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Original paper

The expression profile of *LmTRYP*, *LmTRYR*, and *LmHSP 83* genes in treatment failure clinical isolates of *Leishmania major*

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ABSTRACT. Cutaneous leishmaniosis (CL) is one of the important neglected tropical diseases caused by *Leishmania* spp. such as *L. major*, *L. tropica* in the Old World. In recent years, some reports of treatment failure in patients with CL have been reported worldwide. Therefore, in this study, we assessed *LmHSP 83*, *LmTRYR*, and *LmTRYP* gene expressions in treatment failure clinical isolates of *L. major*. After sampling from the cutaneous lesions, DNA was extracted and then genera verification and species identification was done using ITS1-PCR-RFLP method. A part of each sample was used in order to RNA extraction and cDNA synthesized. *LmHSP 83*, *LmTRYR*, and *LmTRYP* gene expressions were assessed using SYBR Green real-time PCR. The treatment failure clinical isolates had the mean expression of 5.55±1.67, 247.024±23.54, and 1.204±2.14 for *LmHSP 83, LmTRYR*, and *LmTRYP* , respectively less than the same genes in treatment response isolates (*P*=0.001). This study recommended the other mechanisms may involve in response to treatment in treatment failure clinical isolates of *L. major*.

Keywords: cutaneous leishmaniosis, glucantime, antimonial, treatment failure

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Introduction

Leishmaniosis is a tropical parasitic disease caused by the protozoans belonging to the genus *Leishmania* with an extensive variety of clinical forms, and transmitted to humans by *Phlebotomus* and *Lutzomyia* sandflies. Cutaneous leishmaniosis (CL) is the most common form, with a worldwide incidence of 0.6–1,0 million cases per year. CL is caused by *L. major*, *L. tropica*, and *L. aethiopica* in the Old World [1].

According to the World Health Organization (WHO), CL is considered to be a neglected tropical disease and it represents a significant public health problem worldwide [2]. CL is endemic throughout many developing countries, including Algeria, Afghanistan, Iraq, India, Brazil, Pakistan, Peru, Saudi Arabia, Syria, and Iran [3].

There is no vaccine against this important vector-borne disease, so far; therefore, chemotherapy is considered the best strategy to control this disease [3]. Pentavalent antimonial are the first choice drugs for the treatment of CL [4]. However, many recent studies reported increasing cases with clinical resistance or treatment failure (TF) to pentavalent antimonial. Drug resistance is a multifactorial phenomenon that may be generated by the genomic or molecular mechanisms. So, understanding the molecular and biochemical reasons for this phenomenon is very vital [5].

Heat shock protein (HSP) 83, as a molecular chaperone, has critical roles in differentiation, gene expression, signal transduction pathways, the regulation of its own synthesis, and pathogenesis [7]. Another important molecule in *Leishmania* spp. is tryparedoxin peroxidase (TRYP) that protects the parasite against oxidative stress [8]. In a study conducted by Frézard et al. [9], it was indicated that *TRYP* is associated with resistance in leishmaniosis. Trypanothione reductase (TRYR) is another important molecule involving in the parasite thiol metabolism [10] and *Leishmania* viability [11].

Given that the expression of different genes can play significant roles in drug resistance, we proposed to assess the expression profile of *LmHSP 83, LmTRYP*, and *LmTRYR* genes in TF clinical isolates of *L. major*.

Materials and Methods

Sampling

Samples were collected from patients referred to the Navab-e-Safavi Health Center in Isfahan, Iran from May 2018 to January 2020. The isolates obtained from the patients with CL with no response to treatment were considered as treatment failure (TF). The exclusion criteria included treatment interrupted and co-therapy. For gene expression analysis, three clinical isolates with treatment response (TR) profile were considered as reference samples. Therefore, all samples obtained from the cutaneous lesion were maintained in RNA*later* solution (Ambion, Inc., Austin, TX) for next experiments.

Detection

Each scraping sample was placed on the slides, fixed by methanol (Merck, Germany), stained with Giemsa (Merck, Germany). Microscopic examination was performed out by 1000× magnifications to find the amastigotes. The isolates which were containing amastigotes were considered for species identification using ITS1-PCR RFLP.

Molecular identification

DNA was extracted using a DNA extraction kit (GeneAll, South Korea) according to the manufacturer's recommendations. In order to genus verification and species identification, ITS1-PCR RFLP was done [12,13]. The PCR product of 300-350 bp shows *Leishmania* spp. and the pattern of two fragments with 140 and 220 bp in length identifies *L. major.*

RNA extraction and cDNA synthesis

Total RNA was extracted according to the protocol of the RNA extraction kit (Vivantis, South Korea). Then, all samples were treated with DNase I (CinnaGen, Iran, Tehran). The cDNA was synthesized using the cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions, and then stored at –20 °C for the next steps.

Gene expression analysis

The relative gene expressions of *LmHSP 83*, *LmTRYR,* and *LmTRYP* were analyzed in triplicate using SYBR Green real time PCR by thermocycler (StepOne, ABI, USA) using the specific primer pairs: LmHSP83-F: 5'-GACCTGCCGCTGAACA TCTCG-3' and LmHSP83-R: 5'-TCCTCCTTGTT CTCCGCCACC-3' for *LmHSP 83* gene expression; LmTRYR-F: 5'-CGAC TTTAGCTTCGTGTGCC CG-3' and LmTRYR-R: 5'-CGATGCTCTTGGTC TTGTCGGC-3' for *LmTRYR* gene expression; LmTRYP-F: 5'-CGACTTTAGCTTCGTGTGCCC G-3' and LmTRYP-R: 5'-CGATGCTCTTGGTCT GTCGGC-3' for *LmTRYP* gene expression. The thermal reaction was programmed as follows: 94°C for 10 min as an initial denaturation, followed by 40 cycles at 94°C for 10s and 60°C for 60s. The melt curve analysis using temperature increments of 0.2°C every 30s was done to determine the amplification of the predictable product. The GAPDH was used as endogenous control using the specific primer pair of GAPDH-F: 5'-AGGACATT CTCGGCTTCACCAA-3' and GAPDH-R: 5'-GCC CCACTCGTTGTCATACCA-3' [12]. The fold change was calculated as below:

$$\Delta\Delta CT = \Delta CT_{test}\ (CT_{target\ gene} - CT_{GAPDH}) - \Delta CT_{reference}\ (CT_{target\ gene} - CT_{GAPDH})$$

Statistical analysis

Data were analyzed using SPSS ver-21.0 software by the Chi-square for comparative of mean gene expression of each gene in both groups of treatment failure and treatment response and the One-way ANOVA test for significant analysis between the gene expressions of each gene in all isolates. All data were representative of three independent trials. A *P*-value of 0.05 was considered to be statistically significant.

Table 1. Information of the patients with cutaneous leishmaniosis mentioned in this study

Response to treatments	Sex	Age	Lesion size (mm)	No of lesions	Duration of the disease (weeks)	Sample code
TR	M	9	5.3×7.6	1	4	Lm1
TR	F	31	8.4±2.1×10.2±1.4	2	2	Lm2
TF	F	27	10.7×10.4	1	48	Lm3
TR	M	12	7.9×10.5	1	4	Lm5
TF	M	8	1.4×3.1	1	8	Lm7
TF	M	32	2.3±0.9×5.6±1.2	4	6	Lm12
TF	M	3	50	2	20	Lm16
TF	M	42	30	2	16	Lm18

M: male, F: female, TR: treatment response, TF: treatment failure

Ethical statement

The informed consent was written by all the participant patients. This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Approval ID IR.SSU.MEDICINE.REC.1398.294).The experiments in this study were done according to the requirements of the Helsinki Declaration.

Results

Patients

The patients (five) with TF isolates were included in this study; also in order to gene expression analysis, three isolates with response to treatment (TR) were added. The mean number of lesions in patients with treatment failure pattern was two and in patients with treatment response was 1.33. The mean size of lesions was 6±2.28×4.33±3.62 mm in TF isolates and 9±1.81×6.66±2.16 mm in TR isolates (Tab. 1).

Molecular identification

The ITS1-PCR RFLP method identified that all isolates had fragments of 140 and 220 bp after *Hae* III restriction enzyme digestion, identified as *L. major* (Fig. 1).

LmHSP 83 gene expression

The mean expression of *LmHSP 83* gene in the TF clinical isolates was 5.55±1.67 lower than that in TR isolates (*P*=0.005). The gene expression of *LmHSP 83* in each included isolate in this study is present in figure 2.

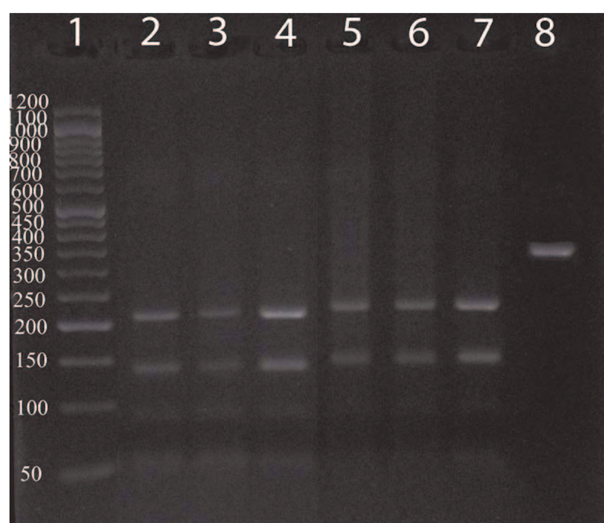

Figure 1. Agarose gel electrophoresis for ITS1-PCR RFLP analysis. Lane 1: 50 bp DNA ladder, lanes 2 to 6: PCR products digested with *Hae* III resulted in two fragments of 140 and 220 bp, which represented *L. major*, lane 7: positive control (*Leishmania major*, MRHO/IR/75/ER), lane 8: undigested ITS1-PCR products from the positive control (*Leishmania major*, MRHO/IR/75/ER)

LmTRYR gene expression

The mean relative expression of *LmTRYR* gene in the isolates with TF pattern was 247.024±23.54 times lower than the mean relative expression of this gene in TR isolates (*P*=0.0001). The gene expression of *LmTRYR* in each included isolate in this study is present in figure 3.

LmTRYP gene expression

The mean relative expression of *LmTRYP* gene

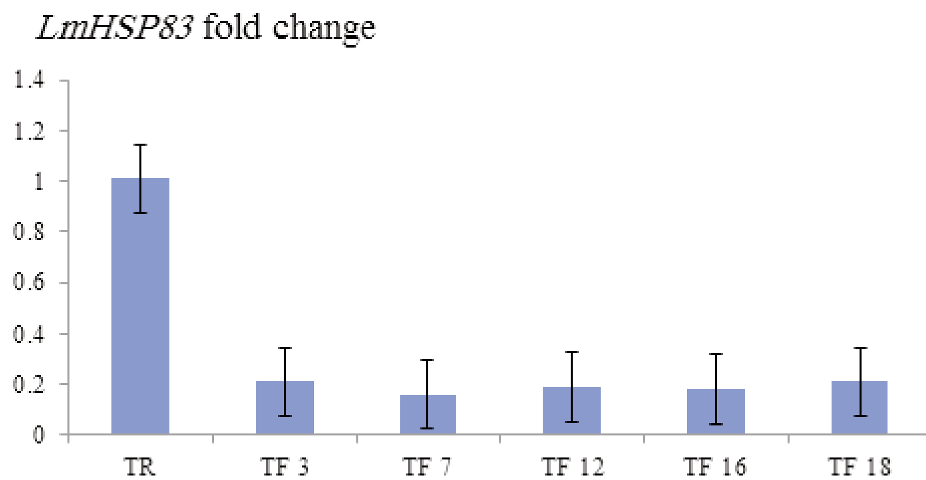

Figure 2. The mean fold change of *LmHSP 83* gene in all treatment failure isolates of *Leishmania major*. The reference sample is related to the mean fold change of *LmHSP 83* in three treatment response clinical isolates of *L. major*

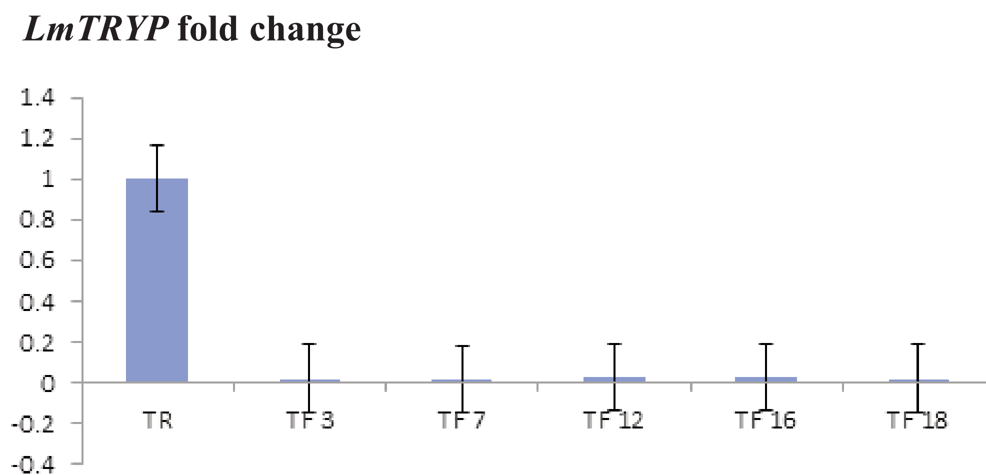

Figure 3. The mean fold change of *LmTRYR* gene in all treatment failure isolates of *L. major*. The reference sample is related to the mean fold change of *LmTRYR* in three treatment response clinical isolates of *L. major*

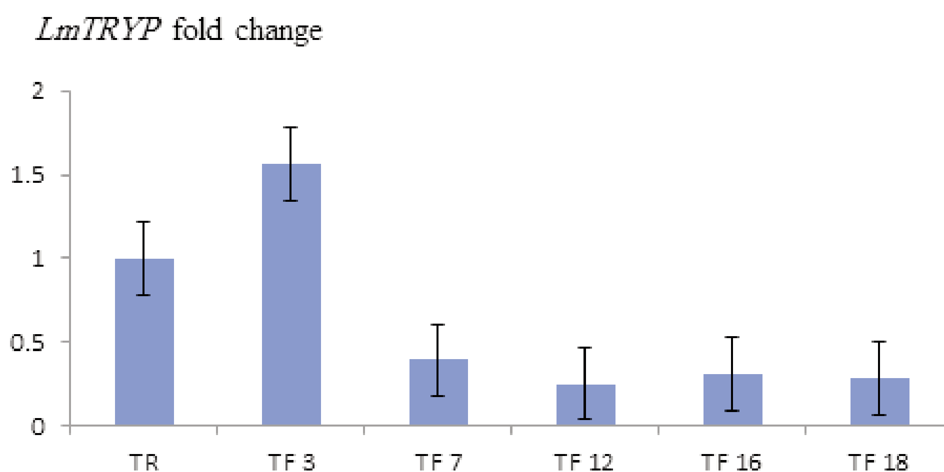

Figure 4. The mean fold change of *LmTRYP* gene in all treatment failure isolates of *L. major*. The reference sample is related to the mean fold change of *LmTRYP* in three treatment response clinical isolates of *L. major*

in TF clinical isolates was 1.204±2.14 times lower than the mean relative expression of this gene in TR isolates (P=0.002). The gene expression of *LmTRYP* in each included isolate in this study is present in figure 4.

Discussion

In this study, *LmHSP 83*, *LmTRYR* and *LmTRYP* gene expression analysis were assessed in TF clinical isolates of *L. major*. We showed that *LmHSP 83*, *LmTRYR* and *LmTRYP* genes expression in the TF isolates had respectively 5.55±1.67, 247.024±23.54, and 1.264±2.14 fold changes lower than those in TR sample.

The isolates in patients with CL and no response to the standard treatment were considered treatment failures [14]. The nature of the resistance to the treatments in the parasite is not well understood. The incidence of non-response to treatment is not clear, and according to various studies, the incidence varies in different districts. Ashutosh et al. [15] reported that the rate of drug resistance to glucantime is 30–60% in visceral leishmaniosis in different areas of India. In some studies in Iran, the rates of drug resistance in CL caused by *L. major* and *L. tropica* are reported as 12 and 6.3% in Mashhad [16] and Kashan [17], respectively. Pentavalent antimonial is still the first choice for the treatment of leishmaniosis but nowadays, there are concerns for developing resistance against this class of drugs. Drug resistance is a multifactorial phenomenon. The response to the treatment relates to the parasite, the host, and the interaction between parasite and host [18]. There are more studies regarding no treatment response in visceral leishmaniosis [19]. However, the studies in the field of treatment response in CL is less and therefore, it is necessary to investigate the related mechanisms in no response to treatment in patients with CL. Some studies showed that a few proteins play as a key role in the development of resistance.

Approximately 2.8% of the total protein in *Leishmania* belongs to the HSP 83. HSP 83 in *Leishmania* spp. is homolog with HSP 90 in mammalian which involves in apoptosis and drug resistance [20–22]. This is opposite of our results that TF clinical isolates of *L. major* had lower expression of *LmHSP 83*. In our study, we showed that *HSP 83* in TF isolates had less expression in comparison with TR isolates. Based on our knowledge, one of the reasons in the isolates with less expression of *HSP 83* is overexpression of Histone 1 (H1) [23]. Therefore, we recommend studying the gene expression of *H1* in the TF isolates alongside with *HSP 83*.

The *LmTRYP* gene expression in this study showed lower expression in TF clinical isolates. TRYP is one of the important proteins in oxidoreductases cascade in *Leishmania* spp. [24]. This protein is member of a huge family in bacteria to mammals involved in defending against antioxidants [25]. Antimonial drugs, as the important treatment against leishmaniosis, make oxidative stress in macrophages, where the parasite resides [26], but TRYP with its antioxidant activity has critical role in destroying hydroperoxide created inside the macrophage and then it results in no response to treatment [27]. In our study, just one of the TF isolates (Lm3) showed overexpression of *TRYP*. In agreement with our study, the results of the studied by Wyllie et al. [27], Das et al. [28], Gómez et al. [29], Andrade and Murta [30], and Henard et al. [31] showed that over expression of *TRYP* is an important mechanism to cause therapeutic failure.

The relative expression of *TRYR* gene in our study in TF3, TF7, TF12, TF16 and TF18 isolates decreased by 1000, 5000, 787.40, 12364 and 9564 times, respectively. TRYR is a new drug target in parasites inhibited by antimonial drugs. Various studies showed that changes in the expression of these proteins in the parasite cause different responses to the sodium stibogluconate (antimony). In a study conducted by Kazemi-Rad et al. [32], the expression of multidrug-resistance protein A (MRPA), aquaglyceroporin (AQP1), TRYR, gamma-glutamylcysteine synthetase (GCS-γ) and, ornithine decarboxylase (ODC) genes in *L. tropica* with resistant and susceptible parasites were investigated and the results indicated that AQP1 gene in TF isolates was significantly decreased when compared with susceptible parasites, but the MRPA, TR, GCS-γ, and ODC genes were increased in the *L. tropica*-resistant parasites, which is in contrast to our results.

In conclusion, all three genes mentioned in this study, including *LmHSP 83*, *LmTRYR*, and *LmTRYP* were identified in TF3, TF7, TF12, TF16, and TF18 isolates and showed significantly reduced expression when compared with TR isolates. For more accurate conclusions, an experimental design with more samples is required. It seems that other molecular mechanisms could be related to treatment

failure. This can indicate the complex mechanisms of drug resistance or treatment failure.

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