments preferentially expressed in the “Y+M” library of *S. globosa*

Clone name	Size (bp)	BLAST database match	Identity (%)	Score	E-value
Y+Mcontig4	352	<i>Bacillus polymyxa</i> strain PW2 large subunit 23S ribosomal RNA gene	98	571	10 ⁻¹⁶⁰
Y+Mcontig7	492	pG1 protein (<i>Lactobacillus jensenii</i> 269-3)	99	842	0
Y+Mcontig19	247	<i>Paenibacillus pabuli</i> partial 16S rRNA gene, partial 23S rRNA gene and ITS1, strain US132	98	321	10 ⁻⁸⁴
Y+Mcontig20	562	pG1 protein (<i>L. jensenii</i> 269-3)	98	920	0
Y+Mcontig26	715	Uncultured bacterium 16S ribosomal RNA gene	99	1273	0
Y+Mcontig119	496	<i>Paenibacillus</i> 16S ribosomal RNA gene, partial sequence	99	860	0
Y+Mcontig156	559	<i>Paenibacillus</i> sp. Fars-87 16S ribosomal RNA gene, partial sequence	100	793	0
Y+Mcontig161	625	<i>P. polymyxa</i> strain PKB1 glutamine amidotransferase	97	1007	0

Table 4 Relative abundance of differential gene expression determined using real-time RT-PCR

Clone name	Phase ^a	Target C_T	Tublin C_T	ΔC_T ^b	$\Delta\Delta C_T$ ^c	$2^{-\Delta\Delta C_T}$ [*]	RA ^d
M+Ycontig63	M	27.58±0.16	25.48±0.11	2.10±0.04	0.00±0.04	1	
	Y	22.48±0.23	24.42±0.01	-1.94±0.23	-4.04±0.23	16.45	↑16.45
M+Ycontig5	M	24.89±0.08	25.48±0.11	-0.59±0.03	0.00±0.03	1	
	Y	12.77±0.48	24.42±0.01	-11.65±0.49	-11.06±0.49	2134.97	↑2134.97

^a M and Y represent the mycelial and yeast forms, respectively. ^b ΔC_T =target transcript C_T - β -tubulin C_T (normalization of C_T for target gene relative to β -tubulin C_T). Statistical analysis of normalized expression levels between mycelial and yeast forms. The levels of each target genes differed significantly (U -test, $P<0.05$). ^c $\Delta\Delta C_T$ =mean $Y\Delta C_T$ -mean $M\Delta C_T$; the mean value for the $M\Delta C_T$ was used as a calibrator to set the baseline for comparing the mean differences in the ΔC_T values of the yeast form. ^{*} Normalized target amount relative to mycelial form. ^d The relative abundance of the corresponding mRNA expressed by the yeast form compared with that of the mycelial form. “↑” and “↓” indicate up-regulation and down-regulation of gene expression by the yeast form compared with that of the mycelial form, respectively

4 Discussion

Sporotrichosis is one of the most common diseases caused by deep fungal infection. The symptoms of sporotrichosis include localized nodular lesions, bumps, and chronic ulcers at the point of entry through small cuts and abrasions in the skin as well as along lymph nodes and vessels (Schubach *et al.*, 2008). The incidence of sporotrichosis is increasing worldwide, particularly in regions with tropical and temperate climates (Schubach *et al.*, 2008). For example, the incidence of sporotrichosis in the South Central Highlands of Peru ranges from 48 to 60 cases per 100 000 persons and is the highest among children aged 7–14 years, approaching one case per 1000 persons (Pappas *et al.*, 2000). The annual incidence of sporotrichosis (6.2%) in Jilin Province in China is relatively high (Wang *et al.*, 1998), and sporotrichosis represents approximately 2.2% of 10 982 cases of dermatomycosis in the Chengdu area of China (Rang *et al.*, 1999).

The genotypes of *S. schenckii* are diverse (Mesa-Arango *et al.*, 2002). For example, de Beer (2003) discovered that *S. schenckii* represents more than one species, and Marimond (2007) identified at least six putative species, including four clinically relevant species as follows: *S. schenckii sensu stricto*, *S. brasiliensis*, *S. globosa*, and *S. lutei*. *S. globosa* causes sporotrichosis in China, and genes encoding proteins such as SSCMK1 and PhoSs differ from those identified using a cDNA subtraction library. Therefore, to address this urgent public health issue in China, in particular, it is important to identify the genes that determine pathogenicity during the transition from the mycelial to the yeast form of *S. globosa*.

To address this issue, the SSH technique was employed to identify genes differentially expressed during the transition from the hyphal to the yeast form. The M+Ycontig5 was the most highly represented SSH sequence and was differentially expressed by a factor of 2134.97 by the yeast form. The predicted amino acid sequence of the M+Ycontig5 is 98% similar to that of the cell cycle checkpoint kinase (CHK1)-like protein of *Helicoverpa armigera*. CHK1 is a serine/threonine protein kinase, evolutionarily conserved, and plays pivotal roles in DNA repair, cell cycle arrest, and in inducing cell death (Chen *et al.*, 2004). For example, entry into mitosis is inhibited by

the activation of CHK1 kinase in response to damaged DNA in late S and G2 phases of the cell cycle, which protects fungi against attack from macrophages or neutrophils (Martinho *et al.*, 1998; Furuya *et al.*, 2010). Furuya and NiKi (2010) found that activation of CHK1 is sufficient to induce hyphal differentiation of *S. japonicus*, which promotes survival in the continuously changing nutritional environment and imparts motility upon this otherwise nonmotile organism (Sipiczki *et al.*, 1998). Thus, the CHK1 signaling pathway arrests the cell cycle in response to checkpoint activation and promotes hyphal differentiation to avoid or mitigate sources of stress. Su and Cheng (2013) studied *Candida albicans* and found that CHK1 affects the reproduction and formation of chlamydospores and hyphae and mediates tolerance to environmental stressors. The present findings support the hypothesis that the M+Ycontig5 might encode a specific entry point required for the dimorphic transition.

The sequence of the M+Ycontig63 is identical to that of the gene encoding the 18S small subunit ribosomal RNA of *Ophiostoma stenoceras*, and its expression was increased by a factor of 16.45 in the yeast form of *S. schenckii*. *O. stenoceras* is an ophiostomatoid fungus, which is a teleomorph of secondary conidia on substrate mycelium, and is included in the *S. schenckii*–*O. stenoceras* complex (de Meyer *et al.*, 2008). Moreover, evidence indicates that 18S and 28S RNAs (Fleischmann *et al.*, 2004) mediate the transient upregulation of RNA polyadenylation, suggesting a possible role in regulating the polymorphic phenotype of *C. albicans*. The data presented here suggest that 18S and 28S rRNA genes may be expressed in the mycelial and yeast forms of *S. schenckii*.

In summary, the results of the present study suggest that the dimorphic transition of *S. schenckii* is mediated through complex mechanisms. The expression levels of differentially expressed mRNAs identified here may be required for the dimorphic transition and pathogenicity. The transcription of these genes might be induced by HSP70 (Huang *et al.*, 2007).

Quantitative comparisons using β -tubulin to normalize expression levels were difficult. One disadvantage is the dependence on a standard that is expressed at variable levels. Despite the advances in

studying the molecular genetics of *S. schenckii*, progress is restricted, in part, due to the absence of a completely sequenced and annotated genome. The physiological functions of these genes require further study.

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Compliance with ethics guidelines

Qing-bi HU, Yu HE, and Xun ZHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题目: 中国球形孢子丝菌双相 cDNA 消减文库构建及其差异基因的筛选和验证

目的: 为进一步研究中国球形孢子丝菌的双向转换机制,明确目的差异基因对我国球形孢子丝菌形态和双相转换的影响,探讨可能参与的信号传导通路,以期较全面地探讨孢子丝菌双相转换相关的分子机制,也为筛选抗真菌药物研究提供新的靶位和切入点。

创新点: 以中国球形孢子丝菌双向转换为立足点,应用抑制性消减文库成功构建的高特异性的中国球形孢子丝菌酵母相(Y)和菌丝相(M)的正反 cDNA 基础上,对中国球形孢子丝菌双相转换相关基因进行生物信息学分析并对部分有意义的差异表达基因进行验证。

方法: 本实验通过抑制性消减杂交技术构建中国球形孢子丝菌酵母相和菌丝相的正反 cDNA 消减文库,并对差异基因进行生物信息学分析,获得部分有意义的差异表达基因,再通过实时荧光聚合酶链反应(PCR)技术验证目的基因在双相中的差异表达。

结论: 通过抑制性消减杂交技术对中国球形孢子丝菌的正向和反向两个文库进行筛选,获得可能参与球形孢子丝菌细胞壁合成、信号转到、新陈代谢类等相关的片段重叠群(contigs),结果表明中国球形孢子丝菌在双相型转换中存在明显表达差异基因,同时利用相对定量的实时荧光定量技术对其中两个基因(细胞表面分子类基因细胞周期检测点激酶 CHK 和结构基因类核糖体 RNA 基因)进行验证,认为它们可能与双相转换有关。

关键词: 球形孢子丝菌;双相;抑制性消减杂交技术(SSH);实时荧光 PCR 技术