Identification of proteins in promastigote and amastigote-like stages of *Leishmania tropica* by a quantitative proteomic approach

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Research

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Abstract

Background Leishmania parasites differentiate into the infective metacyclic form in the sand-fly and following is transformed to the amastigote in the host cells. The aims of this study were to identified differentially regulated proteins in the metacyclic and amastigote-like stages of L. tropica, and investigate their potential role in differentiation and pathogenesis molecular mechanisms.

Methods The samples were cultured and identified by using PCR-RFLP technique. We employed We employed Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) to identify differentially regulated proteins between the metacyclic and amastigote-like stages of L. tropica.

Results A total 176 and 155 distinct proteins were identified in metacyclic and axenic amastigote, respectively. Of these, 65 proteins were altered at least 2 folds. Proteins that were up- or down-regulated included energy production, lipid and amino acid metabolism, heat shock proteins, mRNA processing proteins, glycolytic and translational activity proteins. Several enriched GO terms were identified via biological process analyses, which “metabolic process” (GO: 0044281, P-Value: 6.52e-5), and “translation” (GO: 0006412, p-value: 5.01e-14) were disclosed as top category in up and down-regulated proteins, respectively. Also, the KEGG analysis indicated “metabolic pathways” and “ribosome” term as the top pathways in up and down-regulated proteins, respectively.

Conclusions This study provides the first quantitative analysis of protein expression during differentiation from metacyclic to amastigote-like of L. tropica. In conclusion, anabolic pathways were down-regulated, whereas catabolic pathways were up-regulated during L. tropica differentiation in order to parasite survival in host macrophages, in which these changes can be used as novel potential targets for the infection management.

Background

Parasites of the genus leishmania cause a wide range of disease called leishmaniasis from cutaneous lesions to fatal visceral leishmaniasis. Leishmania species are affecting 12 million people worldwide with 1.5-2 million new cases each year [1, 2]. These parasites have a dimorphic life cycle including extracellular and flagellated promastigote within its vector and an intracellular and non-motile amastigote form within the infected macrophages of their vertebrate hosts. Each of promastigote and amastigote forms are adapted to residue in the different environment include midgut of the sandflies and hydrolytic environment of the phagolysosomes for a long time, respectively. Differentiation from promastigote to the amastigote accompanied by several morphological and biochemical changes which basically depends on the expression of stage-specific proteins [3].Yet, there is no vaccine for leishmaniasis and the control of these protozoa relies only on chemotherapy. The first-line of treatment relies on pentavalent antimony (sbv) compounds and drug resistant parasites emerging worldwide [4] such as Iran. L. major and L. tropica are the causative agents for cutaneous leishmaniasis in Iran and some of the neighboring

countries [5, 6]. Since the parasites regulate gene expression mainly at post-transcriptional stages, “OMICS” approach including genomics, proteomics [7, 8], metabolomics [9] along with bioinformatics analysis [10] is thought to yield critical insight into the mechanisms of stage differentiation, species differences, virulence and drug resistance [11–13].To understand the differences during transformation and between the molecular levels, there are several reports about the proteome of promastigotes and amastigotes forms of L. major, L. infantum, L. donovani and L. Mexicana. Most of these investigations used 2DE map to detect global differences of different life stages of different species [14], which may be due to post-translational modifications (PTMs) affect charged amino acids [15].Therefore, gel free approaches provide a significant resource to higher proteome coverage and more precise quantitative information. SWATH is a recently developed label free quantitative method, in which data independent acquisition is coupled with peptide spectral library match [16, 17]. So far, no differentiation of promastigote into amastigote-like study has been reported in L. tropica. In this study, we have employed label-free quantitative proteomics approach (SWATH) to identify differentially regulated proteins during promastigote and amastigote-like stages of in Iranian isolates of L. tropica.

Methods

Sample collection

A total of five *Leishmania tropica* isolates were collected from patients in Bam city of Kerman province which is endemic region for cutaneous leishmaniasis caused by *L. troica.* This study was approved by Ethics Committee of Shahid Beheshti University of Medical Sciences (Ethical code: IR.SBMU.MSP.REC.1395.351). Informed consent was received from all participating patients in the present study. The identities of the isolates were performed by using PCR-RFLP technique, in which the internaltranscribed-spacer-1 (ITS1) region of the parasites’ ribosomal-RNA gene was amplified, followed by *HaeIII* digestion of the resulting amplicons. To carry out the PCR, we used the primers L1TSR (5′-CTGGATCATTTTCCGATG-3′) and L 5.8 (5′-TGATACCACTTATCGCACTT-3′) as the forward and reverse primers, respectively.

Cell culture and differentiation of *L. topica*

Primary isolates initially were grown on NNN medium and for mass culture, parasites were transferred to RPMI1640 medium (Gibco, Germany) supplemented with %10 FBS (Gibco, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Germany) in 25˚C. Promastigotes were cultured with repeated medium for 6-10 days for achievement the metacyclic promastigotes phase. During this time, the numbers of parasites were counted with light microscope. Then, the parasites of stationary phase divided into two aliquots that the content of one aliquot (10^7 metacyclic form/ml) were centrifuged at 3500 rpm for 20 min at 4˚C and then were washed 3 times with sterile PBS (pH: 7.4) and collected in -70˚C for the protein extraction. The content of other aliquot was used to achieve amastigote-like parasites. To generate amastigote-like form, the change of cell’s environment condition was necessary. After centrifugation of metacyclic promastigotes in 3500rpm for 20min at 4°C, they were washed with

PBS (pH: 7.4). Then, these cells were placed in RPMI1640 and Schneider's Drosophila medium (pH: 3.5-4.4) supplemented with 20-25% FCS (Gibco, Germany), 200 U/ml penicillin, and 200 µg/ml streptomycin (Gibco, Germany) and maintained at 35°C with 5% CO_2 for 96- 120 h. Every day during this period, the cells were examined for observation of lack of flagella and spherical form of cells using Giemsa staining with an optical microscope. After the amastigote-like cells were obtained, 10^7 cells/ml were centrifuged at 2500rpm, 10min, washed with PBS (pH: 4, 37°C) and stored in -70˚C for the protein extraction.

Protein extraction and SWATH-MS analysis

The 1x10 ^{7} cells/ml (each of metacyclic and amastigote-like form) were harvested and dissolved in lysis buffer (containing 8MUrea, DTT, Tris-Hcl, Glycerol, Tween-20 and 1x protease inhibitor cocktail) and incubated for 2h at room temperature. The cell extract was centrifuged at 15,000 g for 15min at 4˚C to remove the cell debris. Protein concentration of supernatant was measured using Bradford assay. The soluble protein extracts were precipitated and stored at -70 ˚C in single-use aliquots. Protein precipitation was carried out in the following steps: i) the proteins were reduced as they spent a long time in DTT in our lysis procedure, ii) 50 mM Tris pH 8.0 was added to the proteins, iii) the proteins were alkylated with 15 mM iodoacetamide for 30min in the dark, iv) the reaction was quenched by adding 10 mM DTT, v) the proteins were precipitated by adding 6 volumes of -20 ˚C acetone and vortex, vi) the protein samples were incubated at -80 ˚C overnight, vii) then the samples were centrifuged at 13,000 rpm for 5min at 4˚C and the supernatant was removed, viii) the pellet was washed 3 times with 6 volumes of -20 ˚C methanol, centrifuged at 13,000 rpm for 2min at 4˚C and the supernatant was removed and ix) finally, the pellet was air-dried. LC-MS/MS was performed at PhenoSwitch Bioscience, laboratory in Sherbrooke, Canada, using ABSciex Triple TOF 5600 instrument (ABSciex, Foster City, CA, USA) equipped with an electrospray interface with a 25 µmiD capillary and coupled to an EksigentµUHPLC (Eksigent, Redwood City, CA, USA). All experiments were done at three replicates. Samples were resuspended in 4M Urea and 25 mM Tris (pH 8.0). Twenty micro grams of each sample was reduced for 15min at 65 °C with 10mM DTT and alkylated for 30 min at room temperature in the dark with 15 mM iodoacetamide. Proteins were digested with 1µg of Trypsin/LysC overnight at 37 °C with agitation. Samples were acidified with 2% formic acid and the peptides were purified by reversed phase SPE. For the IDA mode, the source voltage was set to 5.2 kV and maintained at 225 °C, curtain gas was set at 27 psi, gas one at 12 psi and gas two at 10 psi. For the SWATH mode, the source voltage was set to 5.5 kV and maintained at 225 °C, curtain gas was set at 25 psi, gas one at 16 psi and gas two at 15 psi. Separation was done on a reversed phase HALO C18-ES column 0.3 mm i.d., 2.7 µm particles, 150mm long (Advance Materials Technology, Wilmington, DE) which was maintained at 60 °C. For the 60min (IDA) and 120min (SWATH) LC gradient, the mobile phase consisted of the following solvent A (0.2% v/v formic acid and 3% DMSO v/v in water) and solvent B (0.2% v/v formic acid and 3% DMSO in EtOH) at a flow rate of 3 µL/min. To generate an ion library for the analysis of the samples, we ran the ProteinPilot software on the 12 IDA Wiff files with the combined proteins from the *L. major* taken from the Uniprot database. This ion library was used in the Peakview software (ABSciex) to quantify the proteins in each of the samples, using 3 transition/peptide and 6 peptide/protein maximum. A peptide was considered as adequately measured if the score computed by

Peakview was superior to 1.5 and FDR < 1%. Statistical analysis was done by using t-test. Proteins fold with differences in greater than 2 and *p*-value< 0.05 were identified as significant altered proteins during metacyclic promastigotes differentiation to axenic amastigotes.

Gene ontology and pathway analysis

TriTrypDB (The Kinetoplastid Genomics Resource) (http://tritrypdb.org/tritrypdb/) was used for gene ontology analysis of differentially expressed proteins between two stages based on biological process, molecular function and cellular component. TriTrypDB is an integrated database providing access to genome-scale datasets for kinetoplastid parasites, and supporting a variety of complex queries driven by research and development needs [18]. Pathway analysis of differential expressed proteins were performed by string (http://string-db.org) online database [19].

Protein interaction network analysis

All proteins which had significantly different expressions (up-regulated, down-regulated) in amastigote-like form compared with metacyclic stage were selected for protein-protein interaction network construction. Analyzing the network properties of protein-expression data might reveal the organizational pattern of protein expression in disease, which might in turn help us to identify new potential drug targets. Protein- protein interaction network was constructed by using STRING database, was visualized using the Cytoscape 3.6.0 software [20]. CytoHubba plugin in Cytoscape were selected for high degree (hub) proteins in obtained network. Molecular Complex Detection (MCODE) used to analyze the characteristics of the networks. The MCODE algorithm is used to find densely connected regions (modules) and then to recognize seed nodes as a complex with the highest weighted vertex in each module [21].

Results

LC-MS/MS (SWATH-MS) results

The significant differentially expressed proteins (fold change>2 & p-value<0.05) in the two developmental stages (metacyclic promastigotes and axenic amastigotes) were selected by statistical analysis. As shown in Fig.1, a total 176 and 155 distinct proteins was identified in metacyclic and axenic amastigote stage, respectively, which 81 and 60 proteins of them was expressed uniquely in metacyclic and axenic amastigote-like form, respectively. A total of 65 common proteins were differentially expressed in the two stages as up-regulated and down-regulated proteins, and detailed properties of them present in Table 1. Among differential proteins, 19 and 46 proteins up-regulated and down-regulated during differentiation of *L. tropica* isolates, respectively (Fig.1). One of the differential expressed proteins is hypothetical and its functions in *Leishmania* still remain to be elucidated. Further database mining indicated that the differentially expressed proteins could be classified into 18 groups based on cluster of orthologous groups of proteins (COG) function classification (Fig.2). The COGs classification in the two developmental stages revealed that the up-regulated proteins were foremost involved in energy

production and conversion cluster and down-regulated proteins were more involved in translation, ribosomal structure and biogenesis.

Gene ontology enrichment analysis results

Gene ontology (GO) of total 65 significant differential expressed proteins (up and down regulated) based on cellular component, biological process, and molecular function was performed by the kinetoplastid genomics resource database (TriTrypDB). According to gene ontology analysis of up-regulated proteins, the metabolic process (GO: 0044281), response to stress (GO: 0006950) and catabolic process (GO: 0009056) with 12.55%, 12.28% and 10.96%, had the highest frequency among other biological processes, respectively. Transmembrane transporter activity (GO: 0022857) and ATPase activity (GO: 0016887 and cytoplasm (GO: 0005737), were of high frequency in molecular function and cellular component, respectively (Fig. 3a, 3b and 3c). Most of the down-regulated proteins were involved in metabolic process (1.06e-3) and translation (5.01e-14) (Fig.4a). In the molecular function classifications, most of them are *associated with ion binding (8.37e-4) (Fig.4b). According to the subcellular distributions, down-regulated proteins were enriched in the cytoplasm (26.02% frequency) and organelles (20.79% frequency) which implies that most of the down-regulated proteins in axenic amastigote are cytoplasmic proteins (Fig.4c).* Total 81 and 60 proteins were uniquely expressed in metacyclic and axenic amastigote stage, respectively. Gene ontology enrichment analysis of metacyclic-specific proteins indicating that translation and response to stress, and ion biding, and cytoplasm had the high frequency in biological process, molecular function and cellular component analysis. In addition, translation, molecular function and cytoplasm were the significant GO terms in biological process; molecular function and cellular component of amastigote-specific expressed proteins.

Pathway analysis results

Differentially expressed proteins (up-regulated and down-regulated) were also selected for KEGG pathway analysis. The pathway enrichment analysis was performed using the STRING online database. The pathway enrichment analysis revealed that the most critical pathway of up-regulated and down-regulated proteins involved in *L. tropica* differentiation included metabolic pathways and ribosome, carbon metabolism and glycolysis/gluconeogenesis, respectively (Table 2, 3).

Protein interaction network analysis results

The PPI network of the significantly expressed proteins (compared between metacyclic and amastigote like of Iranian isolates of *L. tropica*) contain 53 nodes and 323 edges (Fig. 5a). Nodes represent the proteins from our list and others that directly interact with them. Connections contain direct interaction partners and interconnections. In order to simplify the connection patterns, interactions for the nodes with the greatest degrees (hubs) was selected. Cytoscape plugin (CytoHubba) analysis revealed the top 10 great number of close interconnections that can be seen with darker color (Fig. 5a). The hub nodes included ENOL, LmjF.35.1180, LmjF.32.0450, LmjF.17.0083, LmjF.15.0200, LmjF.35.0420, LmjF.25.1170, PGKC, LmjF.24.0040 and LmjF.36.0940. Further analysis of complex by MCODE revealed 3 modules for

the network. The seed nodes (yellow nodes in each module) of these complexes included LmjF.07.0510, LmjF.28.2420 and LmjF.24.1630. The orange nodes (6, 3 and 1 nodes in modules 1, 2 and 3, respectively) are the hub proteins that present in modules (Fig.6).

Discussion

We aimed to identify proteins differentially expressed between metacyclic and amastigote-like stages of Iranian isolates of L. tropica. To this end, we used a quantitative proteomic approach to compare proteins of metacyclic and amastigote-like form of L. tropica. To date, several studies have been reported about Leishmania differentiation. Rosenzweig et al. (2008) [15], Lahav et al. (2011) [22], and Saxena et al. (2007) [23] are examples of studies of Leishmania promastigote to amastigote differentiation that employ high-throughput transcriptome and proteomic analyses. In this study, a total of 176 and 155 proteins were detected in metacyclic and amastigote-like form, respectively. Among these 65 significant differential expressed proteins, 46 were down-regulated in amastigote-like form, including 6.25% proteins having unknown function. One of down-regulated proteins (E9AD27) in our results, has also been identified as common protein between L. major, L. tropica and L. infantum from Iranian isolates in the work of Hajjaran et al., by 2DE/LC-MS approach [24]. At present study, another protein (Q4QFL8) has also decreased in amastigote-like form vs metacyclic form of L. tropica. This protein also reported as a differentially expressed protein between meglumine antimoniate sensitive and resistant in promastigote of L. tropica isolated from Iranian anthroponotic cutaneous leishmaniasis patients [25]. Among the 19 proteins over-expressed in amastigote-like form, we identified an uncharacterized protein with non-predicted function. Among up-regulated proteins, energy production and conversion function has the highest percentage, and protein folding/response to stress and lipid metabolism are ranked second in our results. Also, the energy production and conversion cluster included dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, ATP synthase subunit beta, glycosomal malate dehydrogenase and ATPase alpha subunit proteins, which all of them involved in energy generation in the parasite. Malate dehydrogenase catalyzes the oxidative deamination of L-glutamate to α-ketoglutarate, which this enzyme activity links amino acid metabolism with carbohydrate metabolism pathway [26]. Finally, this linked pathway leads to energy production. In addition, according to Rosenzweig et al., results, catabolic pathways that lead to energy production were also up-regulated during the differentiation stages. Specifically, tri-carboxylic acid cycle and respiratory chain activity observed highly correlated expression in amastigote-like stage [15]. Malate dehydrogenase is another up-regulated protein relate to energy production that required for performing the gluconeogenesis process in amastigote forms. Gluconeogenesis is essential process for amastigote proliferation within macrophages [27]. One of the main molecules for perform the gluconeogenesis is NAD-dependent glycerol-3-phosphate dehydrogenase that increased of its level was reported by previous studies. During these processes, the reduced NAD required for this process is provided by the glutamate dehydrogenase [28, 29]. Amastigote forms of L.mexicana and L. major exhibit increased amino acid oxidation activity by using elevated glutamate dehydrogenase activity [28]. In the present study, ATP synthase subunit beta protein up-regulated in amastigote-like stage of L. tropica, and this may be essential for parasite differentiation.

Lipid transport and metabolism involved proteins include succinyl-CoA: 3-ketoacid-coenzyme A transferase, conserved hypothetical protein and possible 3-ketoacyl-CoA thiolase up-regulated also in amastigote-like forms in our analysis. Succinyl-CoA: 3-ketoacid-coenzyme A transferase is a Key enzyme for ketone body catabolism that amastigote form uses these sources for energy production in absence of glucose in macrophage environment. In general, amastigote forms provide their required energy through fatty acid oxidation by elevated TCA enzymes activity and differentiating parasites shift from glucose to fatty and amino acid oxidation and from glycolysis to gluconeogenesis [9, 17, 30]. As mentioned, the proteins involved in amino acid transport and metabolism were also up-regulated in this study. In a quantitative proteomics study using host-derived amastigotes was also observed these changes in metabolic activities. The other up-regulated protein was in terms of protein folding/ response to stress. This cluster includes putative heat shock protein DNAJ, putative lipophosphoglycan biosynthetic protein and mitochondrial chaperonin HSP60. A number of amastigote-specific protein were also observed as heat-shock proteins including HSP60 and HSP70 in Nugent et al., study on L. Mexicana differentiation [31]. Comparative proteomic studies have already shown that proteins involved in stress response are differentially expressed among promastigotes and amasigotes from L. donovani [3] and L. infantum [32]. In addition, the up-regulated response to stress activity must mean that amastigote form struggles with the oxidative stress to survive in host environment. Kinetoplastid membrane protein (KMP)-11 is another up-regulated protein in our analysis. KMP-11, a hydrophobic protein, which is involved in the interaction of pathogen-host, and its expression is increased in amastigote forms, which has been reported to be associated to lipophosphoglycan (LPG) [33]. According to Mukhopadhyay et al., study, the expression of KMP-11 was decreased along with parasite virulence as a function of the time of the subculture in L. donovani [34]. It was also reported in several independent experiments that the isolation of a Sb (III) resistant L. infantum cell line always correlated with a high decrease in the KMP-11 protein [35]. Among down-regulated proteins during differentiation from metacyclic to amastigote-like form of L. tropica, translation/ribosome structure and biogenesis category was important cluster, which included 40S ribosomal protein S3a, 60S ribosomal protein L18, 40S ribosomal protein S6, Putative 60S ribosomal protein L23, 40S ribosomal protein S24, 40S ribosomal protein S2, Putative 60S ribosomal protein L7, Putative 60S ribosomal protein L17, Putative 60S ribosomal protein L21, 60S acidic ribosomal protein P2, Putative 60S ribosomal protein L13a, 40S ribosomal protein S4 and Putative 60S ribosomal protein L7a. All of these proteins are ribosome subunits that involved in translational activity. In summary, our results in agreement with in vivo studies indicate that abundance of translation machinery proteins, translational activity and protein synthesis decreased in parasites undergoes differentiation from promastigote to amastigote [22, 28, 36]. Of course, these results seem logical because amastigotes are less active than promastigotes. In line with this, amastigote growth and energy consumption are also slower rather than promastigotes [37]. Our investigation revealed that during differentiation, the abundance of mRNA processing / replication related proteins decreased, therefore we propose that this decreasing must result in the reduced growth of amastigote. RNA helicases are the proteins that catalyze RNA un-winding and are necessary for diverse biochemical processes such as mRNA splicing, ribosome assembly and translational initiation [38]. In general, anabolic functions for example translational activity and glycolytic pathways were down-regulated, whereas catabolic functions such as lipid and amino acid metabolism

were up-regulated in amastigote-like form, which is in keeping with the existing studies. Tubulin alpha chain is one of down-regulated proteins that is a fundamental component of the cytoskeleton which is responsible for cell shape and is involved in cell division, cilliary and flagellar motility and intracellular transport. Alpha tubulin gene expression varies according to the morphological changes suffered by Leishmania in its life cycle. Down-regulation of proteins related to motility in amastigote-like is in agreement with the non-motility observed in this stage. Herein, we also investigated PPI network of differentially expressed proteins via bioinformatics approach. Protein-protein interaction (PPI) network analysis has a high growth in parasite studies to facilitate introducing novel potential drug targets [10, 39, 40]. As it is shown in Table 1, there are 65 changed expression proteins (including up and down regulated proteins) related to the metacyclic and amastigote-like forms of L. tropica. Since PPI network analysis is a powerful approach in categorization and ranking of the drug target candidate and potential biomarker for a certain disease, here the PPI network of the significant different regulated proteins are constructed (Fig. 5a). Topological analysis of the networks leads to rank of the nodes based on their centrality properties in network [10, 41]. By degree centrality value using Cytohubba plugin in Cytoscape software, the top 10 node selected as important hub proteins (Fig. 5b). The hub proteins can be recommended for new potential drug targets in disease. According to Fig. 5b, ENOL has highest degree and this protein can be thought of as a potential drug target. Enolase is described as an important enzyme in glycolysis and glyconeogenesis as two important cellular pathways. Glycolysis play important roles in ATP supply and glyconeogenesis is crucial for the virulence and viability of Leishmania parasite. Further, it can regulate cell morphology and vesicle trafficking by cytoskeleton system. Furthermore, enolase enzyme is available in secretome and leishmanial parasite surface. Based on the surface enolase, plasminogen receptor can probably play a role in virulence and invasiveness of parasites [10, 42]. It must be pointed out that further investigation are required using western blotting or real time PCR to validate the results of this study. In the present study, another hub protein with a role in energy metabolism is phosphoglycerate kinase (PGKC) and LmjF.25.1170 (ATP synthase subunit beta). Among other hub proteins, LmjF.32.0450, LmjF.17.0083, LmjF.15.0200, LmjF.35.0420, LmjF.24.0040 and LmjF.36.0940 involved in translation and are as constituents of ribosome. Therefore, manipulation and controlling of translation process in L. tropica could be as an approach in differentiation of parasite and also as a potential drug target to cutaneous leishmaniasis therapy. The other hub protein is LmjF.35.1180, NADH-fumarate reductase that this enzyme and other mitochondrial enzymatic activities of L. major and L. donovani promastigotes and amastigotes were investigated. The presence of NADH-fumarate reductase was demonstrated in digitonin-permeabilized L. major promastigotes and mitochondria of L. major and L. donovani promastigotes and amastigotes. The data of Chen et al., indicate that fumarate reductase is an obligatory component of the respiratory chain of the parasite [43]. Since the enzyme is an important component in the intermediate metabolism in the Leishmania parasite and is absent in mammalian cells, it could be a potential target for antileishmanial drugs. Module is a part of a network with closely part of proteins, which having specific biological function [44]. Determined modules of network can provide new insight about different roles of proteins. As shown in Fig. 6, there are three modules for whole network. Functional enrichment analysis of these modules showed that ATP synthesis, glycolysis/gluconeogenesis, biosynthesis of amino acids, pentose phosphate pathway, TCA cycle,

translation and gene expression are the main affected pathways by differentially expressed proteins. Since module number 1 has the highest number of important hub proteins, and proteins of this cluster involved in the translation and gene expression pathway, it can be concluded that the protein production process is the most important pathway altered during parasite differentiation. In the study, further analysis of modules by MCODE revealed seed nodes in modules. The seed nodes of these modules included LmjF.07.0510, LmjF.28.2420 and LmjF.24.1630. In the protein-protein network, each node (protein) with corresponding gene expression value is regarded as “seed node.” For a seed node i, this node and its neighbors j within the shortest distance k, form a connected subnetwork with n nodes [45]. These seed nodes can serve as candidate drug and vaccine for cutaneous leishmaniasis caused by L. tropica.

Conclusion

This study presents an initial attempt at making comparisons between the global protein expression patterns of two distinct life stages of L. tropica species in Iranian isolates. Using a quantitative proteomics approach (SWATH-MS), we showed that protein expression profiles modulated different in two developmentally forms of L. tropica. Also, several important proteins signatures introduced in sand-fly and mammalian host of L. tropica such as parasite biology, infectivity and pathogenesis factors which would be useful in the field of biomarker and drug discovery process. Finally, quantitative proteomics approach plays a crucial role in introducing metabolic pathways related to stage-specific of Leishmania parasite.

Abbreviation

SWATH-MS:Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry; L:Leishmania; ITS1:intrnaltranscribed-spacer-1; NNN:Novy-Nicolle-Mc Neal; MCOE:Molecular Complex Detection; COG:cluster of orthologs groups; GO:gene ontology; PPI:protein- protein interaction

Declarations

Ethics approval and consent to participate

This study was approved by Ethics Committee of Shahid Beheshti University of Medical Sciences (Ethical code: IR.SBMU.MSP.REC.1395.351). Informed consent was received from all participating patients in the present study.

Consent for publication

Not applicable.

Availability of data and materials

Data of this study are included in the article and the primary data can be provided from the corresponding author.

Competing interests

The authors declared that there are no conflicts of interest.

Funding

Not applicable.

Author's contributions

All authors conceived and designed the study; MA and NAD participated in experimental work, collection and analysis of data and drafted the manuscript; NA provided the expertise and critically reviewed the manuscript. All authors interpreted the data, revised and approved the final manuscript.

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Tables

The list of differential expression proteins based on fold change>2 and p-value<0.05 during evelopmentally process from metacyclic promastigotes to amastigotes like in Iranian *L. opica* isolates

FC AT/MT	Uniprot IDs	Protein name	Gene name	Peptides
Up-regulated proteins list during metacyclic conversion into amastigote-like form of *L. tropica*				
12.91	E9ACW0	Putative heat shock protein DNAJ	LMJF_27_2400	DIVHELPVPLEAFYCGK
7.53	E9ADS8	Putative lipophosphoglycan biosynthetic protein	LMJF_29_0760 (LPG3)	MLDILVNSLYTNR
2.05	E9AF45	Kinetoplastid membrane protein 11	LMJF_35_2210 (KMPII-1)	FAELLEQQK , LDRLDEEFNRK , EHSEHFK
3.83	Q4Q1M0	Chaperonin HSP60, mitochondrial	LMJF_36_2030	IQSIHSLLPALNHVVR, TGVTIVR,KIQSIHSLLPALNHVVR, AVAAVATTLGPK
7.28	Q4Q1R4	Putative universal minicircle sequence binding protein	LMJF_36_1610 (UMSBP1)	CGEAGHMSR
41.32	Q4Q1Y2	Putative 40S ribosomal protein S18	LMJF_36_0940	SLTLIPDHFQHIVR,FKIPDWFLNR,TEHLSSSMVDTRAGTLTAEELEKIAEIIADPAK, HAYGLR
13.02	Q4Q3V3	Succinyl-CoA:3-ketoacid-coenzyme A transferase	LMJF_33_2340	SGNLVFR, QTGGQIIR, GPGGAMDLVASGSR
21.18	Q4Q5P6	Putative 26S proteasome regulatory subunit	LMJF_32_0390	VAGLLLGR, HTNDEAIATFLAAIAR
22.5	Q4Q822	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	LMJF_28_2420	GLVVPVIR, LGLMSPFVK, NLIEDPAR
6.68	Q4Q931	Putative 40S ribosomal protein S33	LMJF_28_2420 (S33-1)	ENDMLSLMETER, GNVTQVR, LMAEAGSPDYNR
78.77	Q4Q9X6	ATP synthase subunit beta	LMJF_25_1170	IFNVLGDAIDQR, VAQSALTMAEYFR, GHGGFSVFAGVGER,FTQANSEVSALLGR, TVIIMELINNVAK

3.0	Q4QAB9	**Uncharacterized protein**	LMJF_24_2110	ALENPVNLDK, MEFVIDR, NEAAFQDVGIEYYR
20.39	Q4QD34	Phosphoglycerate kinase (PGKC)	LMJF_20_0100	SALPTIQK, EGGSCILMSHLGRPK, VLGAGYAGYLMEK
2.73	Q4QDF0	Glycosomal malate dehydrogenase	LMJF_19_0710 (Gmdh)	RDPALAELAK, GSATLSMAEAGAR,VQVAGTEVVK, DPALAELAK, LLGVSLLDGLR
5.04	Q4QGX4	Putative pretranslocation protein,alpha subunit	LMJF_11_1050	QANWLMSLKPMLAVLPEIEKPR
6.15	Q4QJF1	ATPase alpha subunit	LMJF_05_0500	VDAGAPNIVSR, SPVNYNLLTGFK, FVALFNQK, VVNPLGHEVPVGL, AVDTMIPIGR
2.0	Q9U0V9	Possible 3-ketoacyl-CoA thiolase	LMJF_23_0690 (L7836.03)	LDDFTFPCLFAK, KHPDFGK
14.28	E9ACG7	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	LMJF_03_0200	YGLTGAVFSR, GAFEFQGQK, CTGAVVGQQPFGGSR, GYFVEPTIIETK
9.32	E9AFE7	Putative cystathione gamma lyase	LMJF_35_3230	NNLHGGMLWFEVK, VGITDGFVR, NNLHGGMLWFEVK, NNLHGGMLWFEVK
down-regulated proteins list during metacyclic conversion into amastigote-like form of *L. tropica*				
8.28	O62591	Probable eukaryotic initiation factor 4A	LMJF_01_0770	HNLIQGLVLSPTR, VLVTTDLVAR,HNLIQGLVLSPTR, ESLTLEGIK
28.72	Q4FX73	40S ribosomal protein S3a	LMJF_35_0400	NVLSDALVR, FTVQEVQGR, EWYDVVAPANFEK
4.81	Q4QEB3	GMP reductase (GMPR)	LMJF_17_0725	IGVGPGSICITR, LIVGAAIGVK, GPLAPILK
2.47	Q4QG98	60S ribosomal protein L18 (RPL18-A)	LMJF_13_0560	GVDLTGISK, AAPIAVVVGDVLDDVR
2.84	E9AD27	Putative calpain-like cysteine peptidase	LMJF_27_0500	SIFLPLNTFLK, AELQRAVLKAQNAK, NATAIQDLEEALNDR
26.07	E9AD53	Putative small GTP-binding protein Rab1	LMJF_27_0760	LLLIGDSGVGK, DFADSLGIPFLETSAK
3.47	E9ADF9	Putative glycosomal	LMJF_27_1810	VAYPLEHIPGALTHAVAGHPNNVIFLTNDAFGVMPPVAR, NLTAPELVQWALK,

		phosphoenolpyruvate carboxykinase		GALCVL SYAK, KGDVTVFFGLSGTGK, GVFNIEGGCYAK
17.39	E9ADX3	Tryparedoxin	LMJF_29_1150 (TXN2)	MPWLALPFEDRK
3.17	E9AE57	Putative fumarate hydratase	LMJF_29_1960	HGGFYLGSIGGPAAILAK, YFAHQAR, YVEEVEVFGR
2.12	E9AEB3	ATP-dependent 6-phosphofructokinase	pfk	TAIELSR, TIDNDLAFSHR, FGGTILGSSR,HLHFNPSETSIGIVTCGGICPGLNDVIR, EMVDTLVR
67.7	E9AEL4	Putative ATP-dependent DEAD-box RNA helicase	LMJF_35_0370	TASFVIPVLEK, VHILVATPGR, GFEKPSPVQEEAIPVALQGK, HIPGLEVMVTTGGTTLR, ELALQTAQVTK, NVNFEEYALR
4.36	E9AEU1	Putative NADH-dependent fumarate reductase	LMJF_35_1180	LGGNSLLECVVFGK, AATILQK, ATSGINAWGTR, LALIGGGTGVAPMLQIVR, LIGCPEANVMATLK
6.1	E9AF23	40S ribosomal protein S6	LMJF_35_2010	LFNLSR, GAIGFNTFR, RGAIGFNTFR,RVQLQDYR, VGDQPIEGVTDTTAPR
2.83	E9AFK3	Putative 60S ribosomal protein L23	LMJF_35_3790	VLNAVIIR, ISTHAPAIV, NLYVISVK
36.1	Q4Q090	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	PGAM	VALQGASLVDDALK, MFVTMDR, SAEITEAAIEALK, VALQGASLVDDALK
2.64	Q4Q124	Adenosylhomocysteinase	LMJF_36_3910	AGVFFLPK, VAALHLAHVGAK, DISLAEWGR, EHVEIKPQVDR, VKDISLAEWGR, FDNLYGCR
7.9	Q4Q1D2	40S ribosomal protein S24	S24E-2	TTGFGLIYDDLASLK
2.8	Q4Q1F5	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex		LTITPIPMPALSPTMEK, WFQHFHDAMENPLSLLL
2.49	Q4Q1X7	Putative 40S ribosomal protein S10	LMJF_36_0980	FFFTEGVIACK
7.71	Q4Q230	**Uncharacterized protein**	LMJF_36_0480	KSPIMSK, LMDQSLPVYDDVVTGVGR
2.59	Q4Q2H7	Putative vacuolar ATP	LMJF_34_3670	ITWNYIR, NIVTFYEEAQR, TCLVANTSNMPVAAR, EEELQEIVQLVGK

		synthase catalytic subunit A		
7.61	Q4Q3U8	Putative heat shock protein	LMJF_33_2390	YNLHFNPQHPLIR, GLLPDWLR, EELTANLGTIAGSGSK
2.74	Q4Q4U1	Dihydrolipoyl dehydrogenase	GCVL-2	ALTGGVEYLFK, AAQLGLK, AVGTEDGFVK
3.42	Q4Q5P0	40S ribosomal protein S2	LMJF_32_0450	GTGIVAAPVPK, THGNLIMATFYALR
8.42	Q4Q6E1	Putative vacuolar-type proton translocating pyrophosphatase 1	LMJF_31_1220	QFQDPEVAEGR
3.81	Q4Q9H4	Putative 60S ribosomal protein L7		KILQLLR, AVEPYIAYGYPSLATVR
35.55	Q4Q9M4	Succinate--CoA ligase [ADP-forming] subunit alpha, mitochondrial	LMJF_25_2130	VIVQGMTGK, VVGGVSPK, VIVQGMTGK, AGTFHTK
3.93	Q4Q9R2	Polyprenol reductase	EnCR	DLGPQIGYR, ELESMFVHK, FSHPTMPMR
2.22	Q4Q9V1	GTP-binding nuclear protein	LMJF_25_1420	LILVGDGGTGK, SNYNFEKPFVWLAK, VCDNIPIVLVGNK
5.74	Q4Q9Y0	Putative cytochrome c oxidase VII	LMJF_25_1130	IPNPFAYSFK, VWAPATTLAEYR
5.22	Q4QAG8	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	LMJF_24_1630	AITMEILAGR, LGANSLLDIVVFGK, GEGGYLVNSEGER, SPVWNSNLIEALELR
18.71	Q4QAX6	Putative 60S ribosomal protein L17	LMJF_24_0040	HVQVDQAAR, SVVAMMSLLK
2.0	Q4QEI9	Elongation factor 1-alpha	LMJF_17_0080	IGGIGTVPVGR, GITIDIALWK, FESPKSVFTIIDAPGHR, SVFTIIDAPGHR, EHALLAFTLGVK, STATGHLIYK
7.48	Q4QEM2	Paraflagellar rod protein 2C	LMJF_16_1425	AQLLEHLVELVADKFR, TLGQLVYK

14.07	Q4QEX4	Putative 60S ribosomal protein L21	LMJF_16_0460	GVGVIINKPVR, TGIVWNVTPR, VGDYVDVVADSAVR
33.52	Q4QF62	60S acidic ribosomal protein P2	LMJF_15_1203	AVHIDVDQATLAFVMESVTGR, ASPSQADVEAICK
23.38	Q4QF80	Tryparedoxin peroxidase	TRYP1	GLFIIDPHGMLR
6.15	Q4QFF2	Putative ribonucleoprotein p18, mitochondrial	LMJF_15_0280	FCAMMDLMEEMQHR, FCAMMDLMEEMQHR, NCPPDLETYNATLQK
19.25	Q4QFG2	Putative 60S ribosomal protein L13a	LMJF_15_0200	APSDVFVR, HRPEIIVIDLK, HRPEIIVIDLKDHVLGR, CEQLNIAGTEIR
2.34	Q4QFL8	Enolase	ENOL	HIDEPLPILMEAIEK, LPVPCFNVINGGK
181.11	Q4QFP8	Putative small myristoylated protein-3	SMP-3	ISFEANPIAK, DNGNGLLFR
5.92	Q4QG31	40S ribosomal protein S4	RS4	LRECLPLLVIIR, AVIVTGGANR, ECLPLLVIIR,DLNNLQVTVPK, MNVIQER,DASGAEFATR
3.49	Q4QGA9	**Uncharacterized protein**	LMJF_13_0450	SPEFDAIYEQQQK
2.77	Q4QGC5	Tubulin alpha chain	LMJF_13_0280	LIGQVVSSLTASLR, IHFVLTSYAPVVSAEK, EIVDLALDR, QLFNPEQLVSGK, AVCMIANSTAIAEVFAR
2.59	Q4QGN9	Glucose-6-phosphate isomerase	PGI	AVLHVALR, HFVALSTNTEK, PSNSILVNALTPR, QVNLEETIFIIASK
22.93	Q4QIP1	Putative 60S ribosomal protein L7a	LMJF_07_0500	APLAVVTGLQEVTR, WPTFVTMQR,TATCVALTDVNAEDEATLK

FC: Fold Change, AT: amastigote of *L. tropica*, MT: metacyclic of *L. tropica*

Table 2. The KEGG pathways of up-regulated proteins involved in the conversion of *L. tropica* metacyclic forms into the amastigote-like forms

Pathway ID	Pathway Description	Gene NO.	FDR	Matching Proteins (IDs)
1100	Metabolic pathways	7	0.0017	LmjF.03.0200,LmjF.05.0510,LmjF.19.0710,LmjF.20.0100,LmjF.23.0690, LmjF.25.1170,LmjF.28.2420,
1110	Biosynthesis of secondary metabolites	4	0.0211	LmjF.19.0710,LmjF.20.0100,LmjF.23.0690,LmjF.28.2420
1200	Carbon metabolism	3	0.0251	LmjF.19.0710,LmjF.20.0100,LmjF.28.2420
20	Citrate cycle (TCA cycle)	2	0.0364	LmjF.19.0710,LmjF.28.2420
280	Valine, leucine and isoleucine degradation	2	0.0364	LmjF.23.0690,LmjF.33.2340

FDR: False Discovery Rate

Table 3. The KEGG pathways of down-regulated proteins involved in the conversion of *L. tropica* metacyclic forms into the amastigote-like forms

Pathway ID	Pathway Description	Gene NO.	FDR	Matching Proteins (IDs)
3010	Ribosome	11	4.13E-10	LmjF.07.0510, LmjF.13.1230, LmjF.15.0200, LmjF.15.1207, LmjF.16.0460, LmjF.24.0040, LmjF.32.0450,LmjF.35.0420,LmjF.35.3800,LmjF.36.0990,LmjF.36.2870
1200	Carbon metabolism	9	1.76E-08	LmjF.12.0530, LmjF.14.1160, LmjF.24.1630, LmjF.27.1810, LmjF.29.1960, LmjF.29.2510, LmjF.32.3310,LmjF.36.2660,LmjF.36.6650
10	Glycolysis / Gluconeogenesis	7	2.19E-08	LmjF.12.0530, LmjF.14.1160, LmjF.27.1810, LmjF.29.2510, LmjF.32.3310, LmjF.36.2660, LmjF.36.6650
1110	Biosynthesis of secondary metabolites	11	2.19E-08	LmjF.12.0530, LmjF.14.1160, LmjF.17.0725, LmjF.24.1630, LmjF.25.1770, LmjF.27.1810, LmjF.29.1960,LmjF.29.2510,LmjF.32.3310,LmjF.36.2660,LmjF.36.6650
1100	Metabolic pathways	14	2.51E-07	LmjF.12.0530, LmjF.14.1160, LmjF.15.1040, LmjF.17.0725, LmjF.24.1630, LmjF.25.1770, LmjF.27.1810, LmjF.29.1960, LmjF.29.2510, LmjF.32.3310, LmjF.34.3670, LmjF.36.2660, LmjF.36.3910,LmjF.36.6650
20	Citrate cycle (TCA cycle)	5	6.25E-06	LmjF.24.1630,LmjF.27.1810,LmjF.29.1960,LmjF.32.3310,LmjF.36.2660
620	Pyruvate metabolism	4	0.000217	LmjF.27.1810,LmjF.29.1960,LmjF.32.3310,LmjF.36.2660
190	Oxidative phosphorylation	3	0.0185	LmjF.24.1630,LmjF.31.1220,LmjF.34.3670
1230	Biosynthesis of amino acids	3	0.037	LmjF.14.1160,LmjF.29.2510,LmjF.36.6650
30	Pentose phosphate pathway	2	0.0395	LmjF.12.0530,LmjF.29.2510
260	Glycine, serine and threonine metabolism	2	0.0395	LmjF.32.3310,LmjF.36.6650

FDR: False Discovery Rate

Figures

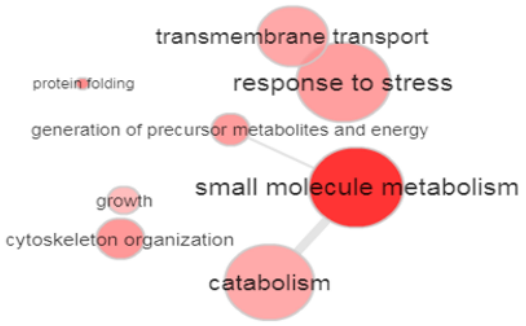
Figure 1

The number of protein profile, differential and uniquely protein expression between metacyclic promastigotes and axenic amastigotes of Iranian L. tropica isolates (U, uniquely expression; up-reg, up-regulated; down-reg, down-regulated)

Figure 2

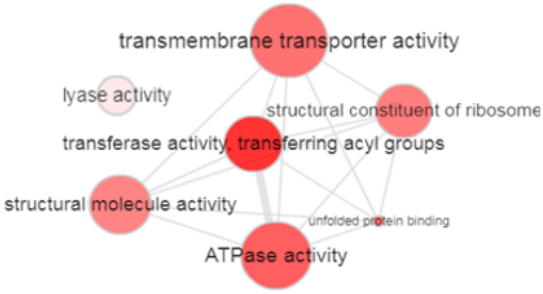
COG (clusters of orthologous groups) function classification coverage of the protein sequence. A total of 8 and 10 groups of all up-regulated & down-regulated of differentially expressed proteins were clustered by orthologous groups, respectively. PTM: post-translational modification

a)



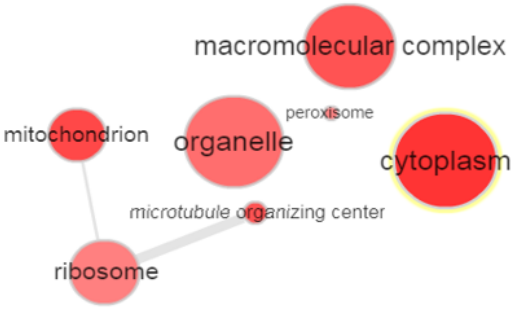
term ID	description	frequency	p-value
GO:0006457	protein folding	1.69%	4.25e-3
GO:0006950	response to stress	12.28%	1.07e-2
GO:0040007	growth	2.67%	4.33e-2
GO:0055085	transmembrane transport	7.02%	1.57e-2
GO:0007010	cytoskeleton organization	3.97%	7.48e-3
GO:0006091	generation of precursor metabolites and energy	3.09%	9.34e-3
GO:0044281	small molecule metabolic process	12.55%	6.52e-5
GO:0009056	catabolic process	10.96%	1.76e-2

b)



term ID	description	frequency	p-value
GO:0003735	structural constituent of ribosome	2.68%	2.71e-2
GO:0005198	structural molecule activity	3.27%	3.25e-2
GO:0016746	transferase activity, transferring acyl groups	2.89%	3.32e-3
GO:0022857	transmembrane transporter activity	5.87%	3.32e-3
GO:0051082	unfolded protein binding	0.49%	1.86e-2
GO:0016887	ATPase activity	4.56%	1.09e-2
GO:0016829	lyase activity	3.60%	6.06e-3

c)

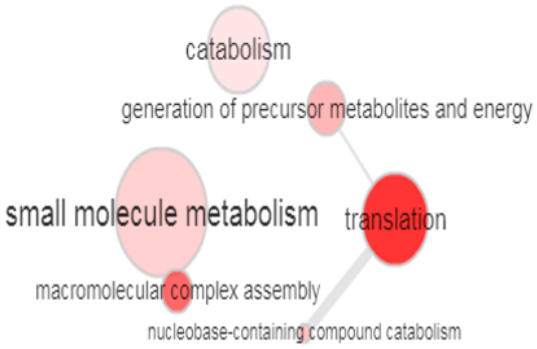


term ID	description	frequency	p-value
GO:0005739	mitochondrion	2.16%	6.48e-3
GO:0032991	macromolecular complex	14.01%	8.74e-3
GO:0043226	organelle	20.79%	1.82e-2
GO:0005737	cytoplasm	26.02%	3.67e-3
GO:0005815	microtubule organizing center	0.35%	8.79e-3
GO:0005777	peroxisome	0.22%	3.56e-2
GO:0005840	ribosome	4.20%	3.02e-2

Figure 3

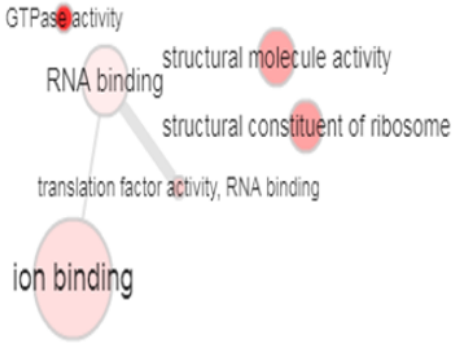
Gene ontology enrichment analysis of up-regulated proteins during conversion of metacyclic promastigotes into the amastigote like of Iranian isolates of L. tropica based on: a) biological process, b) molecular function and c) cellular component by TriTrypDB (kinetoplastid Genomics Resource)

a)



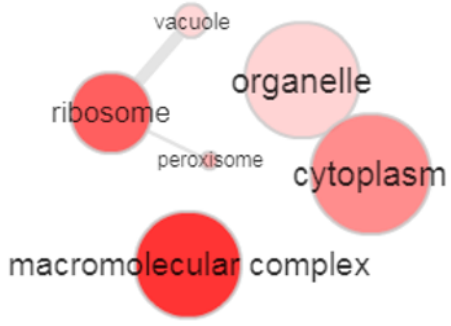
term ID	description	frequency	p-value
GO:0009056	catabolic process	4.82%	2.08e-2
GO:0065003	macromolecular complex assembly	1.50%	6.82e-8
GO:0044281	small molecule metabolic process	15.14%	1.06e-3
GO:0006091	generation of precursor metabolites and energy	1.94%	1.74e-5
GO:0034655	nucleobase-containing compound catabolic process	0.76%	1.72e-4
GO:0006412	translation	5.69%	5.01e-14

b)



term ID	description	frequency	p-value
GO:0003735	structural constituent of ribosome	2.68%	2.35e-9
GO:0003924	GTPase activity	1.14%	8.2e-20
GO:0005198	structural molecule activity	3.27%	6.56e-9
GO:0008135	translation factor activity, RNA binding	0.91%	2.38e-5
GO:0043167	ion binding	33.49%	8.37e-4
GO:0003723	RNA binding	5.28%	1.35e-2

c)



term ID	description	frequency	p-value
GO:0005840	ribosome	4.20%	4.32e-9
GO:0032991	macromolecular complex	14.01%	2.73e-10
GO:0043226	organelle	20.79%	5.10e-3
GO:0005737	cytoplasm	26.02%	1.06e-6
GO:0005777	peroxisome	0.22%	1.33e-4
GO:0005773	vacuole	0.46%	7.88e-3

Figure 4

Gene ontology enrichment analysis of down-regulated proteins during conversion of metacyclic promastigotes into the amastigote like of Iranian isolates of L. tropica based on a) biological process, b) molecular function and c) cellular component by TriTrypDB (kinetoplastid Genomics Resource)

Figure 5

Whole Connected Component of the PPI Network of differential expressed proteins that were visualized by Cytoscape software. a) The nodes are layout by degree value (darker to bright brown nodes corresponded to hub proteins). b) The hub proteins along with their degree (D)

Figure 6

the modules of whole PPI network. The hub proteins of our analyses are presented in modules 1, 2 and 3 that are shown in orange color. Seed nodes in each module are also shown by yellow color