Identifying differentially expressed genes in trophozoites and cysts of *Acanthamoeba* T4 genotype: Implications for developing new treatments for *Acanthamoeba* keratitis

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Abstract

*Acanthamoeba* T4 genotype is the most prevalent genotype associated with amoeba keratitis. *Acanthamoeba* keratitis therapy is difficult due to transformation of trophozoite to cyst stage, which hinders the treatment of the disease. Although encystation assists the organism to survive against the chemotherapeutic compounds, the precise mechanism of encystation remains poorly understood. The purpose of this work was to identify differentially expressed genes in *Acanthamoeba* T4 genotype which might be useful for understanding of the encystment process and may thus help develop more efficient treatment. The mRNA profile of trophozoite and cyst of *Acanthamoeba* T4 genotype isolated from a soft contact lens wearer were analyzed using a cDNA amplified fragment length polymorphism (cDNA-AFLP) technique. Subsequently, a real time reverse transcriptase-PCR was performed to validate the cDNA-AFLP results. Three genes, heat shock protein 70 (hsp70), actin-I and elongation factor-1alpha (EF-1\(\alpha\)) were differentially expressed during *Acanthamoeba* differentiation. An *in silico* result predicted that transformation of trophozoite to cyst could be mediated through their cooperation with the protein partners interaction. Taken together, our experimental and bioinformatics findings suggested potential functions of hsp70, EF-1\(\alpha\) and actin-I in differentiation of *Acanthamoeba* T4 genotype which may be useful in the design of an efficient therapeutic strategy in AK.

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**Keywords:** *Acanthamoeba* keratitis; cDNA-AFLP; Genes modulating trophozoite-cyst transformation; *In silico*; Real-time RT-PCR

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Introduction

The genus *Acanthamoeba*, as one of the most prevalent free-living amoebae is a causative agent of serious human diseases: granulomatous amoebic encephalitis (GAE), and *Acanthamoeba* keratitis (AK). *Acanthamoeba* has been classified into 18 genotypes (T1–T18) based on rRNA gene sequences (Qvarnstrom et al. 2013). The T4 genotype has been frequently reported as a predominant cause of AK across the world (Magshood et al. 2005; Niyiyati et al. 2009b). AK is a severe, vision-threatening infection which is associated with corneal trauma (Khan 2001) and contact lens wearing (CLW). The growing popularity of contact lenses led to increasing of AK cases in Iran (Rezeaian et al. 2007). AK patients initially suffer from severe ocular pain, red eye and photophobia. The late sign of this infectious disease is characterized by ring-shaped stromal infiltrate (Schuster and Visvesvara 2004a). AK can even lead to blindness in 15% of untreated cases (Visvesvara et al. 2007).

The *Acanthamoeba* life cycle is divided into two stages: the trophozoite and the cyst. The trophozoite performs phagocytosis, movement (Bowers and Korn 1968) and cellular division. The phenotypic change of the trophozoite into the cyst (encystment) occurs under adverse situations (Cordingly et al. 1996; Sriram et al. 2008; Lloyd et al. 2001). During the encystment process metabolic and morphogenetic changes occur in the amoeba resulting in the production of a double-walled shelter. The cyst walls are mostly made of proteins (Hirukawa et al. 1998; Weisman 1976) and carbohydrates (Tomlinson and Jones 1962). One of the most significant issues is the activation of the dormant cysts leading to relapse in *Acanthamoeba* infections during effective therapy (Clarke et al. 2012; Syam et al. 2005). Several studies suggested that to achieve a valuable treatment strategy, understanding the molecular mechanisms underlying *Acanthamoeba* differentiation is a prerequisite (Schuster and Visvesvara 2004b). Previous investigations have reported various genes implicated in *Acanthamoeba* encystation including serine protease (Lorenzo-Morales et al. 2005; Moon et al. 2008b) glycogen phosphorylase (Lorenzo-Morales et al. 2008), autophagy protein 8 (Moon et al. 2009), autophagy protein 16 (Song et al. 2012), protein kinase C (PKC) like super family genes (Moon et al. 2012), xylose isomerase and cellulose synthase (Aqeel et al. 2013).

To gain more insight into the encystation process of the *Acanthamoeba* T4 genotype, we identified differentially expressed genes that might be involved in *Acanthamoeba* T4 genotype encystment by a cDNA AFLP approach. The results of the cDNA AFLP were confirmed using a real time RT-PCR. These findings contribute to understanding the biology of *Acanthamoeba* as well as designing a successful chemotherapy agent to treat AK.

Material and Methods

Sampling and amoeba characterization

Amoeba origin

This study was approved in the Ethical Committee of Tehran University of Medical Sciences, Iran. A total of 39 patients including 10 males and 29 females with an average age of 26 ± 4 years, suspected to suffer from infectious keratitis were referred from Farabi Eye Hospital (Tehran, Iran) to the Department of Parasitology, School of Public Health, Tehran University of Medical Sciences (TUMS). Corneal scrapings and soft contact lenses were examined for *Acanthamoeba* isolation as previously described (Rezeaian et al. 2007). Five out of 39 patients were diagnosed with AK. One isolated amoeba was successfully axenized and used in this study (Fig. 1). This patient was a 22-year-old man with a history of soft contact lens wear and complained of eye pain, excess tear production, photophobia, foreign body sensation and decreased visual acuity.

![Fig. 1](image_url). Light microscopic images of trophozoites and cysts. (a), Trophozoites of *Acanthamoeba* T4 genotype in proteose peptone-yeast extract-glucose medium (b), cysts of *Acanthamoeba* T4 genotype after adding encystation buffer (magnification: ×400, scale bar: 30 μm).
Amoeba cultivation and genotyping

In order to perform differential gene expressions between the trophozoite and the cyst stages, the trophozoites were grown axenically in proteose peptone–yeast extract-glucose medium (PYG) [(proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), glucose 1.5% (w/v)) at 25 °C. The isolated trophozoites were genotyped (GenBank access. no. JQ903617) based on the Diagnostic Fragment 3 (DF3), a region of small subunit rRNA gene (Niyati et al. 2009a).

Encystation condition

The trophozoites in late exponential phase were used for encystment studies. The encystment was allowed to proceed under optimal conditions according to Bowers and Korn protocol with slight modifications (Moon et al. 2008a). Briefly, trophozoites were grown in post-log-phase and washed with encystment medium then incubated in the encystment medium including 95 mM NaCl, 5 mM KCl, 8 mM MgSO4, 0.4 mM CaCl2, 1 mM NaHCO3 and 20 mM Tris–HCl [pH 9.0] at 25 °C for 72 h. In order to remove the remaining trophozoites, the pellets were resuspended in 1 x Phosphate Buffered Saline (PBS) containing sodium dodecyl sulfate (SDS) (0.5% [wt/vol] final concentration). Subsequently, the number of cysts was calculated by light microscopic examination.

cDNA amplified fragment polymorphism (cDNA-AFLP)

Total RNA extraction

Total RNA extraction was carried out from 2 x 10^6 trophozoites during the log-phase and 2 x 10^7 cysts using Tripure reagent (Roch, Mannheim, Germany) according to the manufacturer’s instruction. Briefly, the cells were resuspended in 1 ml Tripure reagent and destroyed by vortexing with glass beads 5 times, 45 s each, with cooling on ice for 15 s in between. The cell lysates were mixed with chloroform vigorously and centrifuged at 12,000 g for 15 min at 4 °C. Then, the upper colorless phase was precipitated by adding isopropanol and washed with 75% ethanol. Finally, the pellets were incubated at room temperature and dissolved in DEPS-treated water. The quantity and quality of RNA were analyzed using Nanodrop (ND-1000, Thermo scientific Fisher, US) and gel electrophoresis, respectively. To avoid any genomic contamination RNA were treated with DNase (Qiagen, Hilden, Germany).

cDNA-AFLP

cDNA-AFLP was conducted as previously described (Saffari et al. 2009). Briefly, single strand cDNA (sscDNA) was synthesized with 10 μg of total RNA, 20 pmol/μl random hexamer (Fermentas, Burlington, Canada), 20 pmol/μl Oligo(dT) (Fermentas, Burlington, Canada), 10 mM of dNTP mix (Fermentas, Burlington, Canada) incubated at 65 °C for 5 min following by addition of 20 U Ribolock RNase inhibitor (Fermentas, Burlington, Canada), 4 μL of 5 x reverse transcriptase buffer containing Tris–HCl (pH 8.3) (Fermentas, Burlington, Canada) and 200 U RevertAid premium Reverse Transcriptase (Fermentas, Burlington, Canada), then incubated at 25 °C for 10 min followed by 60 min at 50 °C. The integrity of cDNA was checked with primers of 18S rRNA gene (Table 1). The PCR condition was an initial denaturing step at 96 °C for 4 min and 30 repetitions of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 72 °C for 7 min. Double strand cDNA (dsDNA) was generated using 40 U DNA Polymerase I (Fermentas, Burlington, Canada), 1.5 U Ribonuclease H at 16 °C for 2 h, followed by purification with High Pure PCR Cleanup Micro Kit (Roche, Mannheim, Germany). Double restriction digestion of purified dsDNA was undertaken using 5 U EcoRI and 5 U MboI (Fermentas, Burlington, Canada) at 37 °C for 2 h. Digested dsDNA products were ligated by AFLP adaptors (AD) (Table 1), 8 μg AD EcoRI, AD MboI and 4 μg ad EcoRI, ad MboI. Ligation was performed in a final volume of 60 μl; it was carried out in following conditions: 1 min at 55 °C, decreasing to 10 °C over 45 min (i.e. 1 °C per 1 min) then 6 U T4 DNA Ligase (Roche, Mannheim, Germany) was added to reaction and incubated at 4 °C overnight. Having done purification with High Pure PCR Clean up Micro Kit (Roche, Mannheim, Germany), pre-amplification with pre-amp primers (Table 1) was performed according to this program: 30 cycles of 94 °C, 30 s, 64 °C, 30 s, and 72 °C, 45 s and final extension at 72 °C for 7 min. In order to get optimal conditions in sensitive amplification, different dilutions of pre-amplified products were used. Sensitive primers consist of the adaptor sequences with an extra nucleotide at the 3’ end (Table 1). Products resulting from sensitive amplification were separated on 10% non-denaturating PAGE and stained with silver nitrate. Finally, the gels were sealed and scanned and checked for selection of differentially expressed TDFs. Ten TDFs either downregulated or upregulated in the cyst compared to trophozoite stage, were selected for further analysis.

Identification of differential gene expression

Isolation, cloning and sequencing

The appropriate TDFs were cut out and extracted from PAGE. The eluted DNA was re-amplified by selective primers (Table 1) in 30 cycles with the selective primers from resultant profile. After checking PCR products on 2% agarose gel, they were cloned into a pGEM-T Vector System I (Promega, Madison, WI, USA). Recombinant plasmids were subjected to sequencing using universal primers (T7 promoter and SP6) (Bioneer, Seoul, Korea) to identify the cloned TDFs. Homology searches were conducted in non-redundant nucleic and protein databases BLAST. (http://www.ncbi.nlm.nih.gov/BLAST/).
Table 1. A list of primer and adaptor sequences used in cDNA-AFLP and real time RT-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA AFLP adaptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD EcoR 1</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
</tr>
<tr>
<td>ad EcoR 1</td>
<td>5’-AACGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
</tr>
<tr>
<td>AD Mbo I</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
</tr>
<tr>
<td>Ad Mbo I</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
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<tr>
<td>cDNA AFLP primers</td>
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<td></td>
</tr>
<tr>
<td>pr EcoR 1</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
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<tr>
<td>Pr Mbo I</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
</tr>
<tr>
<td>S1 EcoR 1</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
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<tr>
<td>S2 EcoR 1</td>
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<td>S1 Mbo I</td>
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<td>S1 Mbo I</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
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<tr>
<td>S2 Mbo I</td>
<td>5’-ACCGACGTCGACTATCCATGAAGAATTC-3’</td>
<td></td>
</tr>
<tr>
<td>Real time RT-PCR primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HkF HkR</td>
<td>5’-TCCTGACCACTGGCGTTCATTTAGC-3’ 5’-GCAGATGCTTTTGCAGAAGT-3’</td>
<td>183 bp</td>
</tr>
<tr>
<td>HspF HspR</td>
<td>5’-GACTTCCTTGCCGAGACCGTA-3’ 5’-CCCGCCTACTCCAAGACT-3’</td>
<td>128 bp</td>
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<tr>
<td>ActF ActR</td>
<td>5’-CAGGGCATACACCCTCGATAG-3’ 5’-CATGTACGTCGCCATCCAG-3’</td>
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<tr>
<td>EfF EfR</td>
<td>5’-GTGGACTATTCGCTATTAGC-3’ 5’-TGGACACCGAGGGTGAAG-3’</td>
<td>107 bp</td>
</tr>
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</table>

Real time PCR analysis

To confirm differences in expression patterns of identified genes between the trophozoite and cyst, a real time reverse transcriptase-PCR (RT-PCR) was conducted. Target gene primers were designed corresponding to identified gene sequences by primer3 version 0.4.0, (http://frodo.wi.mit.edu/) (Table 1). cDNA was synthesized from 1 μg of total RNA using QuantiTect® Reverse Transcription (Qiagen, Hilden, Germany) according to manufacturer’s instruction. This experiment was carried out in triplicate with 20 μL volumes using iQ SYBR green Super mix (Takara, Shiga, Japan), in an RT-PCR machine (Corbett, CG-3000, Australia). The thermocycling condition was set as follows: 95°C for 5 min, then 48 cycles of 95°C for 5 s and 60°C for 34 s. The relative expression level of each gene was determined by comparing the cycle thresholds (C Ts) of the target genes with 18S rRNA as a housekeeping gene using 2^-ΔΔCT method and REST 2009 software as previously described (Pfaffl et al. 2002). Serial dilutions of first-strand cDNAs were used for calculation of the efficiencies of the primer sets on real-time PCR. We found that the efficiencies of the different primer sets were similar.

In silico study

To analyze the protein–protein interactions, the proteins were mapped with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 9.1 (http://string-db.org/). The predicted networks were constructed on physical and functional interactions of Entamoeba histolytica which is in the same order as Acanthamoeba.

Results

cDNA-AFLP

The isolated Acanthamoeba was shown to belong to the T4 genotype when analyzed by 18S rRNA gene sequences. The morphological characteristics of the trophozoites and the cysts are shown in Fig. 1a, b. Differential mRNA expression profiles using cDNA-AFLP were resolved on a non-denaturing PAGE (Fig. 2). Several differentially expressed TDFs were observed. Each desirable TDF could contain different transcripts due to restriction enzyme digestion in cDNA-AFLP. Therefore, isolated TDFs were cloned and different recombinant plasmids (at least three colonies form each hand) subjected to sequencing. The BLAST search showed that three sequences exhibited significant homology to genome database. Three genes, heat shock protein70 (hsp70), actin-1, elongation factor 1-alpha (EF-1α) were identified which differentially expressed in the trophozoites compared to cysts. Also, three unknown genes were detected with low mRNA expression levels in trophozoite form. Our BLAST search showed that distinct TDFs can identify the same transcript. Table 2 shows the details of each sequencing result including GenBank accession numbers and DNA sequence of identified genes.
Validation of cDNA-AFLP findings

In order to validate the determined genes via cDNA-AFLP, their mRNA expression levels were analyzed using a real time RT-PCR technique on trophozoites and cysts samples. We found significant upregulation of hsp70 11.76-fold, actin-I 10.63-fold and EF-1α 22.73-fold in the trophozoites compared to cysts (P < 0.001) (Fig. 3). In addition, three unknown genes were dramatically downregulated in the trophozoites versus cysts (data not shown).

Predicting regulators in the Acanthamoeba differentiation

To get further insight into biological activities of hsp70, EF-1α and actin-I we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. As shown in Fig. 4, several important regulatory proteins were potentially associated with the expressed genes obtained from the cDNA AFLP approach.

Discussion

Although antimicrobial drugs can be successful in the treatment of AK infection, it can induce encystment which can lead to several relapses (Kumar and Lloyd 2002; Yang et al. 2001). To date, several studies reported the differential mediating factors in Acanthamoeba (Moon et al. 2011; Fouque et al., 2012). In the present work, we identified dysregulation of hsp70, actin-I, and EF-1α in Acanthamoeba life cycle via a cDNA-AFLP strategy. Based on the literature these genes appear to be potential novel markers in Acanthamoeba differentiation. In addition, we isolated the TDFs, which were upregulated in cysts compared to the trophozoite stage, but sequencing analysis did show no significant homology with known genes in any genome database. These results are quite reasonable, since the Acanthamoeba genome has not yet been completely identified (Anderson et al. 2005). Further
work is therefore needed to identify these genes potentially specifically expressed in the cyst stage.

Our results demonstrated that hsp70 was upregulated in the trophozoite. Heat shock proteins (HSPs) are expressed in a wide range of living organisms such as parasites, rodents and human beings. It is well documented that these proteins are not only induced in response to stimulations, but their expressions are depended on cellular differentiation. Silva et al. (1998) showed a high level expression of hsp70 during conversion of bradizoites of Toxoplasma gondii to tachyzoites. Furthermore, several lines of evidence suggested that other factors including genetics, epigenetics, protein-protein interaction and gene-environmental interaction could be key elements in the HSPs regulation. For instance, SWI and SNF proteins are large and well-studied families of remodeling complexes. The SWI/SNF as one of the most important genetic factors is involved in distinct biological processes such as controlling cell-cycle progression, DNA-damage repair and survival, which probably reflected the uniquely activated phenotype (Liu et al. 2012). De La Serna et al. (2000) reported that the induction of hsp70 was depended on the SWI/SNF components, BRG1 and BRM proteins.

Various studies showed that DNA methylation as an epigenetic factor has a significant function in gene expression and development. Most recently, Gan et al. (2013) studied DNA methylation in relation to chicken hsp70 gene expression. They suggested that the expression of hsp70 could be correlated with the DNA methylation pattern in the hsp70 promoter. Despite the fact that deregulation of the hsp70 has been reported in different life cycle stages in protozoa, to our knowledge, this is the first report that determined the upregulation of hsp70 in trophozoite of Acanthamoeba. Our results, in agreement with previous studies, suggest that hsp70 expression in response to genetic and epigenetic factors could play critical roles in the establishment and life maintenance of a wide range of living organisms.

In addition, we detected an upregulation of EF-1α in the trophozoite. The EF-1α is essential for the enzymatic delivery of aminoacyl tRNAs to the translational machinery (Yager and von Hippel 1987). Also, another relevant function attributed to this protein is its capability to bind to the mRNAs and the cytoskeleton (Liu et al. 2002). In fact, many lines of evidence support the involvement of EF-1α in cytoskeletal membrane trafficking and processing as an actin/microtubule-binding protein (Edmonds et al. 1996). Bouyer et al. (2009) studied protein expression in trophozoite and cyst forms of A. castellani using two-dimensional gel electrophoresis. They could detect Actophorin as an actin-binding protein specifically in the trophozoite stage. Based on the nature function of such proteins during the life cycle, it is quite reasonable that expression of these biomolecules is vital for any stage in living organisms. However, the rate of their expression will vary according to cellular and environmental circumstances. Consistent with this fact, Norman et al. (2010) determined that human B-actin (ACTB) and EF-1α promoters had stable activities during long-term culture of undifferentiated human embryonic stem cells (hESCs). However, they found significantly reduced activities of the promoters during embryoid body (EB) differentiation. Therefore, these findings, in line with our data, suggested that the modulation of EF-1α could be a key element in Acanthamoeba differentiation.

Furthermore, we observed upregulation of actin-I in the trophozoites as compared to cysts. Actin-I is a major feature of the cytoskeleton in eukaryotic cells and is involved in various cellular processes such as differentiation, regulation of cell growth and motility (Pollard and Cooper 2009). Pervious studies reported that protein expression of trophozoites and cysts of Acanthamoeba showed prominent differences in actin dynamics (Bouyer et al. 2009; Leitsch et al. 2010). The expression status of actin has been studied in distinct organisms such as Giardia lamblia (Castillo-Romero et al. 2009) and Balamuthia (Siddiqui et al. 2010). The encystation of some organisms such as A. castellani is associated with profound morphological modifications and is controled by actin regulatory pathways (Dudley et al. 2008). Initially, down-regulation of actin was demonstrated during the encystment of Acanthamoeba castellani by Jantzen (1981). Recently, Leitsch et al. (2010) reoprted that actin was slightly down-regulated during early level of encystment. These results are
in accordance with our findings in which transcription of actin-I gene was 10.63 times lower in the cysts compared to the trophozoite stage.

Finally, in order to speculate a potential biological pathway by which the aforementioned genes act in cellular developments, we performed an in silico study using the STRING program. Various interacting protein partners were predicted. In order to get more specific protein partners in the Acanthamoeba, we changed the required confidence (score) of the STRING program from medium (0.4) to high (0.7). So, under this condition Plastin and Dnaj proteins were predicted to interact either directly or indirectly with actin protein. The Dnaj family proteins act as molecular chaperones, alone or in interaction with hsp70 partners, and are involved in vital cellular processes such as, protein folding, refolding and degradation (Craig et al. 2006). The interaction of Plastin as an actin-bundling protein seems to be crucial for fundamental cellular functions such as cell morphology, and cell motility. Consequently, our experimental and computational analysis suggested that hsp70, EF-1a and actin-I might be potential regulatory elements in the Acanthamoeba life cycle processes.

In conclusion, through this study, we identified regulation of hsp70, actin-I and EF-1a genes in different stages of Acanthamoeba. Also, the biological significances of identified genes were searched using STRING databases. These predicted network proteins would be valuable tools for understanding the mechanism(s) by which Acanthamoeba could underlay encystation. Taken together, identification of genes which are involved in the Acanthamoeba differentiation would improve our knowledge and may help developing novel efficient chemotherapy to treat AK.

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