

RNA Polymrase II gene expression in clinical *Leishmania major* isolates with no-response-to-drug pattern

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ABSTRACT

Cutaneous leishmaniasis (CL) is one of the most important infectious diseases in the world and is increasing day by day. No effective vaccine has been made against this disease, so far. An important issue with this disease is the development of drug resistance or no response to drug, which is going to spreading that its mechanism has not yet been completely identified. The main aim of this study was to assessment the expression of *RNAPII* gene in no response to drug and susceptible isolates of *Leishmania major*. The patients with CL from the central and North of Iran were considered for this study. The samples were transferred in RNAlater solution and stored in -20 °C. RNA extraction and cDNA synthesis were performed. The gene expression analysis was done with SYBR Green Real Time PCR. Written informed consent was filled up by patients and then information forms were written based on Helsinki declaration. Statistical analysis was done with SPSS (16.0; SPSS Inc, Chicago) using independent t-test. $P \leq 0.05$ was considered significant. It was observed that the gene expression of *RNAPII* in the no response to drug isolates was lower than that the one in drug sensitive isolates. A change in the expression of *RNAP II* in no response to drug isolates of *L. major* can indicate the potential role of this gene in the related mechanism.

Keywords: Cutaneous leishmaniasis; *Leishmania major*; no-response to drug; RNA polymerase II; gene expression

1. INTRODUCTION

Leishmaniasis is caused by protozoa belonging to the kinetoplastida called *Leishmania* spp. that is one of the most important health problems in the world [1]. This disease has been reported from more than 100 countries, so far [2, 3]. More than 14 million people are affected by this disease and about 2 million new items are annually reported. Also, over 350 million people are at risk of this disease [4]. The most common form of the disease is cutaneous leishmaniasis (CL) that is endemic in Algeria, Afghanistan, Sudan, Saudi Arabia, Iran, Syria, Iraq, Brazil, and Peru [5, 6].

The first line treatment of leishmaniasis is antimonial compounds especially pentavalent antimonial compounds. Unfortunately, antimonial resistance isolates have been reported from all over the world especially in endemic areas [7-9]. Some of the known mechanisms in drug resistance in leishmaniasis include gene

duplication, gene deletion, gene expression, and aneuploidy [10, 11]. Gene expression changing of the involved gene in clinical isolates have an important role in drug resistance, i.e. decreasing in *Aquaglyceroporin 1 (AQPI)* gene expression make increasing in antimonial resistance [12], although there are some documents with controversy [13, 14]. In the last cases, we encounter the other involved mechanisms and genes. However, the upstream of the gene expression in *Leishmania* parasite is RNA Polymerase II (RNAPII). The RNAPII function is generation of mRNAs from various genes in *Leishmania* spp. [15]. Therefore, the gene expression of *RNAPII* may be an important role for mRNAs expression regulation in no response to drug isolates. In this study, we assessed the gene expression of *RNAPII* in no response to drug isolates obtained from different loci from Iran by SYBR Green Real Time PCR.

2. MATERIALS AND METHODS

Samples and sampling.

The clinical isolates obtained from patients referring to Navab Safavi Health Center, Isfahan and Golestan Health Center, Iran from October 2015 to December 2017. The diagnosis of CL was done by microscopic observation. The *ITS1*-PCR-RFLP method was also used for species identification [14]. The restriction enzyme of *HaeIII* was used for RFLP analysis. Also, in each run of PCR, ddH₂O and *L. major* (MRHO/IR/75/ER) were used as negative control and positive control, respectively. Four isolates were detected as no response to drug that in this study were named

as Lm2 to Lm5. The isolates also were collected in RNAlater solution (Merck, Darmstadt, Germany) and stored at -20 °C for next steps. An antimonial sensitive isolate was considered as the standard sample (Lm1) that was assessed in each reaction with the antimonial no response to drug isolates.

Ethics.

This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences in Yazd with the code of IR.SSU.MEDICINE.REC.1396.154. Each patient was informed about the project, and after the agreement, the written informed

consent was designed in order to participate in this study. Sampling was done based on Helsinki declaration.

RNA extraction and cDNA synthesis.

The RNA was extracted from all included isolates using the total RNA extraction Kit (Vivantis, Malaysia) based on the manufacturer’s instruction. The quantity and quality of the extracted RNAs were analyzed using spectrophotometer and 1% agarose gel electrophoresis (Thermo Fisher Scientific, USA), respectively. Then, cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) based on the manufacturer's instruction.

Target gene and primers.

The gene expression of *RNAPII* was performed in the mentioned isolates in this study using SYBR Green Real-time PCR by the specific primer of RNAPII-F 5'-CGAAGCTGAGCATGAAAGAGGTG-3' and RNAPII-R 5'-

GCCCCACTCGTTGTCATACCA-3' that were designed in this study and assessed bioinformatically by BLAST [16, 17]. *GAPDH* was considered as the endogenous control by the specific primer pair introduced by Eslami et al, 2016 [14].

Real-time PCR.

Amplification was performed in a final volume of 20 µl containing 10 µl SYBR Green I master mix, 200 nM each primer, 2 µl cDNA using Step One Real Time thermocycler (Applied Biosystem, USA). The reaction temperature included 95 °C for 10 s for the first denaturation and followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The melting curve analysis was done the program of 60 to 95 °C with increasing rate of 0.3 °C/s.

Analysis.

Gene expression analysis was performed using the 2^{-ΔΔCT} method. All reactions were repeated in triple.

3. RESULTS

The ITS1-PCR-RFLP showed all samples were *L. major* (Figures 1 and 2). Given Figure 1 shows the samples were *Leishmania* spp. with the expected fragment size of around 350 bp. Figure 2 shows the samples with a pattern similar to *L. major*. The gene expression level or Relative Quantitative (RQ) of *RNAPII* in all isolates is shown in Figure 3. As shown in Figure 3, the RQ of all no response to drug isolates is lower than the sensitive isolate.

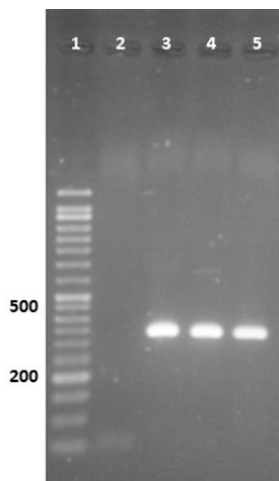


Figure 1. Agarose gel electrophoresis for analyzing of the PCR product of ITS1-PCR in order to *Leishmania* spp. detection. The expected amplicon for *Leishmania* spp. was 300-350 bp. Lane 1: 50 bp DNA ladder, lane2: negative control, lane3: positive control with the standard strain of *L.major* (MRHO/IR/75/ER), lane 4, 5: some samples with fragment with the size of *L. major*.

In this study, we showed that the gene expression of *RNAPII* in no response to drug *L. major* isolates was lower than the sensitive one. Based on our knowledge, it is the first report for the gene expression level in no response to drug isolates of *L. major*. The gene of *RNAPII* encodes the RNA polymerase II that its major function is the generation of mRNAs and most of the small nuclear RNAs (snRNAs) [15]. Eslami et al. [15] showed that *RNAPII* had different mutations among the clinical isolates with various molecular characterization of ITS1 region. The mentioned isolates had different ITS1-PCR-RFLP pattern that was similar to

Crithidia spp, although the sequence of *RNAPII* in some was similar to *L. major* and in some was similar to *Crithidia* spp.

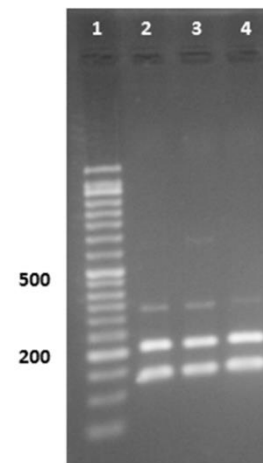


Figure 2. Agarose gel electrophoresis for analyzing of the PCR product of ITS1-PCR-RFLP in order to *Leishmania* spp. identification. The expected fragments after *Hae*III restriction enzyme digestion was 220 and 110 bp for *L. major*. Lane 1: 50 bp DNA ladder, lane2: positive control with the standard strain of *L. major* (MRHO/IR/75/ER), lane 3, 4: some samples with pattern resemble to *L. major*.

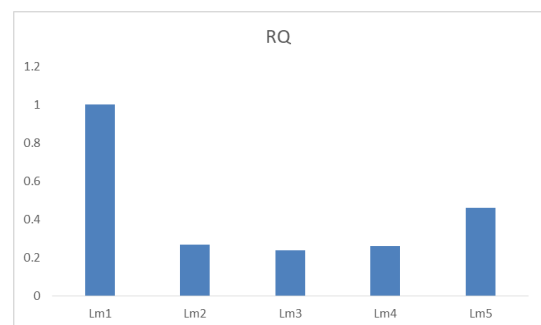


Figure 3. The gene expression of *RNAP II* from clinical isolates of *Leishmania major*. Lm1 isolate is the standard or reference isolate that is drug-sensitive to antimonials. Lm2 to Lm5 are the no-response to drug isolates against antimonials.

Also, the patients harboring the mentioned isolates had different phenotypes of CL, i.e. duration of the CL was very short even without any treatment. The results of Eslami et al. [18] and the

ones in this study showed that *RNAPII* gene expression may have an special role in no response to drug *Leishmania* spp. isolates. *RNAPII* encodes RNA polymerase II that has special role to synthesize pre-mRNA and also U-rich short RNAs regarding spliceosomes and therefore processing of pre-mRNA to translatable mRNAs [19]. Based on our knowledge, the proteins involving drug responses in *Leishmania* spp. comprise Tryparedoxin [20], MAPK1, HSP 90, HSP70 [21], AQP1 [14], Tryparedoxine peroxidase 6 [22], JBP1 [23, 24], etc.

Based on our knowledge, the main drug resistance in *Leishmania* spp. is changing in gene dosage, i.e. higher copy number variations, higher gene dosage [25]. LRR protein is an important gene in antimonial resistance in *Leishmania* spp. [26]. This gene is located in chromosome 6.

The gene expressions in *Leishmania* spp. is under regulation of RNA Polymerase II. We showed in this study that *RNAPII* gene expression is lower than the sensitive isolate. It may have two different reasons. First, although we know that the over expression of the genes is related to the regulation by *RNAPII* in antimonial resistance of *Leishmania* spp., there are some documents [27, 28] that show the RNA stability has an especial role in over expression of the genes involving in no responsibility to drug isolates.

There are some documents that showed the over expression of a gene in the absence of a promoter may result from either gene duplication following multicopy arrays of identical/semi-identical

genes or supernumerary chromosomes [29-33]. The last has been involved in drug resistance in *Leishmania* [10, 34]. This is in agreement with our results. It is obviously clear that drug resistance is resulting from the involved gene with over expression. But because *RNAPII* had low expression in no response to drug isolates, therefore some other mechanisms may help to over expression of the involved gene in drug resistance. Aneuploidy is one of the mentioned mechanisms that make higher copy number and therefore higher transcript levels [35]. The parasite can adapt itself by aneuploidy. This can affect also on phenotype, virulence, and drug response.

The *RNAPII* is located in chromosome 31. Based on our knowledge, chromosome 31 in *Leishmania* is exceptional because has no aneuploidy [36]. Therefore, genomic variation and adaptation are regarding other chromosomes such as 6 harboring *LRR* that higher expression is resulting in aneuploidy. However, it seems that some genes use aneuploidy for adaptation and gene expression regulation and therefore some other mechanisms such as posttranscriptional ones are involved [37, 38]. We know that the copy number of chromosome 31 is >2 [39]. Aneuploidy or multi coping of the genes makes heterogeneity [40], but it seems that chromosome 31 without aneuploidy make itself more conserved than the other chromosomes. It may show the importance of chromosome 31 that has been conserved from more than 40 million years ago [41].

4. CONCLUSIONS

It can be said that the occurrence of drug resistance in the *Leishmania* parasite is due to different molecular variations, each of which has a special role, and these changes can vary in different strains of *Leishmania* and in different geographical regions. Due

to the increasing spread of the disease in different parts of the world and the spread of drug resistance, it is important to investigate and identify the cause of no responsibility to drug.

5. REFERENCES

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