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Brief communication (Original)

Differentially expressed genes of *Naegleria fowleri* during exposure to human neuroblastma cells

Supathra Tiewcharoen[a], Jundee Rabablert[b], Sittiruk Roytrakul[c], Waravee Wiyawuth[d], Nat Malainual[a], Virach Junnu[a], Kumchol Chaiyo[a, e], Prasert Auewarakul[e]

[a]Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, [b]Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, [c]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani 12120, [d] Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, [e]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Background: Free-living amoeboflagellate, *Naegleria fowleri* causes acute fulminant primary amoebic meningoencephalitis (PAM). Interaction of *N. fowleri* with human cells is essential for the cytopathogenic process before phagocytosis and tissue invasion.

Objective: In order to understand the pathogenesis mechanisms of *N. fowleri*, subtractive cDNA library was used to analyze overall induction in gene expression of *N. fowleri* during exposure to the human neuroblastoma cells.

Methods: Co-cultivation of *N. fowleri* and human neuroblastoma SK-N-MC monolayer cultivation was performed. Subtractive cDNA libraries of inoculated *N. fowleri* at 15, 30, 45, 60, and 120 minutes were constructed. The PCR amplified products were cloned into *E. coli*. The specific clones were selected and further sequenced. Nucleotide sequences were compared with those deposited in the Genbank using BlastX. Significant probabilities were considered when E-value was less than 10^{-4}. The induction of several gene expressions was validated by real-time RT-PCR.

Results: Extensive changes in gene expression of *N. fowleri* during the interaction with the human neuroblastoma SK-N-MC *in vitro* were detected. One hundred twenty clones were obtained. Among these, five clones containing DNA sequence homologue to known genes were identified. These genes included acetyl Co-A synthetase, 18s ribosome RNA, naegleria pore B precursor, isocitrate dehydrogenase, and pyruvate kinase. Real-time quantitative RT-PCR indicated that the expression level of all five genes was up-regulated within 1 hour after exposure. The expression level of acetyl Co-A synthetase increased and reached 7-times significantly greater than that of the control while pyruvate kinase, isocitrate dehydrogenase, naegleria pore B precursor, and 18s ribosome RNA transcripts increased from 2- to 4-fold, respectively.

Conclusions: A defined set of genes in *N. fowleri* that was differentially transcribed after contacting with the SK-N-MC monolayer cells was identified. The transcription profiles unique for amoebic cell may help elucidate the transcriptional framework of *N. fowleri* pathogenicity and serve as a basis for identifying transcriptional virulence factors.

Keyword: cDNA subtraction, human neuroblastoma cells, *Naegleria fowleri*

Free-living *Naegleria fowleri* amoeba, causative agent of acute primary amoebic meningoencephalitis (PAM), are fatal pathogens of humans and animals [1]. Pathogenesis occurs from the invasive amoebae entering the nervous system through the olfactory nerve, causing extensive inflammation, hemorrhage, and subsequently necrosis.

Although brain is the target organ of *N. fowleri*, the studies concerning cytopathogenesis of *N. fowleri* in the brain are still lacking, especially on the genes responsible for its virulence that corresponds to the real time situation. This information is essential to understand cytopathogenesis of *N. fowleri* and could represent the degree of degeneration of brain tissue

Correspondence to: Jundee Rabablert, Department of Biology, Faculty of Science, Silpakorn University, Sanamchan Palace, Nakhon Pathom 73000, Thailand. E-mail: jundee04@gmail.com

[2]. In the present study, we aimed to study specific virulent genes by subtractive hybridization technique in a co-culture model of *N. fowleri* and human neuroblastoma monolayer cells. Human neuroblastoma cells were selected due to their specificity characteristics [3-7].

The subtractive cDNA hybridization is a technique that enables researchers to compare two populations of mRNAs. Differentially expressed gene profiling using cDNA subtraction has been an ideal tool to identify novel genes and transcripts of low abundance [8-11]. Owing to the unavailability of genome sequence data at the time, the subtraction cDNA library approach was therefore used to identify the transcriptional changes during the initial step of *N. fowleri* attached to SK-N-MC cells. Numerous *N. fowleri* gene expression changes upon contact to neuroblastoma were detected. Real-time RT-PCR of selected genes was carried out on RNA samples prepared from the amoeba subjected to human brain cell exposure.

Materials and methods

Naegleria fowleri cultivation

Free-living *Naegleria fowleri* amoebae (strain Khon Khan) were cultured at the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. Trophozoites were grown axenically in batch culture at 37°C in Nelson medium supplemented with 5% heat-inactivated fetal bovine serum. The virulence of *N. fowleri* amoebae was maintained with Vero cells. Molecular characteristic of *Naegleria* amoeba was confirmed by specific primers: internal transcribed spacer (ITS) and specific virulent *nfa1* primers.

Human neuroblastoma cultivation

Human neuroblastoma monolayer cells, SK-N-MC, obtained from a Caucasian female patient with Askin's tumor from metastasis at supra–orbital area, was purchased from Cell line Service, Germany in 2006. The cells were cultured in tissue culture T 75-cm^2 flasks containing Dulbecco's Modified Eagle Medium and HAM's F-12 (DMEM: HAM'S F-12) nutrient mixture without antibiotics. The SK-N-MC cell was incubated in 37°C plus 5% CO_2 incubator for 3 days. The completed monolayer of SK-N-MC was approximate 1×10^7 cells.

Host-pathogen interaction

Human neuroblastoma cells were used as a cytopathogenesis model of *N. fowleri* as mentioned in our previous report [12]. Briefly, 2.5×10^6 SK-N-MC were seeded to T75 culture flasks containing Dulbecco's modified Eagle's medium–HAM-F12 (1:1, vol/vol) and incubated at 37°C and 5% CO_2 for 5 days to obtain completed monolayer culture. The SK-N-MC was then washed with a medium mixture without serum. Amoebae at the mid-logarithmic growth phase were added to the SK-N-MC monolayer at ratio of 1:1 and incubated at 37°C to allow adherence of the amoebae. The amoebae were incubated with the SK-N-MC for 30 minutes, and non-adherent amoebae were removed by aspiration. The monolayer with adhered amoebae was further incubated for 2 hours until the monolayer was completely lysed. The amoebae after interaction with the monolayer were referred to as primed amoebae.

PCR-based cDNA subtractive hybridization

Total RNA from control and primed amoebae after contact with SK-N-MC was isolated with NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instruction. The double-stranded cDNAs were synthesized from 1 ìg total RNA of each group with a Smart PCR cDNA synthesis kit (BD Clontech, Mountain View, CA). Then, the cDNA was used in the suppression PCR-based subtractive hybridization using a PCR-select cDNA subtraction kit (BD Clontech). The cDNAs prepared from control and primed amoebae were regarded as driver and tester, respectively. The driver cDNA population was subtracted from the tester cDNA population. The tester and the driver cDNAs were digested with Rsa I. The tester cDNA was separated into two groups, and each was ligated with different cDNA adapters. In the first hybridization reaction, an excess of driver was added to each sample of the tester. The samples were heat-denaturated and allowed to anneal. Because of the second-order kinetics of hybridization, the concentration of high- and low-abundance sequences was equalized among the single-stranded tester molecules. At the same time, single-stranded tester molecules were significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples were mixed together without denaturation. Only the remaining equalized and subtracted single-stranded tester cDNAs

can re-associate forming double-stranded tester molecules with different ends. After filling in the ends with DNA polymerase, the entire population of molecules was subjected to nested PCR with two adapter-specific primer pairs. Then, secondary PCR products were used as templates for PCR amplification of â actin gene, a housekeeping gene, at 18, 23, 28, and 33 cycles to assure subtracted efficiency.

Cloning of subtracted cDNA libraries

Products of these amplified overhangs containing a subtracted cDNA library (6 µl) were ligated into a pGEM-T-easy vector (Promega Corp., Madison, WI). Subsequently, the plasmid was transformed into *E. coli* strain DH5á. Bacteria were cultured in 800 ì l of LB medium and incubated for 45 minutes at 37°C with shaking. After incubation, bacteria were plated onto agar plates containing ampicillin (100 mg/L), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 20 µg/ml), and isopropyl-β-D-thiogalactoside (IPTG, 12.1 µg/ml) and incubated overnight at 37°C. White colonies were selected and identified by colony PCR. DNA bidirectional sequencing was performed and sequences were edited manually to remove contaminants from the vector, primer sequences, and poly A tails. All the inserted sequences were checked for their identity using the BLASTX program against the Genbank non-redundant sequence database.

Quantitative real-time RT-PCR

Total RNA from control and primed amoebae was isolated with Tri Reagent (Molecular Research Center, Inc.), according to the manufacturer's instruction. Total RNA from control and primed amoebae was reverse transcribed using oligo dT 20 primer and ImProm-II Reverse transcription system (Promega, USA). The synthesis of the first-strand cDNA was performed at 42ºC for 60 minutes. The SYBR Green real-time RT-PCR was performed using iCycler iQ Real-time PCR System (Bio-Rad). The experimental cycling profile was set as recommended in the manufacturer's manual for QuantiTect SYBR Green PCR kit (QIAGEN). The real-time PCR results were presented as the relative expression levels of target genes to those of the housekeeping reference gene *Naegleria* â-actin. Data were presented as the means±standard error of the means (n = 3).

Results and discussion

PCR-based cDNA subtraction hybridization

The PCR-based cDNA subtraction method was used to isolate differentially expressed cDNAs from two different cDNA populations called the tester (primed amoebae) and the driver (amoebae). The driver cDNA population was subtracted from the tester cDNA population by hybridization, and the cDNAs present only in the tester population were enriched and PCR-amplified. This one way subtraction approach was used to enrich the primed amoebae cDNAs that were absent in amoebae control. After the subtractive hybridization, 120 cDNAs were cloned and subsequently sequenced. BlastX analyses revealed that five clones were identical to the known *N. fowleri* genes (**Tables 1 and 2**). They are naegleriapore B precursor (NP-B), isocitrate dehydrogenase, NADP-dependent pyruvate kinase, acetyl Co-A synthetase, and 18S ribosomal RNA. The homology analysis indicated that these genes are involved in metabolic processes of carbohydrate, fatty acid, and protein. Nevertheless, antimicrobial function and translation may also involve.

Real time-PCR confirms increased gene expression

Quantitative RT-PCR was used to validate the relative transcriptional level of these five genes, which were predominantly up-regulated upon contact with the host cells. The relatively high expression level of these genes was shown in **Figure 1**. Compared to the control, the range of increased expression varied from two-fold (isocitrate dehydrogenase) to seven-fold (acetyl Co-A synthetase) at 60 min-post exposure. These data re-affirmed the up-regulation of genes identified in our subtraction library.

Table 1. BlastX evaluation of cDNA clones from the subtractive cDNA library of *Naegleria fowleri*

Clone no.	Genbank no.	Name	Score	E value
1	XM_002673287.1	*Naegleria gruberi* isocitrate dehydrogenase NADP-dependent	251	2e-64
4	XM_002681017.1	*Naegleria gruberi* pyruvate kinase	220	6e-55
5	XM_002677147.1	*Naegleria gruberi* acyl CoA synthetase	228	8e-76
6	AF196309.1	*Naegleria fowleri* naegleriapore B precursor (NP-B) gene	300	7e-79
15	U80059.1|NFU80059	*Naegleria fowleri* 18S ribosomal RNA gene	292	6e-86

Table 2. Primers for identification of clones from subtractive cDNA library used in the confirmation of differential expression by qRT–PCR

Genbank no.	Name
XM_002673287.1	Isocitrate dehydrogenase NADP-dependent Forward: AACATCGTCTTATTGATGACATGG Reverse: CCTCGGACTCAACAGTCTTACC
XM_002681017.1	Pyruvate kinase Forward: AACAGTCATGAATACTCGTCTTGC Reverse: CAACAATAAAAATCAATCAATTGTCC
XM_002677147.1	Acyl CoA synthetase Forward: CCGCTGGTGGTTGTATCC Reverse: ACCCTTAATGGCAATTTCACC
AF196309.1	Naegleriapore B precursor Forward: TTGATGTCAATGCTGTCAAGC Reverse: CTTTGGGCAGACATCAACG
U80059.1|NFU80059	18S ribosomal RNA gene Forward: GCGATAATACTTGTTCCCTTCG Reverse: GGCCACTAGAAAAAGCAAACC

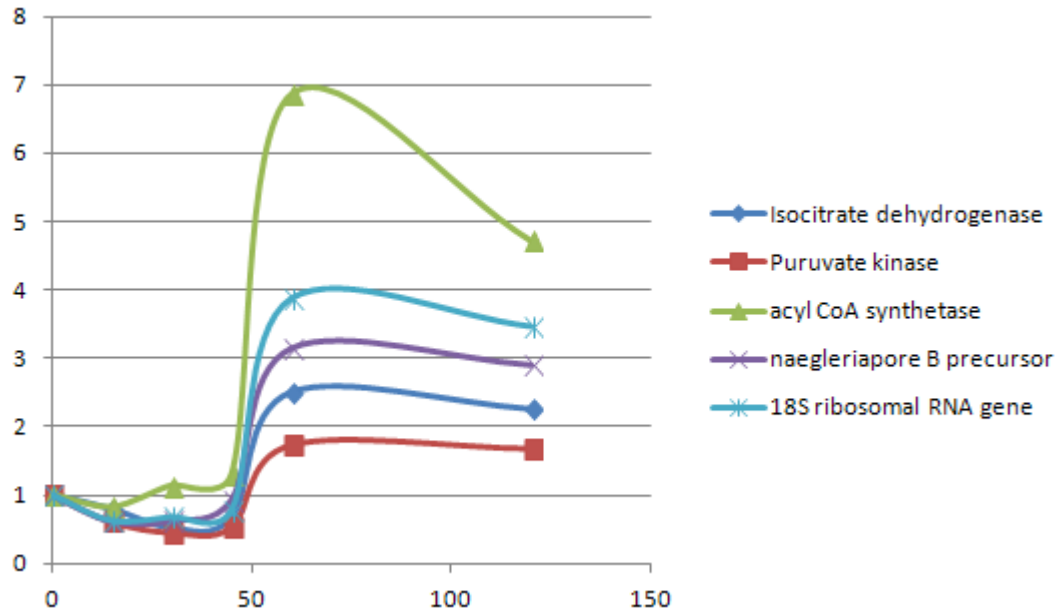

Figure1. Over expression in four pairs of candidate cDNA clones using real-time RT-PCR for genes encoding acetyl Co-A synthetase, 18s ribosome RNA, naegleria pore B precursor, isocitrate dehydrogenase, and pyruvate kinase after contacting with SK-N-MC at 0, 15, 30, 45, 60, and 120 minutes.

Isocitrate dehydrogenase (ICDHs) catalyzed oxidative decarboxylation of isocitrate to α-ketoglutarate and required either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [13]. NADPH is an essential reducing agent equivalent to the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [14]. Both are important for the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. In this study, predominance of ICDHS was found to be consistent with the previous report of Tiewchareon et al. [15], which demonstrated the ICDHs in zymogram pattern of *N. fowleri*. We have confirmed that the activity of ICDHs involved both structural and functional proteins during

cytopathogenesis activity of *N. fowleri*. Several researchers studied the correlation of ICDHs with pathogen organisms. Zemanová et al. [16] revealed that ICDHs were new targets to identify *Leishmania donovani* complex, the causative agents of human cutaneous and visceral leishmaniasis. The phylogenetic analysis of a concatenated alignment of gene (263 kb) and the functions played a role on respiratory system and electron transport chain of *E. coli*. The ICDHs activity was also reported in metabolic regulation analysis of ICDHs gene knockout *E. coli* based on 2D electrophoresis followed by MALDI-TOF mass spectrometry and enzyme activity measurements. The results showed that the abolition of ICDHs activity significantly affected the respiratory system and electron transport chain, as evidenced by the significant down regulation of proteins encoded by the genes *nuoE, nuoH, cydA,* and *cyoA* in icd mutant of *E. coli,* compared to the parents [17]. In this report, our *in vivo* study indicated that ICDHs activity appears to be modulated through enzymatic glutathionylation and deglutathionylation during oxidative stress [18].

Pyruvate kinase (PK), catalyzes the final step in glycolysis converting phosphoenolpyruvate to pyruvate, is a central metabolic regulator in most organisms [19]. We found that pyruvate kinase activity decreased after the neuroblastoma cells co-cultured with *N. fowleri*. This finding was in agreement with the report of Feksa et al. [20] that the diminution of pyruvate kinase activity may contribute to the brain damage in patients suffering from Malaria disease.

In our results, acetyl Co-A synthetase increased after the cDNA subtraction between neuroblastoma cells and *N. fowleri*. It was consistent with the report of Hu et al. [21] who demonstrated that acetyl Co-A increased in pulmonary cells after being infected with *Crytococcus neoformans.* Acetyl Co-A is a central metabolite in the balance between carbohydrate metabolism and fatty acid catabolism. Regulation of the balance may be a specific adaptation to the host environment by *N. fowleri,* as suggested by the abundance of transcripts for enzymes mediating the production and utilization of acetyl Co-A during neuroblastoma infection. However, the mechanism of *N. fowleri* on pathogenesis of brain damage has been unknown. Rivière et al [22] found that an acetyl Co-A synthetase gene encoding a cytosolic enzyme (AceCS) is essential for viability of *Trypanosoma brucei,* a protozoan parasite responsible of human sleeping sickness, an economically important livestock diseases. It was suggested that acetyl Co-A synthetase gene may be involved viability of *N. fowleri* during its contact to the host.

Naegleriapore B precursor genes encoded naegleriapores A and B proteins that potently display pore-forming activities that kill both prokaryotic and eukaryotic target cells [23, 24]. This implies that the discharged cytolytic factor may facilitate invasion of the amoeba into the brain. Moreover, rapid killing of the effector cells of the immune system by amoebic pore-forming peptides may explain why the human defense system is, at least in several cases, unable to prevent the invasive process. Accordingly, in the fatal conversion from a free living, bacteria-hunting amoeba to the parasite that assaults the human body, the cytolytic armament particularly the pore-forming peptide may play a pivotal role [25].

In addition, we found specific small-subunit (18S) rRNA gene. It has been previously reported that the 18S rRNA gene was used for identification of clinical specimens infected with *Acanthamoeba* and *Naegleria* [26, 27]. Although the relationship between the 18 s rRNA genotypes and the *Naegleria*-infected neuroblastoma cells is still in question, certain relation between specific small-subunit (18S) rRNA gene of *N. fowleri* and brain cytopathogenesis should not be overlooked.

Further functional analyses of these genes may gain an insight into the process that occurs during the cell adherence and directly elucidates the mechanisms of cytopathogenesis in the target cell.

Conclusions

Subtraction hybridization cDNA of *N. fowleri* trophozoites after contacting with SK-N-MC monolayer cells showed a rapid increase in expression of acyl Co-A synthetase, 18s ribosome RNA, naegleria pore B precursor, isocitrate dehydrogenase, and pyruvate kinase transcripts. The expression of these genes may be the key event that enables *N. fowleri* to invade into the brain cells.

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