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白色念珠菌之致病基因的鑑定

Identification of Virulence Genes of *Candida albicans*

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一、中文摘要

黴菌感染在近十年已爲院內感染的主要病原菌之一。黴菌不但造成局部性的表皮感染，還會引起散播性的全身感染。甚至還會造成生命的威脅。目前所用的抗黴菌藥物都有副作用、不能有效控制一些黴菌及引起抗藥性的致病原的問題。

白色念珠菌(*Candida albicans*)是最常引起感染的黴菌之一。它可由酵母菌型細胞轉換爲菌絲型細胞，這種形態的交替和其致病力有關。不會形成菌絲型細胞的 *cph1/cph1 efg1/efg1* 雙重突變體(double mutant)也沒有致病力。因此，由 Cph1p 及 Efg1p 所調控的基因或其產物和白色念珠菌的致病力是息息相關的。

這個研究就是爲尋找白色念珠菌的致病基因(virulence genes)所設計的。前題是：野生種的白色念珠菌(wild-type)有致病力，而 *cph1/cph1 efg1/efg1* 雙重突變體沒有。推論是：若基因只在野生種表現而不在 *cph1/cph1 efg1/efg1* 突變體表現，這些基因可能和白色念珠菌的致病有關；相反地，若基因只在突變體表現，這些基因或其產物可能有阻止白色念珠菌感染的功能。

利用相減雜交法(subtractive hybridization)的技術，相互比對野生種及雙重突變體的 total RNA，我們成功的分離了 991 個只在野生種表現或在野生種表現比在雙重突變體多(Wt – Mutant)之 cDNA clones 及 340 個只在雙重突變體表現或在雙重突變體表現比在野生種多 (Mutant-Wt)之 cDNA clones。接著利用 restriction fragment mapping, DNA 定序,及資料庫比對將之分成類。目前已完成部份

Wt-Mutant 中已分成 11 類是不同之基因群，Mutant-Wt 則有 13 類不同功能之基因群 (Table 2)。

爲了更清楚地瞭解這些基因的作用，我們會將和白色念珠菌致病可能相關的基因突變，再觀察這些突變體的特性。

這個研究所得之基因及其特性不但可以幫助我們對白色念珠菌的致病機制有進一步的了解，而且對抗黴菌新藥物的發展亦有貢獻。

關鍵詞：致病基因，念珠菌，抗黴菌新藥物

English Abstract:

In the past decade, yeast infections have played an increasingly important role in nosocomial infections in the hospital setting. *Candida albicans* is the most frequently isolated fungal pathogen in humans [4].

Candida albicans can switch from a unicellular yeast form into a multicelluar filamentous form. It has been reported that the *cph1/cph1 efg1/efg1* double mutant in *C. albicans* is defective in filamentous growth and is also avirulent in a mouse model [5]. Thus, identifying the genes regulated by the Cph1p and Efg1p in *C. albicans* may reveal important therapeutic targets in addition to the mechanism of pathogenicity.

This study is designed to identify the virulence genes in *C. albicans.* We have compared the patterns of gene expression between the wild-type strain and the *cph1/cph1 efg1/efg1* double mutant in *C. albicans* by the method of subtractive hybridization [3]. The genes expressed in the wild-type strain but less or not in the *cph1/cph1 efg1/efg1* double mutant (Wt-Mutant) may be virulence genes which are potential candidates for targets of antifungal agents. In contrast, the genes expressed in the *cph1/cph1 efg1/efg1* double mutant but less or not in the wild-type strain (Mutant-Wt) may be negative regulators for pathogenicity of *C. albicans*. In the

experiment of Wt-Mutant we have obtained 991 clones, while only 340 clones from the reversed subtraction (Mutant-Wt). Those clones were subjected to restriction mapping, DNA sequencing, and data analysis for identification and classification purposes. Genes in the Wt-Mutant groups that have been sequenced can be classified into 11 different groups according to their known function. And genes in the Mutant-Wt are into 13 groups. Totally, there are 52 different candidate genes in Wt-Mutant and 263 different candidate genes in Mutant-Wt (Table 2). The particular functions of those genes expressing specifically in the virulent strain or the avirulent mutant will be determined by gene disruption approaches.

The knowledge gained from this study will help us to understand more about the pathogenicity of *C. albicans* and also to design better antifungal drugs that block the virulence of *C. albicans* and, potentially, other fungal pathogens.

Keywords：Virulence gene, Candida, antifungal agent

二、緣由與目的

In the past decade, nosocomial infections caused by yeast pathogens have increased dramatically. For instance, the prevalence of nosocomial candidemia increased 27-fold from 1981 through 1993 at National Taiwan University Hospital [2]. In the United States yeast infections rank as the fourth most common cause of nosocomial bloodstream infection [6]. The rise in the prevalence of fungal infections has exacerbated the need for the next generation anti-fungal agents. Many current available anti-fungal agents have several problems, for example, having side effects, being ineffective against new or reemerging fungi, and leading to the rapid development of resistance. To design and develop a new effective anti-fungal agent requires the understanding of the mechanism of pathogenicity.

To establish an infection, fungal pathogens have to survive and divide in the host environment and also invade new tissues and phagocytic cells. The molecule dissection of virulence genes of fungal pathogens has hence focused on adhesion, proteinase secretion, and hyphal formation. Since *C. albicans* is one of the major fungal pathogens in humans and has been established for the molecular study, this study is designed to identify the virulence genes of *C. albicans*. A strain of *C. albicans* with *cph1/cph1 efg1/efg1* double mutant has been shown to lose its virulence in a mouse model [5]. This strain was used for our study to isolate genes regulated by the Cph1p and Efg1p, which control the virulence in *C. albicans*.

三、結果與討論

In order to identify virulence genes of *C. albicans*, we determined the differential gene expression, under the condition that induces wild-type cells into hyphal form, of the virulent wild-type strain and the avirulent *cph1/cph1 efg1/efg1* double mutant by means of the subtractive hybridization [3]. The genes expressed in the wild-type

strain but less or not in the *cph1/cph1 efg1/efg1* double mutant may be virulence genes of *C. albicans*. In contrast, the genes expressed in the *cph1/cph1 efg1/efg1* double mutant but less or not in the wild-type strain may be negative regulators for virulence of *C. albicans*.

The RNA isolations are preceded according to the procedure of Carlson and Botstein [1]. The total RNA from the fungal cells was used for the synthesis of cDNA for the subtractive hybridization described by Diatchenko et.al. [3]. The products from the subtractive hybridization were then subcloned onto plasmid vectors.

In the experiment from which the gene expression pattern of the double mutant was subtracted from that of the wild-type strain, we have obtained 991 clones, while only 340 clones from the reversed subtraction. Eleven clones were picked randomly for sequencing. The raw sequence results were used for sequence alignment by the blast program provided by NCBI. Five of them are *EFG1* gene of *C. albicans* (Fig. 1). Identification of the *EFG1* gene is an excellent indication and positive control that the techniques used in this research have worked as intended since *EFG1* has been deleted in the *cph1/cph1 efg1/efg1* double mutant. Another indication of success is the cloning of the homolog of yeast *STE20* (Table 1). *STE20* is known to be involved in hyphal formation. In the Mutant-Wt groups, identification of Tup1 is the indicator that our experimental has worked accordingly, since Tup1 is known to express higher in the double mutants. The categorization of the cDNA clones was performed by comparing the patterns of restriction enzyme digestions of each clone. The Wt-Mutant has 381 groups, and the Mutant-Wt has 266 groups. Groups with more clones have been sequenced first.

The sequence results were then used for comparisons with Standford Candida Genomic databases and NCBI databases for identification of the genes. For those that do not have a match to Candida genes with known functions, the function of

homologs from other specieses were adapted to our candidate genes. Then, all the candidate genes with assigned functions were classified according to their functions.

So far, there are11 different groups in the Wt-Mutant categories, and 14 in the Mutant-Wt. Totally, there are 52 different genes in Wt-Mutant and 263 different genes in Mutant-Wt. In both categories, there are also genes that match Candida genomic sequence database but without any match genes with known function or homologs. To understand their function in Candida virulence, those genes will be subjected to genetic and functional studies after assessing by northern analysis.

四、計畫結果自評

The identification of the *EFG1* sequence from the clones obtaining from the experiment in which the gene expression pattern of the *cph1/cph1 efg1/efg1* double mutant was subtracted from that of the wild-type strain provides solid evidence that the subtractive hybridization has functioned as it was designed to. Another indication of success is the cloning of the homolog of yeast *STE20* (Table 1). *STE20* is known to be involved in hyphal formation. At the same time, five out of eleven clones chosen for sequencing are *EFG1* indicating that this method is also very sensitive to the level of gene expression. It is likely that the level of expression of *EFG1* is higher in the hyphal form than that in the yeast form. However, it is necessary to confirm the expression (both quality and quantity) of interested genes in wild-type and double mutant cells by the Northern blots analysis.

So far, after restriction mapping, sequence analysis, 52 different genes in Wt-Mutant and 263 different genes in Mutant-Wt have been identified. However, it is worth while to emphasize that in our experimental design, the hyphal formation is used only as a marker for virulence, genes regulates these two pathways do not have

to be complete identical. In fact, it is likely that they only share some genes, for instance, *EFG1* and *CPH1*. Also, due to the limitation of SSH, there will be false positives in our result. Hence, all candidate genes selected for further studies will be assessed by northern analysis before proceed to genetic and functional studies. It will be necessary to use mutagenesis and ovexpression approaches to determine the functions of interested genes. Another interesting result is the groups of genes without match in databases. Those novel genes usually represented unique biological properties of the organisms and are likely provides access to a new area. On the other hands, there are increasing competitions worldwide, a lot of progress has been made (7,8), not no critical breakthrough has happened yet, especially one like our project that provides full-scale screening for virulence-associated genes.

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Clone	BLAST Result	Identical bp/Ali
1	Candida albicans partial hxt6 gene for glucose transporter	280/308(90%)
2	Candida albicans estrogen binding protein (EBP1),NAD(P)H deH(old yellow enzyme)	747/764(97%),679/6
5	Candida albicans EFG1 gene for putative transcription factor	69/80(86%),444/476
8	Candida albicans EFG1 gene for putative transcription factor	191/229(83%)
14	Homo sapiens MAX dimerization protein (MAD) mRNA	52/58(89%)
17	Candida albicans EFG1 gene for putative transcription factor	163/163(100%),162/
19	Candida albicans EFG1 gene for putative transcription factor	164/180(91%)
20	Candida albicans squalene epoxidase (CAERG1) gene	113/130(85%),558/6
22	Homo serine/threonine kinase 24(Ste20, yeast homolog)	90/109(82%)
23	Candida albicans DNA for squalene epoxidase (CAERG1)	83/96(86%)
24	Candida albicans EFG1 gene for putative transcription factor	172/175(98%),242/2

Table 1. Sequence Alignment result of 11 clones from genes expressing higher or only in wild-type cells.

* Both ends of the cDNA have been sequenced.

Table 2. Classification of Virulence-related Genes

Category		No. of genes
A: Genes expressing higher or only in wild-type cells		
	Oxidoreductases	10
	Transferases	7
	Hydrolases	5
	Lyases	3
	Isomerases	2
	Hypothetical	7
	Ribosomal protein	2
	Cell wall	3
	Signal Transduction	9
	Protease	2
	Other	2
TOTAL	11 groups	52
B: Genes expressing higher or only in double-mutant cells		
	Structural	9
	Membrane Protein	12
	Transporter	21
	Metabolism	30
	Mitochondria	16
	Signal Transduction	10
	Cell Cycle	12
	Replication	7
	Transcription	18
	Translation	11
	Ribosomal Protein	31
	Hypothetical Protein	46
	Others	40
TOTAL	13 groups	263