Original Article

Differential Gene Expression of Heat Shock Protein 90 (*Hsp90*) of *Candida albicans* obtained from Malaysian and Iranian Patients

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Abstract

***Background*: *Candida albicans (C. albicans)* has several virulence factors, in particular heat shock protein 90 (*Hsp90*), which is expressed by *Hsp90* gene. The purposes of this study were to assess the expression of *Hsp90* gene in clinical and control isolates of *C. albicans* obtained from different geographical regions (Malaysia and Iran), different temperatures (25°C, 37°C and 42°C) and mice with candidiasis.**

***Methods:* *C. albicans* isolates were cultured onto sabouraud dextrose agar (SDA). The assessment of the expression of *Hsp90* gene was performed using real time-polymerase chain reaction (RT-PCR).**

***Results:* The results showed a significant increase in the expression of *C. albicans Hsp90* gene under high thermal shock (42°C) when compared to other temperatures tested (*P*-value = 0.001). The mean differences in the expression of *Hsp90* gene at 37°C were 0.20 (95% confidence interval (CI) 0.13-0.29) between Malaysian and Iranian controls (P-value = 0.040) and 0.47 (95% CI 0.27-0.60) between Malaysian and Iranian patients (*P*-value = 0.040).**

***Conclusion:* The results demonstrated that the expression of *C. albicans Hsp90* gene varied between Malaysian and Iranian subjects, representing the efficacy of geographical and thermal conditions on virulence gene expression.**

Keywords: *Candidiasis, Candida albicans, gene expression, HSP90 Heat-Shock Proteins, Molecular Diagnostic Techniques*

Introduction

The majority of viable cells ranging from bacteria to mammals respond to high temperature via the production of specific proteins, in particular heat shock proteins (Hsps) (1). Upon heating, the cell represses the transcription and translation processes except for Hsps, initiating its adaptation to new environments. Also, the molecular chaperones are induced by non-thermal stresses such as oxidative stress, low pH and treatment with cytotoxic drugs (2). *Hsp90* is one of the most important molecular chaperones with classical *in vitro* activity of protein folding. However, unlike other molecular chaperones, *in vivo Hsp90* is not necessary for *de novo* protein synthesis; it assists only a small set of proteins to perform correctly their functions (3).

Candida albicans (*C. albicans*) is the most frequent opportunistic yeast of humans. Candidiasis caused by Candida species manifests as mucocutaneous diseases, but it results in systemic infections of immunocompromised patients as well (4). *C. albicans* expresses a panel of virulence factors such as yeast to hyphal transition, surface molecules and extracellular hydrolytic enzymes, which are involved in the development of infections (5). Nowadays, the environmental influences on gene expression of virulence factors are studied by some investigators. Swoboda et al. (6) demonstrated that *C. albicans Hsp90* gene showed 84% identity to Saccharomyces cerevisiae (S. cerevisiae) Hsp82. Although the physico-pathological and genetic roles of *C. albicans Hsp90* has not yet been elucidated, over expression of *Hsp90* gene in S. cerevisiae leads to increase the

virulence of this organism for mice (7), indicating support for its designation as a virulence factor. In addition, immune response to a 47-kDa antigen of *C. albicans* elicits protective antibody responses against systemic candidiasis (8), and the eliciting antigen is conserved at the C-terminal portion of *C. albicans Hsp90* (9). *Hsp90* has also been applied to a phage-displayed vaccine tested in mice, which acquired resistance to systemic *C. albicans* infection (10). Moreover, *Hsp90* of *C. albicans* has been demonstrated to play an important role in the emergence of resistance to azole and echinocandin antifungal drugs (11,12). Because fungal infections in humans are often caused by commensal *C. albicans* strains, they are different from other clinically important phenotypes in terms of pathogenicity at different situations. Expression profiling provides new information about what genes do under various conditions. To our knowledge, little data reported the importance of different geographical conditions and different temperatures on the expression of *C. albicans Hsp90* gene (13). Therefore, the purposes of this study were to assess the expression of *C. albicans Hsp90* gene obtained from Malaysian and Iranian subjects at different temperatures (25°C, 37°C and 42°C) and mice infected by *C. albicans* strains.

Materials and Methods

Fungal isolates

Thirty-two clinical isolates of *C. albicans* collected from Malaysian and Iranian patients with systemic candidiasis and 32 control isolates of *C. albicans* obtained from healthy humans were used for evaluating the expression of *Hsp90* gene. The group study consisted of 32 patients (16 males and 16 females) and 16 age-and sex-matched control individuals (16 males and 16 females). The mean of age in group study was 35.9 years (aged between 18 and 60 years old). The clinical isolates were obtained from the blood of immunocompromised patients with systemic candidiasis, representing the risk factors such as cancer, diabetes mellitus, antibiotic and steroid treatments. The control isolates were isolated from the skin of people who were immunocompetent and had normal immune function. Study protocol was approved by ethics committees of University of Malaya Medical Center (UMMC), Malaysia, and Tehran University of Medical Sciences, Iran. Informed written consent was obtained from all subjects prior to entering the study.

The yeast cells were grown onto sabouraud dextrose agar (SDA) (Merck Co., Darmstadt, Germany) at 37°C for 24 h. The yeasts were identified based on germ tube test, CHROM agar, β-glucosidase test, urease test, cornmeal agar-Tween 80 for chlamydospore production, sugar fermentation and assimilation tests using Rap ID Yeast Plus System (Remel Inc., Lenexa, Kansas, USA). In order to evaluate the effect of temperature alterations on heat shock response, Candida cells were cultured onto SDA for overnight at 25°C, and then transferred to sabouraud dextrose broth (SDB) media in two distinct tubes in a shaking water bath at 110 oscillations per minute at 25°C (low temperature) and 42°C (high temperature) for 45 min. After incubation, Candida cells were harvested by centrifuging at 3000 × g for subsequent step (14).

Evaluation of the expression of C. albicans Hsp90 gene in mice model

Forty-eight six-week-old (25-30 g) female BALB/C mice were purchased from Razi Institute, Karaj, Iran. Eight clinical isolates of *C. albicans* (4 Malaysian and 4 Iranian strains) and 8 control isolates of *C. albicans* (4 Malaysian and 4 Iranian strains) were selected in this study and three mice were considered for each isolate. The yeasts were cultured onto SDA and incubated at 37°C for 24 h. Yeast colonies were removed and washed twice with phosphate-buffered saline (PBS). The yeast suspension was adjusted approximately 500×10^3 cells/mL using hemocytometer slide. The mice were infected by inoculation of 0.1 mL of *C. albicans* suspension through the caudal vein. After three days of infection, the mice were anesthetized by chloroform and the kidneys were aseptically removed from each mouse. The tissue was crushed in physiologic serum and 0.2 mL of the filtrate was cultured onto SDA at 37°C for 24 h. Candida cells were harvested from SDA media and used for evaluating the expression of *Hsp90* gene in real time-polymerase chain reaction (RT-PCR) process. All experiments were performed based on the Veterinary Research Ethics and this study was approved on Society Protection of Cruelty to Animals (SPCA) in Iran.

Quantitative Real-Time PCR (qRT-PCR)

C. albicans isolates were used for the isolation of total RNA according to kit protocol (RNeasy Mini kit, Qiagen, Hilden, Germany). Three microgrammes of RNA were then treated with DNase I based on the protocol Quanti Tect

Table 1: The primers of *Hsp90* and 18S rRNA genes.

Primers	Sequence (5´-3´)
18S rRNA, Forward primer	5´-GCCAGCGAGTATTAACCTTG3´
18S rRNA, Reverse primer	5´-AGGCCTCACTAAGCCATTCA3´
Hsp90, Forward primer	5'-CGATGAATATGCCATGACT-3'
Hsp90, Reverse primer	´5'-TCCATAGCAGATTCTCCAG-3'

Reverse Transcription Qiagen Company (Hilden, Germany) and used for cDNA synthesis using the Taq Man Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed in a 25 µL total reaction volume including 12.5 µL of Power SYBR Green ® PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL of forward primer (400 nM), 1 µL of reverse primer (400 nM), 5 µL of complementary DNA (300 ng) as template and 5.5 µL of double distilled water in Micro Amp Optical 96-well plate (Applied Biosystems, Foster City, CA, USA) on ABI 7300 Real-Time-PCR instrument (Applied Biosystems, Foster City, CA, USA). The conditions were as follows; cycle 1 was repeated 40 times: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min and cycle 2 representing the dissociation level was performed once: 95°C for 15 s, 60°C for 15 s, 95°C for 15 s. In order to show the cDNA bands of *Hsp90* and 18S rRNA genes, the PCR products were run on 1.5% (w/v) agarose gel. A 1 kb DNA ladder (Fermentas Life Science, St. Leon-Rot, Germany) was used as marker at this stage. Data for the *Hsp90* gene was calculated and expressed as a fold regulation compared to the reference gene 18S rRNA for each condition tested, using the standard curve quantification method (15). The sequences of the reference gene primers were obtained from Manolakaki et al. (16) study (Table 1). The qRT-PCR assay was performed independently in triplicate.

Statistical analysis

The analysis of data was carried out using the statistical package for social sciences (SPSS, version 17, Chicago, IL, USA). Independent t and Mann-Whitney U tests were used to compare the difference in the mean expression values of *Hsp90* gene between the groups tested. Differences were considered significant at *P*-value < 0.05.

Results

Results of the expression of *C. albicans Hsp90* gene in different groups under study were illustrated in Tables 2 and 3.

At 25°C temperature, the expression values of *Hsp90* were 0.65 for Malaysian patients and 0.50 for Malaysian controls, whereas the mean gene expression were 0.40 for Iranian patients and 0.32 for Iranian controls. In addition, the mean expression values of *Hsp90* gene at 37°C were 1.75 and 1.20 for Malaysian patients and controls, respectively, whereas the mean gene expression values were 1.00 and 0.66 for Iranian patients and controls, respectively. There were statistically significant differences between Malaysian and Iranian controls (*P*-value = 0.040) as well as Malaysian and Iranian patients (*P*-value = 0.040). In infected mice, the mean expression values were 2.15 and 1.55 for Malaysian patients and controls, respectively, whereas the mean gene expression values were 1.38 and 1.10 for Iranian patients and controls, respectively. At 42°C thermal shock, the mean expression values of *Hsp90* gene were 3.45 for Malaysian patients and 3.03 for Malaysian controls, whereas the mean gene expression values were 3.20 for Iranian patients and 2.98 for Iranian controls. A significant difference was observed between 42°C temperature and other temperatures tested (*P*-value = 0.001).

As shown in Figure 1, the expression of *C. albicans Hsp90* gene increased in Malaysian populations when compared to Iranian populations at different temperatures as well as in mice body (37°C, *in vivo*) when compared to *in vitro* study at 37°C.

The results of the expression of *Hsp90* and 18S rRNA genes from clinical isolates were illustrated in Figure 2. As observed, *Hsp90* and 18S rRNA bands were appeared on the agarose gel after electrophoresis of the clinical isolates of *C. albicans* obtained from human patients and mice kidneys. The locations of *Hsp90* gene and 18S rRNA gene bands were detected about 605 bp and 220 bp, respectively.

Table 2: The statistical results of the expression of Candida albicans *Hsp90* gene in different groups understudy at 25°C, 37°C, 37°C (mice) and 42°C thermal shock.

Temperature	Group	n	*Hsp90* gene expression			
			Mean (SD)	Mean difference (95% confidence interval)	t-statistic (df)	*P*-value
25°C	Malaysian controls	16	0.50 (0.16)	0.10 (0.08-0.17)	0.32 (5)	0.774[a]
	Iranian controls	16	0.32 (0.09)			
	Malaysian patients	16	0.65 (0.20)	0.08 (0.03-0.20)	0.29 (5)	0.768[a]
	Iranian patients	16	0.40 (0.12)			
37°C	Malaysian controls	16	1.20 (0.47)	0.20 (0.13-0.29)	0.53 (5)	0.040[a]*
	Iranian controls	16	0.66 (0.19)			
	Malaysian patients	16	1.75 (0.53)	0.47 (0.27-0.60)	1.10 (5)	0.040[a]*
	Iranian patients	16	1.00 (0.36)			
37°C (mice)	Malaysian controls	4	1.55 (0.50)	0.89 (0.55-1.20)	1.93 (2)	0.241[b]
	Iranian controls	4	1.10 (0.39)			
	Malaysian patients	4	2.15 (1.01)	0.99 (0.73-1.86)	2.90 (2)	0.233[b]
	Iranian patients	4	1.38 (0.48)			
42°C	Malaysian controls	16	3.03 (1.42)	1.02 (0.89-1.68)	3.41 (5)	0.096[a]
	Iranian controls	16	2.98 (1.20)			
	Malaysian patients	16	3.45 (1.64)	1.59 (1.06-1.68)	3.78 (5)	0.120[a]
	Iranian patients	16	3.20 (1.50)			

* Statistically significant difference.
[a] "Independent t-test. Normality assumption for the data was met."
[b] "Mann-Whitney U test. The data were not normally distributed."

Table 3: Comparison of mean difference of the expression of Candida albicans Hsp90 gene in different groups at 25°C, 37°C, 37°C (mice) and 42°C thermal shock.

Temperature	Group	*Hsp90* gene expression		
		Mean difference (95% confidence interval)	t-statistic (df)	*P*-value
25°C	Malaysian patients vs Malaysian controls	0.23 (0.18-0.30)	0.59 (5)	0.819[a]
	Iranian patients vs Iranian controls	0.19 (0.12-0.27)	0.51 (5)	0.729[a]
37°C	Malaysian patients vs Malaysian controls	0.86 (0.47-1.10)	1.89 (5)	0.128[a]
	Iranian patients vs Iranian controls	0.10 (0.06-0.18)	0.34 (5)	0.228[a]
37°C (mice)	Malaysian patients vs Malaysian controls	0.83 (0.60-1.51)	3.06 (2)	0.223[b]
	Iranian patients vs Iranian controls	0.79 (0.39-1.01)	1.90 (2)	0.228[b]
42°C	Malaysian patients vs Malaysian controls	1.51 (0.89-1.96)	3.07 (5)	0.089[a]
	Iranian patients vs Iranian controls	1.42 (0.79-1.78)	2.65 (5)	0.137[a]

[a] "Independent t-test. Normality assumption for the data was met."
[b] "Mann-Whitney U test. The data were not normally distributed."

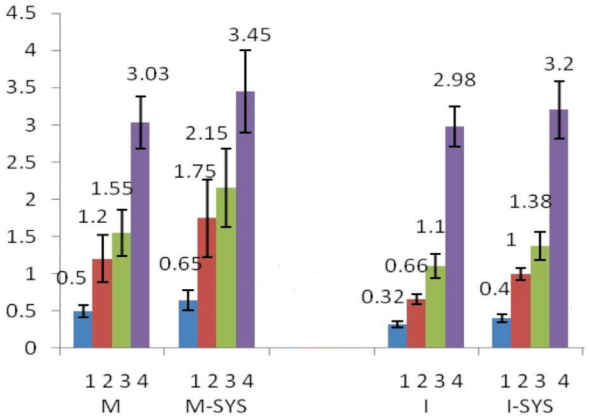

Figure 1: Comparison of *Hsp90* gene expression of clinical and control isolates of *Candida albicans* obtained from Malaysian and Iranian populations at 25°C (**1**), at 37°C (**2**), mice kidneys at 37°C (**3**) and at 42°C thermal shock (**4**). (**M**: Malaysian controls; **M-SYS**: Malaysian systemic patients; I: Iranian controls; **I-SYS**: Iranian systemic patients).

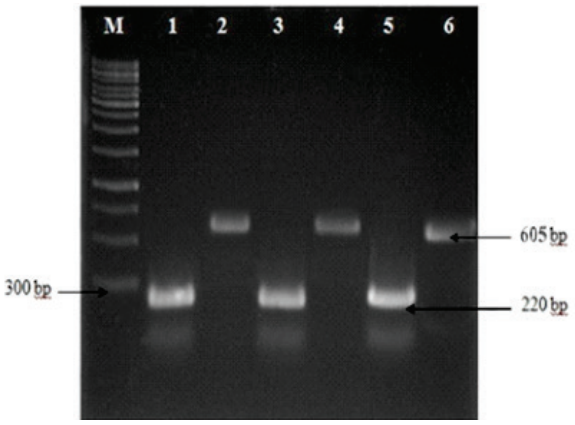

Figure 2: Gel electrophoresis of *Hsp90* (605 bp) and 18S rRNA (220 bp) genes of *Candida albicans* isolates [**M**: 1 kb DNA marker; **1, 3 and 5 lines**: 18S rRNA genes from clinical isolates of Malaysian (1), Iranian (3) and mice (5) populations; **2, 4 and 6 lines**: *Hsp90* genes from clinical isolates of Malaysian (2), Iranian (4) and mice (6) populations].

Discussion

Hsp90 is generally expressed at much higher levels than required for basal function, however, environmental stress can induce global problems in protein folding and thereby overwhelm *Hsp90*'s functional capacity (17). *Hsp90* modulates the phenotypic effects of genetic variation in an environmentally responsive manner (18), influencing 20% of observed natural genetic variation and serving both to maintain phenotypic robustness and promote diversification (19). In order to evaluate the expression of *Hsp90* gene of *C. albicans* strains, we did RT-PCR analysis of *C. albicans* cells in exposure to different geographical conditions, different temperatures and mice model. The results of this study exhibited that the expression values of *C. albicans Hsp90* gene were different in various situations. According to the results, the mean expression values of *Hsp90* gene at 37°C were 1.75 and 1.00 for Malaysian and Iranian patients, respectively, indicating a significant difference between Malaysian and Iranian isolates (*P*-value = 0.040). The explanation could be that genetic and environmental sources play a role in different expressions of *C. albicans Hsp90* gene. The exceptional adaptability of *C. albicans* in various populations is mediated by rapid alterations in gene expression in response to the host and environmental stimuli, such as race, changes in nutrient availability, pH, osmolarity, temperature or attack by cells of the immune system (20). Previous study examined the influence of race on the incidence of candidiasis and found that the incidence was at least four-fold higher among black populations in every age group than white populations (21).

The mean expression values of *Hsp90* gene were 0.65 and 0.40 at 25°C, and 3.45 and 3.20 at 42°C for Malaysian and Iranian patients, respectively. We found a 5.3-fold increase for Malaysian isolates and an 8-fold increase for Iranian isolates in the expression of *Hsp90* gene at 42°C when compared to 25°C, indicating a significant difference between two temperatures tested (*P*-value = 0.001). Previous studies showed a close relationship between temperature and the expression of C. albicans *Hsp90* gene (10,16,22-24). In accordance with our results, Swoboda et al. (6) demonstrated that *Candida Hsp90* gene was heat shock inducible and its expression was strongly induced at higher temperature, such as 45°C (about nine folds), but a change in temperature to 37°C had lower effect on the level of *Hsp90* mRNA. It has been suggested

that *Hsp90* might be involved in some aspects of growth and differentiation of *Candida* cells following temperature shifts (25). The expression of *Hsp90* gene observed after a temperature shift from 25°C to 37°C would appear to be involved in the germination of C. albicans in the warm-blooded host since the yeasts experience a temperature shift under these circumstances (26). However, it is well-known that forms of stress other than heat shock may evoke the over-expression of *Hsp90* gene in a warm-blooded host (27). O'Connor, Essmann & Larsen (28) showed that the expression of *Hsp90* gene was increased under treatment with 39°C and 17-β-estradiol up to 50% and 75%, respectively. According to the results obtained by Shapiro et al. (23), a medium induction of Hsp was observed with changes in temperature from 30°C to 37°C or exposure to serum at 30°C, but a higher stimulation was seen in response to the combination of serum and elevated temperature.

In infected mice, the mean expression values of *Hsp90* gene were 2.15 and 1.38 for Malaysian and Iranian clinical isolates, respectively. Our results showed that the expression of *Hsp90* gene in mice body (*in vivo* study) was higher than that of 37°C (*in vitro* study). Our findings are in accordance with Carper, Duffy & Gerner (29) and LaFayette (12) studies, who demonstrated the existence of differences in the expression of *C. albicans Hsp90* gene between *in vitro* (37°C) and *in vivo* (mice body) in both systemic and non-systemic isolates. A number of factors are thought to contribute to the virulence of *C. albicans*, but definitive experiments, which establish the relative importance of these factors, have yet to be performed. One potential virulence factor is the ability of this fungus to survive in host body by producing the protective proteins (30). While *Candida* cells enter the mice body or another host from outside environment, they are exposed to several stressful factors, such as temperature changes, and produce *Hsp90* against high temperature. Increased *Hsp90* is known as a defensive mechanism to protect *Candida* cells from harmful effects of heat. Jenkins, Schultz & Matin (31) showed that the oxidative stress responses were the most important factors in the development of experimental systemic infections. These responses were found to be as the consequence of the exposure of *Candida* cells with phagocytes circulating in bloodstream such as neutrophils. Enjalbert et al. (27) reported a reduction in virulence of *C. albicans* by compromising of *Hsp90* function or expression in mice with systemic candidiasis. Hodgetts et al. (7) also showed that over-expression of *Hsp90* gene in *S. cerevisiae* increased the virulence of this fungus in mice and therefore *Hsp90* appears to be a virulence factor of *C. albicans*.

Conclusion

In conclusion, *C. albicans* isolates obtained from Malaysian and Iranian subjects with different geographical situations showed some changes in the expression levels of *Hsp90* gene. We demonstrated remarkable expression of *C. albicans Hsp90* gene between Malaysian and Iranian populations at 37°C in the current *in vitro* study. The highest expression of *C. albicans Hsp90* gene was observed at 42°C (representing significant difference with other temperatures), followed by 37°C (*in vivo*), 37°C (*in vitro*) and 25°C. The results of this study can highlight possible gene-environment interactions in the disease process. Identifying etiologic heterogeneity can be an important step toward analysis of diseases using molecular epidemiology techniques and may eventually lead to improved disease prevention strategies.

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Conflict of Interest

None.

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