Medical genetics

Expression analysis of activated protein kinase C gene (LACK1) in antimony sensitive and resistant *Leishmania tropica* clinical isolates using real-time RT-PCR

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Abstract

**Background** Resistance to pentavalent antimonial drugs has become a serious problem in the treatment of cutaneous leishmaniasis in some endemic areas. Investigations on molecular markers involved in drug resistance are essential for monitoring of the disease. *Leishmania*-activated C kinase gene (LACK1) is involved in multiple central processes such as signal transduction. According to the probable role of the LACK1 gene in antimony resistance, we used real-time reverse transcription–polymerase chain reaction (PCR) to investigate the expression of this gene in clinical *L. tropica* strains, which were resistant or sensitive to meglumine antimoniate.

**Methods** We analyzed the expression level of LACK in 18 sensitive and 14 resistant *L. tropica* isolates collected from patients with anthroponotic cutaneous leishmaniasis. After cDNA synthesis, gene expression analysis was performed by quantitative real-time PCR using SYBR Green. In addition, the full length of the LACK gene from six reference strains was cloned and sequenced then deposited in the NCBI database to confirm our strains.

**Results** Real-time reverse transcription-PCR revealed that the average RNA expression level of LACK in isolates from unresponsive and responsive patients were 0.479 and 4.583, respectively, and expression of LACK was significantly downregulated (9.56-fold) in resistant isolates compared to sensitive ones.

**Conclusion** Results of the present study suggest the probable role of the LACK gene in antimony resistance. Moreover, it can be considered as a potential marker for monitoring antimony resistance in clinical isolates. However, further studies are required to exploit the biological functions of it in antimony resistance.

Introduction

Leishmaniasis is a protozoan infection with worldwide spread particularly in tropical and subtropical countries. *Leishmania* species as arthropod-transmitted parasites can infect and replicate in host macrophages.1 Various species of *Leishmania* have a different clinical manifestation from self-curing cutaneous lesions to the potentially fatal visceral disease. In addition, atypical infections have been reported.2 In Iran, human leishmaniasis occurs from the northeast to the central, west, and covers the entire southeast and west region. Depending on the *Leishmania* species, several forms of disease including anthroponotic cutaneous leishmaniasis (ACL; *L. tropica*), zoonotic CL (*L. major*), and visceral leishmaniasis (*L. infantum*) occur in Iran.3 Cutaneous infection is limited to skin, but some studies show that *L. tropica* can infect visceral organs of humans and dogs.4–6 The mainstay of therapy for leishmaniasis is meglumine antimoniate (Glucantime) in Iran. Unfortunately, during the recent decade, the increase in resistance of antileishmanial drugs has become a serious public health concern, particularly the ACL form induced by *L. tropica* in endemic areas of Iran.7,8 Studies showed that the *Leishmania* parasites have been slowly acquiring mutations and/or modulating in gene expression, leading to decreased susceptibility to the drug. However, other factors such as host immunity might be reasons for treatment failure.9 In this context, determining some biomarkers for...
monitoring drug sensitivity seems essential. Considering that antimony resistance is a major problem in treatment, determining the molecular markers for monitoring the drug-resistant isolates is essential. Availability of the whole *Leishmania* genome and proteome sequence and the development of genomic and proteomic approaches have increased the potential to detect the resistance mechanisms.\(^\text{10}\)

Following a proteomics study on *L. tropica* clinical isolates, which were sensitive and resistant to Glucantime treatment, a number of proteins with different expressions were identified by the mass spectrometry method.\(^\text{11}\) Among them, due to the importance of its physiological performance, the activated protein kinase C receptor (LACK) (*Leishmania* analogue of the receptors of activated C kinase) antigen was selected, and its expression was investigated in RNA levels in *L. tropica* clinical isolates that were sensitive and resistant to Glucantime treatment. Despite the genetic diversity of *Leishmania* species, the 36 kDa LACK antigen is highly conserved among species and expressed in both promastigote and amastigote forms of the parasite.\(^\text{12}\) The LACK as a T-cell epitope is able to stimulate the immune response and generation of T-helper 1 cells; therefore, several studies have turned their attention to the LACK gene as the target for the vaccine.\(^\text{13-15}\) A survey of the LACK DNA vaccine in mice revealed a decrease of the disease and parasite burden with the enhancement of interferon γ production,\(^\text{16}\) while LACK is essential for the progression of infection in BALB/c mice with *L. major*.\(^\text{17}\) There is solid evidence that LACK is required for parasite viability and establishment in the host macrophage.\(^\text{18}\) These proteins were involved in multiple central processes, including signal transduction, RNA processing, and cell cycle control. Recently it has been demonstrated that although it localizes in cytosol, it could be secreted, and the temperature shift from the sandfly vector to the mammalian host induces its secretion. In addition, it can bind and increase plasminogen activation *in vivo*. This function could promote the plasmin formation and might contribute to the invasiveness of the parasite.\(^\text{19}\) Several studies indicate that some genes have a different expression under physiological conditions such as drug resistance, for instance ubiquitin and amino acid permease genes were upregulated in antimony-resistant *L. tropica*,\(^\text{20}\) and downregulation of the calcineurin gene was associated with Glucantime resistance in *L. infantum*.\(^\text{21}\) Despite the importance of the LACK gene and widespread *L. tropica*, particularly resistant isolates, in Iran, in the present study, a sensitive molecular technique (real-time polymerase chain reaction [PCR]) was used to investigate the differential expression of the LACK gene in Glucantime-resistant and -sensitive *L. tropica* field isolates.

### Materials and methods

#### Patients and sampling

This cross-sectional study was conducted from November 2013 to December 2014 in patients with infected ACL in the Khorasan Razavi province/Mashad city located in the north-east of Iran. This area has a high rate of transmission of *L. tropica* throughout the year. Samples were collected from patients with positive smear skin lesions referred to the medical health centers. For gene expression analysis, 32 samples were used including *L. tropica* strains isolated from skin lesions in patients with CL who responded (18 sensitive isolates) and did not respond (14 resistant isolates) to treatment with Glucantime. Resistant cases were defined as patients who received at least three courses of Glucantime® but presented active lesion(s), and sensitive cases were defined as patients who healed after receiving one course of Glucantime.\(^\text{7}\) Furthermore, in the present study, *L. tropica* strain MHOM/IR/10/175 (S175) with IC\(_{50}\) of 6.67 ± 1.15 µg/ml was used as an antimony sensitive reference strain. In addition, two isolates MHOM/IR/10/827 and MHOM/IR/10/439 with IC\(_{50}\) values of 56.67 ± 5.77 and 50.66 ± 3.51 µg/ml, respectively, were considered as resistant reference strains (Kazemi-Rad *et al*. unpublished data).\(^\text{8}\) This study approved by the Institutional Ethical Committee of the School of Public Health, Tehran University of Medical Sciences.

#### Skin lesion biopsy and parasite culture

Samples were obtained from the margin of the cutaneous lesions using a disposable lancet. An amount of ulcer serosity was transferred to RPMI-1640 medium (Gibco, ThermoFisher Scientific), supplemented with 15% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 23 ± 1 °C.

#### Molecular characterization

Promastigotes were harvested in a stationary phase by centrifugation (800 g, 5 min at room temperature) and washed using sterile phosphate-buffered saline. Total DNA was extracted with the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. PCR-restriction fragment length polymorphism (PCR-RFLP) performed using internal transcribed spacer 1 of ribosomal RNA fragment of the parasites, which amplified using the primers (LITSR and 5.8S) and PCR conditions outlined. PCR products were digested with fast digestion HAEIII (BsuRI) enzyme (Fermentas Thermo Scientific), according to the manufacturer’s instructions, and digested fragments were visualized on 3% agarose gels. *Leishmania* species were identified based on obtained patterns alongside reference species including *L. tropica* (GenBank accession no. EF653267).\(^\text{6}\)
Polymerase chain reaction, cloning, and sequencing of the LACK gene
According to the complete sequence of the LACK gene in Leishmania, specific primers were designed for PCR amplification of the 939 bp fragment from genomic DNA. The sequences of primers for LACK are forward 5′-CTAGGAGCTATGAACAGGTGTCACCT-3′ and reverse 5′-CTAGGAATTCGCTCGGCGTCGGAGATGGACC-3′.

In this study, we used six Leishmania reference strains for amplification and sequencing of the LACK gene that are as follows: two resistant and one sensitive L. tropica reference strain isolated from humans; one resistant and one sensitive L. infantum strain isolated from canines; and one L. major strain derived from Rhombomys opimus. For PCR amplification, 1.5 µl DNA was used as a template with 0.5 U PFU polymerase, buffer MgSO4 (×10), 20 mM dNTPs and 10 pm of both the primers. The PCR program included an initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 35 s, 63 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified PCR product (939 bp) was electrophoresed on gel agarose 1.5%. The amplified DNA fragment was excised from the gel and purified using a Gel Extraction kit (Bioneer, Seoul, Korea) according to the manufacturer’s recommendations. In addition, after checking the PCR products, they were cloned into a pGEM-T Vector System I (Promega, Fitchburg, MA, USA). To determine the identity of the isolated LACK gene, recombinant plasmid containing PCR product was subjected to sequencing using universal primers, T7 promoter (Bioneer). Homology searches were performed in non-redundant nucleic and protein databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

RNA extraction and complementary DNA synthesis
Total RNA was extracted from 10⁶ promastigotes of 32 L. tropica isolates during the early stationary phase by Tripure reagent (Roche) according to the manufacturer’s instructions. For elimination of any DNA contamination, DNase1 (Qiagen, Hilden, Germany) was used for 45 min at 37 °C. Complementary DNA (cDNA) synthesis was performed using the cDNA Synthesis Kit (Roche) according to its protocol. cDNA was synthesized using 10 µg extracted RNA, 20 pmol/µl random hexamer primer, 20 pmol/µl oligo-dT and 10 µM of dNTP mix incubated 10 min at 65 °C followed by the addition of 20 U Ribolock, 4 µl of ×5 reverse transcriptase (RT) buffer and 200 U Revert Aid premium RT, and then incubated at 25 °C for 5 min followed by incubation at 50 °C for 60 min. The integrity of cDNA was checked with α-tubulin primers (F: CAGGTTGGTGTCGTCCTTCTGAC/R: TAGCTCGAGCACGAGTGG) as the housekeeping gene, which amplified a fragment of 119 bp. The PCR condition was an initial denaturing step of 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 30 s with a final extension of 72 °C for 7 min.

Real-time reverse transcriptase-polymerase chain reaction analysis
Real-time RT-PCR was conducted to analyze the expression level of LACK gene among the sensitive and resistant L. tropica isolates. The primers were designed by Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/). LACK1 primers including forward (5′-GAACAGAAGGTCACCTGAA) and reverse (5′-AGACCGTAGTCGCTGCCAC) were used for amplification of a 165 bp fragment in the experiments. RT-PCR was performed in 20 µl reactions containing 1 µl cDNA target, 100 nM forward and reverse primers, and 1× SYBR® Premix ExTaq™II (Takara, Tokyo, Japan). The reaction was carried out in duplicate for LACK1 and α-tubulin genes in an Applied Biosystem step one instrument (Applied Biosystem, Foster City, CA, USA). The PCR condition was as follows: an initial denaturation at 95 °C for 3 min, 40 cycles of 10 s at 95 °C and 32 s at 60 °C followed by a melt curve analysis using temperature increments of 0.2 °C every 30 s to ascertain amplification of the expected product.

The relative expression of LACK1 gene was calculated by comparing the cycle thresholds of the target gene with that of the housekeeping gene (α-tubulin) using the relative expression software tool (REST, http://rest.genequantification.info) as previously described.

Analysis of gene expression
The relative gene expression value of LACK1 gene in the L. tropica field isolates was normalized to the internal control gene (α-tubulin) and relative to the sensitive reference isolate S175 using REST (as previously described). All experiments were conducted in duplicate, and the results are expressed as the mean ± SDs. The expression ratio results of the target gene were tested for significance by a pairwise fixed reallocation randomization test and plotted using standard error (SE) estimation via a complex Taylor algorithm, calculated by REST. Samples with P < 0.05 were considered significantly different between the groups.

Results
Internal transcribed spacer 1 polymerase chain reaction-restriction fragment length polymorphism
In the current study, 32 samples were examined, including 18 sensitive isolates, which responded to Glucantine therapy as well as 14 resistant isolates, which did not respond to treatment. After DNA extraction, all the clinical isolates were characterized using PCR-RFLP. After digestion with the HAEIII enzyme, all the samples and the reference strain exhibited three bands (200, 70, and
<50 bp) on agarose gel, which correspond to *L. tropica* (Fig. 1).

**LACK polymerase chain reaction amplification and sequencing**

Full-length LACK gene was amplified from *L. tropica*, *L. infantum*, and *L. major* genomic DNA using two specific primers LACK F/R. Figure 2 shows a band of 939 bp obtained from all species, which were cloned into the pGEM vector. After transformation and screening, positive colonies were confirmed using PCR, and then the recombinant plasmids were subjected to sequencing. Sequence alignment analysis showed that all the sequences had significant similarities to the LACK gene in the database, and then the resultant sequences were deposited in the NCBI database. The accession numbers of six reference strains are: (a) *L. tropica* sensitive isolate (MHOM/IR/04/Sensitive-2M (S175)) JX305924.1; (b) *L. tropica* resistant isolate (MHOM/IR/04/Resist-439) JX305923.1; (c) *L. tropica* resistant isolate (MHOM/IR/04/827) JX289832.1; (d) *L. infantum* sensitive isolate (MCAN/IR/10/Sensitive1) JX305922.1; (e) *L. infantum* resistant isolate (MCAN/IR/10/Resist1) JX305921.1; and (f) *L. major* (MRHO/IR/75/ER) JX289833.1.

**cDNA synthesis**

To evaluate the integrity of synthesized cDNA, PCR was conducted using *α*-tubulin primers as the housekeeping genes. The PCR result was considered positive when a single band of 119 bp was observed (Fig. 3).

**Real-time reverse transcription–polymerase chain reaction analysis**

Real-time RT-PCR was used to determine the differential expression of the LACK gene in *L. tropica* field isolates recovered from infected patients that were responsive and unresponsive to Glucantime. All the clinical isolates were characterized as *L. tropica* using PCR-RFLP. The relative gene expression value was analyzed in 32 clinical isolates, including 18 responsive and 14 unresponsive isolates with respect to reference sensitive isolate S175. Figure 4 shows that the maximum and minimum expression level of the LACK gene is in sensitive and resistant isolates, respectively. Our results revealed that the relative mRNA expression of LACK was increased in 13 sensitive isolates by 10.72-fold to 1.6-fold, while it was decreased in one isolate by −1.677-fold (*P* < 0.05), and in four remaining isolates, it exhibited normal expression comparable to ref-
ference strain S175 ($P > 0.05$). On the other hand, in resistant isolates the expression of LACK was increased in five isolates (4.09–1.91-fold) as well as two standard resistant isolates MHOM/IR/10/827 (1.51-fold) and MHOM/IR/10/439 (3.01-fold). However, the transcript levels of LACK were decreased in four resistant isolates ($-1.83$ to $-4.14$-fold) ($P < 0.05$) and in three remaining isolates did not change significantly ($P > 0.05$) (Fig. 4).

Moreover, the overall average of the transcript level of LACK in isolates from unresponsive and responsive patients was $0.479$ and $4.583$, respectively ($P < 0.05$), and a significant decrease in the expression of LACK (9.56-fold) was observed in resistant isolates compared to sensitive ones (Fig. 5).

Discussion

As drug resistance is one of the main issues in successful treatment, the detection of mechanisms involved in the drug resistance process could be helpful in improving treatment strategies. It can also be advantageous in detecting proper markers for monitoring and diagnosing drug resistance cases and predicting the trend of drug resistance in endemic areas.

Researchers have studied the mechanisms of drug resistance in Leishmania from different aspects. Most of these studies have been done on the resistant laboratory isolates, which became resistant under drug pressure. In recent years, the studies conducted on the resistant clinical isolates suggest that the most likely clinical resistance mechanisms are different from laboratory resistance and the diversity of mechanisms such as apoptosis involved in the natural resistance process.

Nowadays, various methods such as real-time RT-PCR, microarrays, and proteomics have been applied to detect the factors and genes effective in clinical resistance. Only a few studies have been done in Iran in the field of Leishmania resistance to antimonial compounds. In a study conducted by Hadighi et al. on 185 patients infected with ACL in Mashad city, 20 cases (10.8%) were non-responsive to treatment in which the drug resistances of theses isolates were proved in in-vitro conditions in macrophage cultivation. This study revealed no difference between the L. tropica sensitive and resistant strains based on sequencing of the pteridine reductase 1 gene. In a study by Hajjaran et al. in 2012, proteomics tech-
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The technique was used to evaluate protein changes in resistant and sensitive isolates of L. tropica. The results determined an increase in the expression level of activated protein kinase C receptor (LACK). Therefore according to the difference of the LACK protein expression in resistant and sensitive isolates, this gene was suggested to be one of the most probable factors involved in drug resistance.

The LACK is a 36 kDa antigen expressed in all Leishmania species. This protein belongs to the repetitive W40 proteins type and possesses tryptophan-aspartate motifs. Proteins of this type play a major role in important cellular activities such as cellular signaling pathway. According to the conducted studies, LACK plays a key role in the survival and establishment of parasite in host. Even though the immunologic response to this protein is well known and has been repeatedly used in the vaccination studies, the precise mechanism of this protein has not yet been discovered in Leishmania.

According to the probable role of the LACK gene in resistance to antimonial compounds, we investigated the expression of this gene in clinical L. tropica strains, which were resistant or sensitive to Glucantime. First, all the isolates were characterized using PCR-RFLP, then the full length of the LACK gene was cloned and sequenced in six reference strains (L. tropica, L. infantum, and L. major). The BLAST analysis revealed high similarities in all the sequences compared to the reference LACK gene in the database. Then the resultant sequences were deposited in the NCBI database.

To investigate the differential expression of the LACK gene in resistant and sensitive clinical L. tropica strains, real-time RT-PCR was conducted. A total number of 32 clinical isolates were investigated in this experiment, including 18 responsive and 14 unresponsive isolates. After the RNA extraction and cDNA synthesis, transcript levels of the LACK gene were analyzed in all isolates with respect to reference sensitive isolate S175. Analysis on the data obtained from RT-PCR, shows a maximum and a minimum expression level of the LACK gene in the sensitive and resistant isolates, respectively. The results indicate that the average of the RNA level of the LACK gene in isolates from unresponsive and responsive patients was 0.479 and 4.583, respectively, and expression of the LACK was significantly downregulated (9.56-fold) in resistant isolates compared to sensitive ones.

A recent study that used the microarray method on clinical resistant strains of L. donovani, a reduction in the gene expression level of one of the kinase-related genes, mitogen activated protein kinase (MAPK1) was observed in the resistant strains compared to sensitive strains. This reduction was also approved by real-time RT-PCR and Western blotting. Moreover, transfection of LACK and its overexpression in the isolates resulted in an increase in their sensitivity to antimonial compound and suggested the key role of MAPK1 in the cellular death pathway induced by the antimonials.

Similarly, Kazemi-Rad et al. in 2013 reported downregulation of this gene in resistant L. tropica isolate by applying the cDNA-AFLP and real-time RT-PCR methods. Furthermore, in another study Brotherton et al. in 2013 detected a mutation of kinase genes in resistant L. infantum isolate using the next generation sequencing method.

In this line, in the current study the mRNA expression level of the activated protein kinase C receptor (LACK) gene was found to be reduced in clinical resistant L. tropica isolates. The reduction of the expression level of kinase proteins (MAPK), which were reported in the earlier studies could be related to the reduction of the expression of receptors that activated kinase protein. This suggests the probable role of this gene in the cellular death pathway induced by the antimonials and thereby antimony resistance.

Conclusion

In conclusion, we used the real-time RT-PCR approach to investigate the mRNA expression of LACK in L. tropica isolates from unresponsive and responsive patients. Our result revealed downregulation of the LACK gene in resistant isolates compared to sensitive ones, suggesting its potential role as a receptor of protein kinase in the antimony resistance process. Moreover, it can be considered as a potential biomarker for clinical resistance monitoring. Further comprehensive studies are required to exploit the biological functions of it in antimony resistance, which would be helpful to develop effective treatment strategies against antimony-resistant parasites.

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References


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