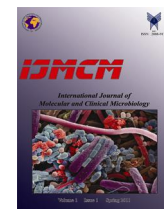


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Investigation of *Candida albicans* EFG1 gene expression in Vulvovaginal Candidiasis and biofilm formation

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ABSTRACT

Candida albicans has the ability to change between yeast and hyphal cells and is known to be a virulence property. Efg1 gene of *C. albicans* is a main transcription factor that plays pivotal roles in biofilm formation. The aim of current study was to investigate the presence of Efg1 gene in *C. albicans* isolates from women with vaginal candidiasis and its impact on biofilm formation. 50 clinical isolates of *C. albicans* that confirmed by PCR-RFLP were used in this study. Total RNA was extracted from *C. albicans* isolates by glass bead and lysis buffer, and cDNA was synthesized using Reverse Transcriptase enzyme. Reverse Transcriptase PCR (RT-PCR) was used to evaluate the expression of Efg1 gene. Biofilm formation was evaluated in 96-well microplate and then tetrazolium reduction was assayed. All data were analyzed using T-test. The result of molecular analysis showed that in 47(94%) isolates, Efg1 gene was present by using PCR, and 12 (25.53%) isolates out of 47, expressed Efg1 gene by the RT-PCR. Results of MTT assay identified biofilm formation in isolates with Efg1 gene expression that successfully form dense biofilm on microplate, whereas in isolates without Efg1 gene expression, the biofilm formation was not detected. Statistical analysis showed that this difference is significant ($P < 0.05$). It can be concluded that the growth of hyphae for biofilm formation is influenced by important genes. It is also suggested that EFG1 gene in *C. albicans* contribute to biofilm formation.

1. Introduction

Candida species particularly *C. albicans* are mainly commensal fungi that reside on mucosal surfaces and in the gastrointestinal and genitourinary tracts (Harriott et al., 2010). One of the important factors contributing to the virulence of *Candida* is the formation of biofilm. *Candida* infection arise as a result of the organism's ability to grow as a biofilm on implanted medical devices such as venous catheters, urinary catheters, and artificial joints (Haswer and Douglas, 1994). *C. albicans*

biofilms comprise of mainly two kinds of cells: small yeast-form cells, and hyphal cells. Extracellular matrix material is clearly evident as well, bound to both yeast and hyphal cells (Harriott et al., 2010).

Biofilm have some advantages for *Candida* such as providing the pathogenesis and evading from host defense. The resistance of biofilm producing *Candida* species to antifungal agents is another major challenge especially in the design of therapeutic and prophylactic strategies. These factors, resulting in high mortality as well

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as economic problem due to prolonged hospital stay (Muni *et al.*, 2012)

Morphogenesis in *C. albicans* is essential to pathogenesis and invasion to host tissue. Morphogenesis in *C. albicans* is defined as transition from unicellular yeast form to filamentous form (irakawa *et al.*, 2014). The growth of hyphae is influenced by environmental factors

Efg1 (enhanced filamentous growth 1) gene of *C. albicans* is a main transcription factor that plays pivotal roles in biofilm formation (Nadeem *et al.*, 2013). Efg1 plays a significant role in hyphal growth and biofilm formation (Srikantha *et al.*, 2000). On the other hand, Efg1 as a transcription factor regulate expression of hyphal genes including HWP1, HYR1, ALS3 (Ramage *et al.*, 2002; Sohn *et al.*, 2003). Hence, *C. albicans* with Efg1 gene has more ability to biofilm formation and pathogenesis. For above reason, identification of genes in *C. albicans* that contribute to pathogenesis and biofilm formation is necessary.

This study is aimed to investigate the expression of the Efg1 gene in *C. albicans* in clinical samples and its role in biofilm formation.

2. Materials and Methods

2.1. Patients

In the cross-sectional study, the total number of 250 vulvovaginal candidiasis specimens was collected from women who referred to health center (Tehran, Iran) during 2015-2016. Symptoms include white-patchy leucorrhoea, itching and vulvar edema. Wet vaginal swabs were cultured on Sabouraud Dextrose agar (Dipco USA). The age range of the study population was 18-55 years with a history of antibiotics and antifungal use as well as underlying conditions such as diabetes. The isolates were confirmed by phenotypic and genotypic assays such as CHROM agar *Candida*, conventional methods and PCR-RFLP (Restriction Fragment Length Polymorphism) technique. This project has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Molecular identification

All isolates were examined by Reverse Transcriptase PCR (RT-PCR) method to

evaluate the expression of Efg1 gene in the following steps.

2.3. RNA extraction

Total mRNA was extracted by glass bead and lysis solution according to previously described method (Bastidas *et al.*, 2009).

2.4. cDNA synthesis

After treatment by DNAase, RNA was converted to cDNA according to cDNA synthesis kit (Vivantis-2 step, malassia) and was carried out as follows: in a sterile and free nuclease microtube, 8µl RNA (3µg) was added to 1µl Random hexamer primer and 1µl dNTP for 5 min at 65 °C. The microtube was placed on ice for 2 minutes to which 2.5µl 10 X Reaction Buffer, 1µl Ribonuclease inhibition (20u/ml) 10mM dNTP and 1µl M-MuIV Reverse Transcriptase (200u/ml) were added in order to make a final volume of 20µl for 5 min at 25 °C and then for 60 min at 42 °C. To inactivate the enzyme reverse transcriptase (M-MuIV), the microtube hold for 10 min at 70 °C, then were transferred immediately to a bowl of ice. cDNA was kept at -20 °C (Roudbarmohammadi *et al.*, 2016).

2.5. RT-PCR Reaction

RT-PCR was carried out according to the manufacture's recommendation. The RT-PCR program was as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min), and extension (72°C for 1 min), followed by a final extension step at 72°C for 3 min. Appropriate negative and positive controls were included in each test. ACT1 was used as a normalized gene (house keeping gene) in all molecular tests for confirmation of PCR. Primers are listed in table 1.

Table 1. EFG1 and ACT1 specific primers for RT-PCR

| Primer name | Sequence (5'→3') | Size (bP) |
|-------------|--------------------------------|-----------|
| EFG1F | 5'- TGAACGGAAATTACAATAACGG -3' | 700 |
| EFG1R | 5'- TTATCTGCTCTTCTGACAACCG -3' | |
| ACT1F | 5'-CCAGCTTTCTACGTTTCG-3' | 200 |
| ACT1R | 5'-CTGTAACCACGTTTCAGAC-3' | |

2.6. Preparation of fungal cell suspension

At first, two strains were grown on Sabouraud dextrose agar medium (SDA Merck, Germany) at 37°C for 18h. Then freshly grown colonies were inoculated into yeast nitrogen base medium (YNB medium; Himedia Co.) containing 100 mM glucose and incubated at 37 °C for 24 h. After that, a few colonies of the yeast were transferred into a test tube containing sterilized PBS, pH=7.2 (Difco). The mixture was then centrifuged (10000g, 10 min). Turbidity of suspension in the cell was compared to 0.5 McFarland standard to estimate cell density and finally, yeast cells were counted and adjusted at 1×10^6 cell/ml. ATCC 10231 strain was used as the standard strain.

2.7. Biofilm formation

1×10^6 cell/ml planktonic yeast cell in yeast nitrogen base medium (YNB) containing 100 mM glucose and 100 μ l was poured into a 96-well microplate. The plate was then shaken in an incubator (Stuart Scientific) of 37°C at 75 rounds per minute for 90 minutes. Yeast cells were attached to the bottom of the well. To remove the unattached cells, the well was washed three times with phosphate-buffered saline (PBS, pH=7.4). In addition, 200 μ l of YNB containing 100 mM glucose was added to each well and stored in a shaking incubator for 48 h. After that, the biofilm formed with 100 μ l PBS was washed again (Sudbery, 2011).

2.8. MTT Assay

The tetrazolium salt 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was used to study living cells as described before. MTT formazan formation was measured at 540 nm using ELISA reader (Memmert, Germany) (Haswer and Douglas, 1994). The results were evaluated regarding to the percentage of viable cells in comparison to the negative control (isolates without Efg1 gene expression). All tests were performed in three independent experiments.

2.9. Statistics analysis

Data analysis was performed by t-test method with SPSS Statistics version 17. The level of statistical significance was set at $P < 0.05$.

3. Results

50 clinical isolates out of 250 were confirmed as *C. albicans* by using PCR-RFLP. 47(94%) isolates out of 50, had Efg1 gene by using PCR in their DNA. Semi quantities RT-PCR result by specific primer showed, 12(25.53%) *C. Albicans* isolates expressed the efg1 gene in mRNA. Moreover, 35 of the isolates had no expression for Efg1 genes in mRNA by RT-PCR reaction (Fig 1)

Data from MTT Assay indicated the isolates with expression of Efg1 mRNA had more adherence capability than those lacking any Efg1 gene expression and this difference was significant ($P < 0.05$). Isolates which expressed Efg1 gene successfully formed dense biofilm on micropalate in MTT assay. Data were expressed as means \pm standard deviations of three independent experiments ($P < 0.05$).

4. Discussion

This study is designed to determine the expression of Efg1 genes in *C. albicans* isolated from vulvovaginal candidiasis for the first time in Iran. Biofilm formation in *candida* species is a unique capacity for virulence and drug resistance as well as the entire of antifungal drug to biofilm structure is very slow and biofilm acts a strong barrier against penetration of drugs. Many factors are involved in formation and stability of biofilm matrix. Among them, genes contribute to morphogenesis switching have important role (Vinitha and Ballal, 2007)

Efg1 gene, such as major transcription factor that regulation hyphal morphogenesis and metabolic in *C. albicans*. The main function of the gene is triggering hyphe formation during biofilm formation process (Yamada *et al.*, 2002; Mosmann., 1983).

Some studies confirmed the role of Efg1 gene in *C. albicans* functions. For example defects in gene expression cause the lack in biofilm formation. Study showed that the mutation in *cst20*, *hst7*, *efg1* and *cph1* in compare with wild strain can not to form biofilm; because of hyphe production is essential for biofilm formation (Nadeem *et al.*, 2013).

The expression of Efg1 gene in hyphal form, biofilm formation, pathogenesis and invasion of *C. albicans* is imperative (Lassak *et*

al., 2011; Theberge *et al.*, 2013; Felk *et al.*, 2002, Peirce *et al.*, 2012).

In this present study, RT-PCR was used as a reliable method to evaluate expression of *Efg1* gene in *C. albicans* isolates and also their capacity to biofilm formation was assessed by using MTT test. According to our finding, of 50 *C. albicans* isolates, 12(25.53%) expressed *Efg1* gene and 35 isolates did not showed any expression by RT-PCR method. The expression of the *Efg1* gene in these isolates may be associated to the role of *Efg1* in adherence the *Candida* to epithelial cells in vagina and pathogenesis. As it was demonstrated, the other genes such as *Sap1* contribute to adherence of *candida* to epithelial cells (Naglik *et al.*, 2008; Kumamoto *et al.*, 2005). Thus other mechanisms may explain the role of virulence of *C.albicans*. In isolates that not expressed *Efg1* mRNA in RT-PCR, expression and presence other functional genes and proteins must be noticed.

A recent analysis by Nobile *et al* showed that *Efg1* is part of a network of six transcription factors that regulates biofilm development in *C. albicans*. The six regulators control each other's

expression; up to five factors bind to each promoter, and in addition each activates its own synthesis that may elucidate the adhesion of isolates with unexpressed *efg1* genes in this study (Nobile *et al.*, 2012).

Other studies evaluated the *ALS1* and *ALS3* gene expression profile in clinical isolates of *C. albicans* in vulvovaginal candidiasis by RT-PCR assay. The result indicated considerable number of the isolates expressing the *ALS1* and *ALS3* genes may have contributed to their adherence to vagina and biofilm formation (Roudbarmohammadi *et al.*, 2016).

Result of MTT assay showed there is a significant difference in biofilm formation between *Candida* isolates with *Efg1* gene expression and the control group that did not showed any expression of *Efg1* gene because of their ability biofilm formation was higher in compared to the control group ($p=0.014$).

It can be concluded that the growth of hyphae for biofilm formation is influenced by important genes. It is also suggested that *EFG1* gene in *C. albicans* contribute to biofilm formation.

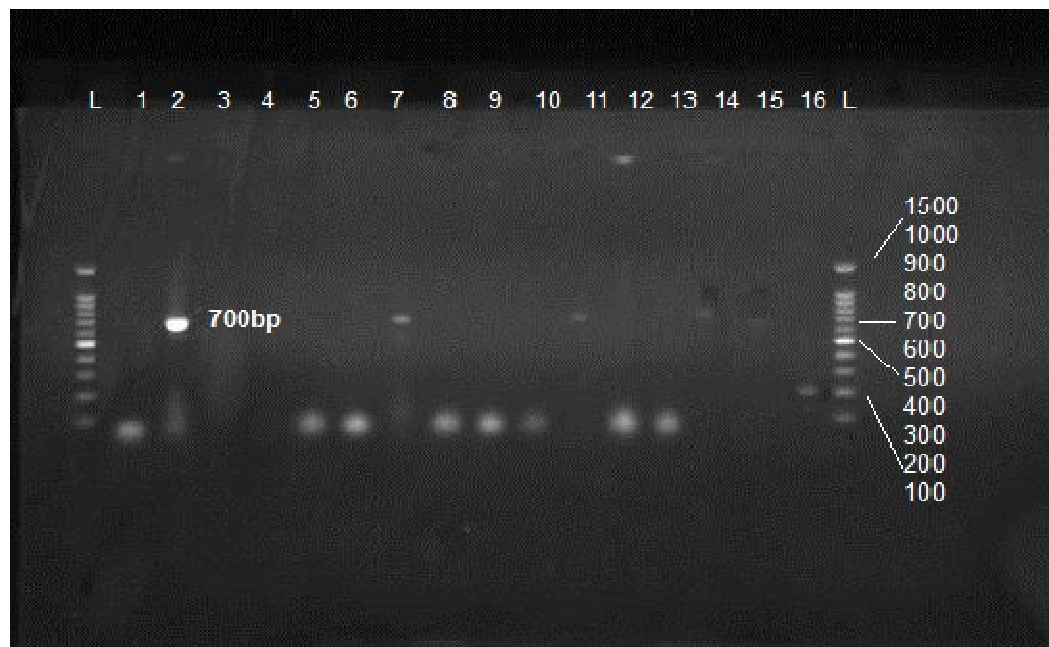


Figure1. mRNA of *C.albicans* isolates extracted and first strand cDNA was synthesized. RT-PCR with *Efg1* specific primers was done. Line 1-13, gene expression of *Efg1* in isolates (700bp); line 14 standard isolate *candida* ATCC10231 ,line 15 Negative control and line 16 *Act1* gene (200bp) ,M:Marker molecular weight (100bp)

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