UNIVERSITI PUTRA MALAYSIA

IMPACT OF GLUCOSE AND HIGH AFFINITY GLUCOSE SENSOR ON PHYSIOLOGICAL RESPONSES IN Candida glabrata

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By

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Faculty : Medicine and Health Sciences

Emerging fungal pathogen, *Candida glabrata* displays its metabolic flexibility by colonizing several site of host niches with different nutrient availability. Glucose sensing and utilization could be particularly important for the regulation of *C. glabrata* metabolic adaptation. Further exploration on the central metabolism pathway could help in advancing the knowledge on the novel antifungal development and overcome the limited choice of antifungal available currently. In line with this objective, the present study embarked on a several efforts in deciphering the role of glucose and glucose sensing in the viability and fitness of *C. glabrata*. The first part of this research outlined the putative genes of *C. glabrata* that are involved in the glucose inducing-Sugar Receptor-Repressor (SRR) pathway by comparing its orthologs found in *Saccharomyces cerevisiae*. Expression of selected key genes was also studied to confirm their response in five different glucose concentrations. For the second part, the phenotypic and physiological response of three strains of *C. glabrata* namely, ATCC2001 (laboratory isolate), Cg 2737 (clinical blood isolate) and Cg 91152 (clinical vaginal isolates), towards various glucose concentrations were studied. These strains were examined under different glucose concentration for their ability to grow, form biofilm, resistance toward amphotericin B (antifungal drug) and hydrogen peroxide (oxidative agent). Clinical isolates of *C. glabrata* were found with the ability to grow in low glucose environment (0.01%) where ATCC2001 strain has failed to survive. Generally, ATCC2001 and Cg 2737 was found to be active in biofilm formation under lower glucose environment (0.01, 0.1% and 0.2%) in comparison to glucose-rich environment (1% and 2%). Besides, low glucose surrounding (0.01, 0.1% and 0.2%) was also found to promote the survivability of *C. glabrata* towards amphotericin B (1 μg/ml), while higher glucose environment (0.2%, 1% and 2%) promotes *C. glabrata* resistance towards hydrogen peroxide. It is speculated that nutrient crisis in lower glucose setting is supposed to direct *C. glabrata* to a less active life cycle and therefore led it to group and form biofilm for nutrient sharing purposes. Lower metabolic rate and lower flux rate of molecules within *C. glabrata* biofilm may also result in the incompetence of

amphotericin B. Nevertheless, the promotion of anti H_2O_2 capability in *C. glabrata* by glucose requires further investigation. These observations have demonstrated the fine tuning of *C. glabrata* physiological behavior towards surrounding glucose levels as low as 0.01%. Besides, the higher expression of high affinity glucose sensor (*SNF3*) explained the ability of clinical isolates to grow in low glucose environment, in comparison to ATCC2001 strain. For the third part of this study, a *SNF3* knockout strain was constructed to study the role of this gene in the physiology of *C. glabrata*, particularly its involvement in the glucose sensing pathway. The *snf3*Δ showed a weaker growth of mutant strain under lower glucose environment (0.01% and 0.1%) in comparison to wild type. However, no different in growth was found when they were subjected to higher glucose concentration surrounding (1% and 2%). In addition, deletion of *SNF3* did not affect the ability of *C. glabrata* to form biofilm but instead disrupt the ability of *C. glabrata* to resist amphotericin B and survive in macrophage. Notably, deletion of *SNF3* resulted in the changes of transcription level for several key genes in the SRR pathway and suggested the shutting down of glucose uptake pathway that under low glucose environment. The disruption of *SNF3* was found to rattle the fitness of *C. glabrata*, particular in low glucose concentration environment, which is crucial for it to thrive in human niches site. This study has highlighted the impact of glucose on the physiology of *C. glabrata* and further decodes the involvement of *SNF3* in mediating the glucose uptake, which contributes to the vitality of *C. glabrata*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN GLUKOSA DAN PENDERIA GLUKOSA BERAFINITI TINGGI ATAS KE GERAK BALAS FISIOLOGI *Candida glabrata*

Oleh

NG TZU SHAN

Oktober 2016

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Sebagai patogen kulat yang baru muncul, *Candida glabrata* telah menunjukkan fleksibiliti metaboliknya dengan menjajah pelbagai lokasi dalam badan manusia yang mengandungi ketersediaan nutrien yang berbeza. Pencernaan dan penderiaan glukosa *C. glabrata* memainkan peranan penting dalam penyesuaian metaboliknya. Penyelidikan selanjut ke atas metabolisme berpusat dapat menyumbang dalam usaha menemui antikulat yang baru untuk menangani isu pilihan antikulat yang terhad. Selaras dengan objektif ini, beberapa usaha telah dijalankan untuk menerokai peranan glukosa dan penderiaan glukosa dalam pertumbuhan *C. glabrata*. Bahagian pertama kajian ini telah berjaya mengenal pasti gen-gen *C. glabrata* yang terlibat dalam proses Sugar Receptor-Repressor (SRR) dengan membandingkan ortholog mereka dalam *Saccharomyces cerevisiae*. Seterusnya, kajian ekspresi gen telah dijalankan untuk mengesah gerak balas gen-gen yang terpilih terhadap kepekatan glukosa yang berbeza. Untuk bahagian kedua, kajian gerak balas fenotip dan fisiologi bagi tiga isolat *C. glabrata*, iaitu ATCC2001 (isolat rujukan), Cg 2737 (isolat klinikal dari darah) dan Cg 91152 (isolat klinikal dari faraj) terhadap pelbagai kepekatan glukosa telah dijalankan. Dalam keadaan kepekatan glukosa yang berbeza, keupayaan ketiga-tiga *C. glabrata* ini dari segi pertumbuhan, pembentukan biofilm, rintangan terhadap amphotericin B (ubat antikulat) dan hidrogen peroksida (ejen oksidatif) telah dikaji. Isolat klinikal *C. glabrata* telah menunjukkan keupayaan mereka untuk bertumbuh dalam persekitaran yang mengandungi kepekatan glukosa yang rendah (0.01%), manakala ATCC2001 gagal bertumbuh dalam persekitaran yang sama. Secara umumnya, pembentukan biofilm oleh ATC2001 dan Cg 2737 adalah lebih aktif dalam persekitaran glukosa berkepekatan rendah (0.01%, 0.1% and 0.2%), jika dibandingkan dengan persekitaran glukosa berkepekatan tinggi (1% and 2%). Selain itu, persekitaran glukosa yang berkepekatan rendah (0.01%, 0.1% dan 0.2%) juga didapati dapat meningkatan rintangan *C. glabrata* terhadap amphotericin B (1 μg/mL). Sebaliknya, persekitaran glukosa berkepekatan tinggi (0.2%, 1% and 2%) dapat meningkatkan rintangan *C. glabrata* terhadap hidrogen peroxida. Krisis nutrien yang dihadapi oleh *C. glabrata* dalam persekitaran glukosa berkepekatan

rendah telah mengurangkan keaktifan cara hidup *C. glabrata*. Oleh itu, mereka terpaksa berkumpul dan hidup dalam biofilm demi perkongisian nutrien yang cekap. Sementara itu, kajian in mengspekulasi ketidakcekapan amphotericin B dalam kepekatan glukosa yang rendah adalah disebabkan oleh kadar metabolisma dan aliran molekul yang rendah dalam biofilm *C. glabrata*. Di samping itu, keupayaan tinggi *C. glabrata* terhadap antihidrogen peroksida dalam persekitaran glukosa berkepekatan tinggi adalah sesuatu yang tidak diketahui dan memerlukan penyelidikan selanjutnya. Data menunjukkan bahawa keupayaan *C. glabrata* dalam mengawal tingkah laku fisiologinya adalah bergantung kepada kandungan glukosa di persekitarannya. Selain itu, ekspresi gen penderia glukosa beraffiniti tinggi (*SNF3*) adalah lebih tinggi dalam isolat klinikal, berbanding dengan ATCC2001. Pemerhatian ini mungkin berkaitan dengan keupayaan isolat klinikal yang mampu bertumbuh dalam persekitaran glukosa berkepekatan rendah, berbanding dengan ATCC2001. Dalam bahagian ketiga kajian ini, mutan *C. glabrata* yang tanpa *SNF3* (*snf3*Δ) telah dihasilkan untuk mengkaji peranan *SNF3* dalam fisologi *C. glabrata*, terutamanya dalam proses penderiaan glukosa. Mutan *C. glabrata* telah menunjukkan kadar pertumbuhan yang lebih rendah dalam persekitaran glukosa berkepekatan rendah (0.01% dan 0.1%), berbanding dengan jenis liar. Walau bagaimanapun, kadar pertumbuhan dalam persekitaran glukosa berkepekatan tinggi (1% dan 2%) adalah sama bagi mutan dan jenis liar. Di samping itu, penyingkiran *SNF3* tidak menjejas keupayaan *C. glabrata* untuk membentuk biofilm. Di sebaliknya, ia menjejaskan keupayaan *C. glabrata* untuk menentang amphotericin B dan pertumbuhan *C. glabrata* di dalam makrofaj. Penyingkiran *SNF3* juga mengakibatkan perubahan transkripsi bagi beberapa gen dalam proses SRR dan dipercayai merencatkan proses pengangkutan glukosa ke dalam sel di persekitaran glukosa berkepekatan rendah. Data yang dikemukakan telah menunjukkan bahawa penyingkiran *SNF3* akan mengganggu pertumbuhan dan kercergasan *C. glabrata*, terutamanya dalam persekitaran glukosa berkepekatan rendah. Kajian ini telah memaparkan impak glukosa ke atas fisiologi *C. glabrata* dan peranan *SNF3* dalam mengkoordinasi pengambilan glukosa untuk pertumbuhan dan kercergasan *C. glabrata*.

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I certify that a Thesis Examination Committee has met on 13 October 2016 to conduct the final examination of Ng Tzu Shan on his thesis entitled "Impact of Glucose and High Affinity Glucose Sensor on Physiological Responses in *Candida glabrata*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This is to confirm that:

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percent
ABC	ATP-binding cassette
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tools
bp	Base pair
cAMP	Cyclic monophosphate
CDG	*Candida* Genome Database
cDNA	Complementary DNA
CFU	Colony forming unit
CHEF	Clamped homogenous electric field
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal electron microscopy
CO_2	Carbon dioxide
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EtBr	Ethidium bromide

FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Gram
GEXSR	Glucose-enhanced oxidative stress resistance
GOF	Gain of function
GPI	Glycosylphosphatidylinositol
h	Hour(s)
H_2O_2	Hydrogen peroxide
JTT	Jones-Taylor-Thornton
LiAc	Lithium acetate
M	Molar
MALDI-TOF	Matrix-assisted laser desorption ionization-time-of-flight
MEGA	Molecular Evolutionary Genetics Analysis
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
MLST	Multilocus sequencing typing
mM	Milimolar
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NCAC	Non-Candida albicans Candida
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards

ng	Nanogram
NJ	Neighbor Joining
nm	Nanometer
NRT	Non-reverse transcriptase
NTC	Non-template control
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide Gel Electrophoresis
PATH	Prospective Antifungal Therapy
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDREs	Pleiotropic drug response elements
PDS	Post-diauxic shift
PEG	Polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
PKA	Protein kinase A
PPP	Pentose phosphate pathway
qRT-PCR	Quantitative real-time polymerase chain reaction
rDNA	Ribosomal deoxynucleic acid
rRNA	Ribosomal ribonucleic acid
REST	Relative Expression Software Tools
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA

RT-PCR	Reverse transcriptase-polymerase chain reaction
SBP	Swi4-Swi6 cell cycle box binding factor
SC	Synthetic complete
SD	Synthetic defined minimal glucose media
sec	Second
SEM	Scanning electron microscopy
SEM	Standard error means
SRR	Sugar Receptor-Repressor
STRE	Stress-responsive element
TAE	Tris-acetate-EDTA
TBE	Tris-Boric acid-EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
TES	Tris.Cl-EDTA-SDS
UMMC	University of Malaya Medical Center
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Volt
w/v	Weight/Volume
WGD	Whole-genome duplication
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
YPD	Yeast extract peptone dextrose
µg	Microgram
µL	Microlitre
µm	Micrometer

μM	Micromolar

GENE NOMENCLATURE

The nomenclature of *Candida* and *Saccharomyces cerevisiae* gene and protein are based on the Gene Nomenclature Guide, published in *Candida* Genome Database (www.candidagenome.org/Nomenclature. shtml) and also *Saccharomyces* Genome Database. An overview of the nomenclature guide is illustrated below:

Items	Descriptions	Example
Gene (loci)	Gene symbols comprise three italic letters (uppercase italic for dominant), and an Arabic number.	*ADE12*
Protein	Proteins are referred to by the relevant gene symbol, non-italic and initial letter uppercase.	Ade12

CHAPTER 1

INTRODUCTION

1.1 General introduction

The advancement of medicine such as organ transplant, medical devices implantation, wide distribution of corticosteroids and antimicrobial drugs does not only result in greater longevity of human but also cause the emergence of opportunistic fungal infections (Perfect & Casadevall, 2006; Collette & Lorenz, 2011). Of the 300,000 known fungal species, only a few fungi are capable of infecting human, including *Candida* species, *Aspergillus* species, *Cryptococcus neoformans*, *Histoplasma capsulatum* and so on (Ricardson, 1991; Perfect, 2016). Among these causative agents of fungal infections, *Candida* appears as the leading cause of opportunistic mycoses and also the fourth most common cause (accounted for 8% to 10%) of bloodstream infections acquired from hospital (nosocomial) in hospitalized patients (Wisplinghoff et al., 2004; Pfaller & Diekema, 2007; Perfect, 2016; Rajendran et al., 2016; Tan et al., 2016). A total of 72.8 - 290 cases of *Candida*-caused infection per million of population per year with high mortality rate (46% to 75%) were reported in United States (Eggimann et al., 2003; Wisplinghoff et al., 2004). Based on these statistics, the number of death due to nosocomial *Candida* bloodstream infection is estimated to be 2800 - 11200 death per year in United States (Pfaller & Diekema, 2007). On top of the high mortality rate, the prolonged hospitalization due to systemic invasive candidiasis has also resulted in costly medical fees, for example, a total of £16.2 million and $2 billion was estimated annually in managing candidiasis patients in the United Kingdom (except Scotland) and the United States, respectively (Wilson et al., 2002; Hassan et al., 2009).

Candidiasis may either be superficial, which involves the skin, hair, nails, oral and vaginal, or systemic, which affects the major body organs such as acute disseminated candidiasis (Molero et al., 1998; Fidel et al., 1999; Silva et al., 2011a). Recently, the incidence of candidiasis has continued to increase, particularly the systemic invasive candidiasis due to the exploited weakness in the human immune system. *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* are among the dominant (>95%) causative agents for candidiasis (Pfaller & Diekema, 2010; Castanheira et al., 2016). Despite the dominance of *C. albicans* (> 50%), the noticeable emergence of non-*Candida albicans Candida* (NCAC) species, especially *C. glabrata* has also been reported in recent epidemiological reports studies (Pfaller et al., 2010; Diekema et al., 2012; Pfaller et al., 2014; Ng et al., 2015).

Glucose is known to be an important and precious source of nutrient for metabolism and growth of most living cells (Towle et al., 2005). The conserved glucose sensing and transportation found between prokaryote and eukaryote suggest the importance of these highly evolved and complete sensory mechanisms in the development of the organism, including pathogenic fungi (Sun et al., 2012). Adaptation through the

sensory mechanism is particularly important for pathogens in order to maintain its viability in various host niches and also to counteract the hostile environment such as microenvironment in phagocytes. In respect to the dynamic and complex environment in the host, the ability to respond to the constantly changing environmental nutrient availability is crucial for the survival of pathogen (Rodaki et al., 2009; Brown et al., 2014), e.g. the sudden nutritional starvation imposed on the phagocytes-trapped pathogen. Therefore, the ability to sense the surrounding nutrient, especially glucose is crucial in contributing to the growth and persistence of pathogen in the human host.

1.2 Problem statement

As an emerging and top two leading causative agent of candidiasis, the high mortality rate of *C. glabrata* fungemia has an important implications for therapy (Diekema et al., 2012). In comparison to antibacterial, there are only limited classes of antifungal agents available for physicians to combat this deadly fungal pathogen, namely polyenes, azoles, and echinocandins (Perfect, 2016). In addition to the innate resistance to azole drug group, the resistant to echinocandins observed in *C. glabrata* has complicated the management of patients (Pfaller et al., 2012). Taking antibacterial drug as an example, the worldwide explosion of antibacterial resistance has renewed the interest in the study of the central metabolism pathway, which has been known to be an unattractive drug target due to the lack of selectivity as most of the metabolic enzymes are conserved between bacteria and human (Murima et al., 2014). Similarly, most of the mainstream antifungals such as amphotericin B, fluconazole, and caspofungin target the cell wall and membrane of the fungal cell (Perfect, 2016). The interruption of cellular metabolism through the disrupted key nutrient sensory and transportation mechanisms, such as glucose sensing has been proved to affect the virulency of pathogenic fungi, *C. albicans* (Brown et al., 2006). Thus, it is essential to have a detailed study on this vital cellular process of the pathogenic fungi for the development of novel antifungal drug

The wide-ranged candidiasis suggests the incredible nutrient responsiveness of *Candida* species to thrive in diverging range of human anatomical site. Therefore, the ability to sense and utilize the glucose, particularly in the nutrient-limited niches is speculated to be important for the fitness of *Candida* species. Several studies have demonstrated the prominent effect of glucose availability in the physiological response and virulence traits of *C. albicans*, including the ability to form biofilm, resistance towards antifungals and oxidative stresses (Rodaki et al., 2009; Uppuluri et al., 2010). In addition, the reduced virulence of *C. albicans* and *Cryptococcus neoformans* in the disseminated murine model as a result of losing their high affinity glucose sensor suggest the vital role of this protein (Brown et al., 2006; Liu et al., 2013). However, little is known on the regulatory effect of glucose and the physiological role of high affinity glucose sensor in *C. glabrata*. Therefore, further investigation on these would serve as a continuous effort in the exploration of novel metabolic-targetted antifungal drug.

1.3 Objectives

In general, this study aimed to fill the gap of knowledge by revealing the regulatory effect of glucose and the importance of glucose sensing mechanism in the physiology of *C. glabrata*. The specific research objectives are as follows:

1. To identify and illustrate the glucose sensing and uptake pathway-related genes of *C. glabrata.*
2. To investigate the impact of glucose levels on *C. glabrata* viability and virulence.
3. To generate selected mutant strain for better understanding of the physiological role of selected genes in *C. glabrata.*

1.4 Thesis outline

This thesis is divided into six (6) chapters and formatted in accordance to the Style 2 as described in the Guideline to Thesis Preparation, Second Edition (June 2013), School of Graduate Studies, Universiti Putra Malaysia. Chapter 1 of this thesis portrays the brief introduction on candidiasis and *C. glabrata*, together with the needs and the objectives of this study. Chapter 2 is the literature review on the current knowledge of the biology of *C. glabrata* and the yeast glucose sensing/transportation mechanism as the subject of this study. Chapter 3 to Chapter 5 are the research chapters that discussed on three specific research objectives of this study. Chapter 6 is the general conclusion and recommendation that summarizes and concludes the findings of this study.

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BIODATA OF STUDENT

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Tzu Shan Ng, Mohd Nasir Mohd Desa, Doblin Sandai, Pei Pei Chong & Leslie Thian Lung Than. (2015). Phylogenetic and transcripts profiling of glucose sensing related genes in *Candida glabrata*. *Jundishapur Journal of Microbiology*. 8:e25177. doi: 10.5812/jjm.25177. (Journal Citation Reports 2015 Impact Factor: 0.655).

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