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ORIGINAL ARTICLE

J-binding protein 1 and *J-binding protein 2* expression in clinical *Leishmania major* no response-antimonial isolates

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Abstract Cutaneous leishmaniasis (CL) is a major disease in many parts of the world. Since no vaccine has been developed, treatment is the best way to control it. In most areas, antimonial resistance whose mechanisms have not been completely understood has been reported. The main aim of this study is gene expression assessing of *J-binging protein 1* and *J-binding protein 2* in clinical *Leishmania major* isolates. The patients with CL from central and north 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 using ΔΔCT. Written informed consent forms were filled out by patients, and then, information forms were filled out based on the Helsinki Declaration. Statistical analysis was done with SPSS (16.0; SPSS Inc, Chicago) using independent *t* test, Shapiro–Wilk, and Pearson's and Spearman's rank correlation coefficients. $P \leq 0.05$ was considered significant. The gene expression of *JBP1* and *JBP2* had no relation with sex and age. The *JBP1* gene expression was high in sensitive isolates obtained from north of the country. The *JBP2* gene expression was significant in sensitive and no response-antimonial isolates from the north, but no significant differences were detected in sensitive and resistant isolates from central Iran. Differential gene expression of *JBP1* and *JBP2* in various clinical resistances isolates in different geographical areas shows multifactorial ways of developing resistance in different isolates.

Keywords Cutaneous leishmaniasis · Drug resistance · *JBP1* · *JBP2*

Abbreviations

CL	Cutaneous leishmaniasis (CL)
ACL	Anthroponotic cutaneous leishmaniasis
ZCL	Zoonotic cutaneous leishmaniasis
JBP	J-binding protein
RNAP II	RNA polymerase II
ITS	Internal transcribed spacer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Introduction

Leishmania is a protozoan parasite with worldwide distribution. It is the agent of leishmaniasis that has wide spectrum of manifestations, including cutaneous leishmaniasis (CL), visceral leishmaniasis (kala-azar), and mucocutaneous leishmaniasis. CL is endemic in many parts of the world, including anthroponotic CL (ACL) and zoonotic CL (ZCL) (Yaghoobi-Ershadi et al. 2004). To date, there is no suitable vaccine, so chemotherapy is the main treatment. Pentavalent antimony (SbV) compound is the first-line

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treatment and is comprised of sodium stibogluconate, meglumine antimoniate, or generic formulations. Unfortunately, though, antimony resistance has occurred, especially in endemic regions (Guerin et al. 2002; Herwaldt 1999).

Drug resistance in CL may be due to several factors, including drug–host immune interaction, pharmacokinetic differentials, and the variant genetics of the agent species of *Leishmania*. The gene dosage may have affective results in response to drugs, including intrachromosomal rearrangements (Mukherjee et al. 2013), whole chromosome copy numbers (Downing et al. 2011), and aneuploidy (Ubeda et al. 2008). Silencing or expression of genes may be another important factor that changes the drug responses that regulate with RNA polymerase II. The final factor is under regulation of DNA modifications and synthesis of base J (β-D-glucosyl-hydroxymethyluracil) in kinetoplastida. Base J commonly localizes at terminator sites (van Luenen et al. 2012) and is critical for terminating RNA polymerase II (RNAP II) transcription which is responsible for transcription of protein-coding genes. Base J is common in either repetitive DNA, such as telomeric repeats (Gommers-Ampt et al. 1993; Genest et al. 2007) or some other regions inside the genome (van Luenen et al. 2012). Biosynthesis of base J is a potential target for chemotherapy of pathogenic kinetoplastids (Borst and Sabatini 2008).

Base J is synthesized by J-binding protein 1 (JBP1) and J-binding protein 2 (JBP2) (DiPaolo et al. 2005; Yu et al. 2007). JBP1 binds base J through the duplex DNA and then affects J levels. JBP2 does not bind to J-DNA (DiPaolo et al. 2005), but acts instead on the SWI/SNF domain (DiPaolo et al. 2005). Both JBP1 and JBP2 belong to TET/JBP subfamily of dioxygenases in that their activity depends on Fe^{2+} and 2-oxoglutarate (Iyer et al. 2009, Tahiliani et al. 2009). The main focus of this study was the relationship between *JBP1* and *JBP2* gene expression in antimonial resistance and susceptible clinical isolates of *L. major*.

Materials and methods

Designing

Nine clinical isolates with antimonial-resistant phenotype were obtained from patients referred to Navab Safavi Clinical Center, Isfahan, and Clinical Center of Golestan, Iran, from October 2015 to September 2018. The CL primary diagnosis was done using microscopic observation of Giemsa-stained slides. The final detection and identification were performed using internal transcribed spacer (ITS) 1-PCR–RFLP. The *L. major* isolates were included in this study. Each patient was informed about the project, and after agreement, was given the written informed consent form to sign in order to participate in this study. Sampling was done based on Helsinki Declaration. Another nine isolates of antimonial-sensitive *L. major* were included in this study for comparison with our interested isolates. Totally, 18 samples were considered for this study, including seven isolates from Navab Safavi Clinical Center, Isfahan, Central of the country (Lm1 to Lm7); and 11 samples from Clinical Center of Golestan, north of Iran (Lm8–Lm18).

Sampling

The samples obtained from the edges of skin lesions were immediately transferred into RNA*later* storage solution (Merck, Darmstadt, Germany) and stored at − 20 °C for later steps. The clinical data, along with characteristics of all included patients, were collected from each patient. All studied patients were observed for 3 months in order to determine whether the *L. major* was antimonial-sensitive or antimonial-resistant. This study was approved by the Ethics committe from Research Vice Chancellor of the Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

RNA extraction and cDNA synthesis

All 18 isolates were included for total RNA extraction using the total RNA extraction kit (Vivantis, Malaysia) based on the manufacturer's instructions. The RNA quality and quantity were assessed using 1% agarose gel electrophoresis and spectrophotometer (Thermo Fisher Scientific, USA), respectively. Then, cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with reverse transcriptase enzyme and primers either oligo dT or random hexamer, based on the manufacturer's instructions.

Target genes and primers

The gene expression of *JBP1* and *JBP2* was assessed using SYBR Green real-time PCR. The GAPDH (Eslami et al. 2016) was considered as endogenous control in gene expression analysis. All primer pairs related to the *JBP1* and *JBP2* were designed using Primer3 software (Table 1) and then checked by BLAST (Altschul et al. 1997, 2005).

Real-time PCR

Amplifications were reacted in a total volume of 20 µl containing 2 µl cDNA, 10 µl SYBR Green I master mix, and 200 nM each of primer (Table 1) using a Step One thermocycler (Applied Biosystem, USA). The thermal conditions of reaction were 95 °C for 10 s in order to first

Table 1 The primer name and their sequences for gene expression of JBP1, JBP2, RNAP II, and GAPDH that were used in this study

Sequences (5′–3′)	Primer name
ATCTTTAACTTCCCGACCGCCA	JBP1-F
CTCGCAGCACAACACCAATGAT	JBP1-R
AGGACATTCTCGGCTTCACCAA	GAPDHL-F
GCCCCACTCGTTGTCATACCA	GAPDHL-R
CTCAACACGATGATCCAACTCTGC	JBP2-F
GCCGCCATCTTCCTCGTTCTTC	JBP2-R

achieve denaturation, followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The final extension was done at 72 °C for 10 min. Specificity of the reaction products was confirmed by analysis of the dissociation curve using melting curve, which consisted of temperatures between 60 and 95 °C with a heating rate of 0.3 °C/s. In addition, the amplicons size in each specific primer pair was confirmed by analysis of the amplicons using 3% agarose gels in 0.5 × TBE, stained with DNA Green viewer and visualized under UV light.

Statistical analysis

Comparisons of *JBP1* and *JBP2* gene expression levels (showed with relative quantification (RQ) of each gene), were done using the 2^−ΔΔCT method. Analysis of other characteristics was done with the independent t-test in the statistical package for the social sciences version 20 (SPSS, Version 16.0; SPSS Inc, Chicago, IL). The Shapiro–Wilk test was used to verify that the data were normally distributed. The Pearson's and Spearman's rank correlation coefficients were used to evaluate the correlation between normalized expression of all studied genes with the age of patients using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA, USA). The $p \leq 0.05$ was considered significant.

Results

The mean size of lesions in patients with no response-antimonial isolates was $11.7 \pm 7.3 \times 8.71 \pm 5.16$ cm^2, but it was $2.9 \pm 3.2 \times 2.7 \pm 3.3$ cm^2 in patients with antimonial-sensitive isolates. The lesion numbers in resistant cases was in the range of 1 (2 cases) to 9 (1 case), and in sensitive ranged from minimum 1 (5 cases) to maximum 4 (1 case). The samples of Lm2, Lm3, Lm5, Lm6, Lm11 and Lm12, Lm13, Lm15, Lm18 were considered as antimonial-resistant isolates. The residual ones were sensitive to antimonial therapy.

The gene expression (RQ) of *JBP1* for all 18 isolates is shown in Fig. 1. Among the no response-antimonial isolates, Lm11, Lm12, Lm13, Lm15, and Lm18 were obtained from the north of Iran and the others were from the central region of the country. Among the sensitive isolates, Lm1, Lm4, and Lm7 were isolated from the central region of Iran and Lm8, Lm9, Lm10, Lm14, Lm16, and Lm17 were obtained from the north of Iran. For calculation of delta–delta CT and then RQ of the mentioned gene, Lm4 was the one isolate that was considered as the standard isolate. The gene expression analysis showed that out of 11 isolates from the north of Iran (Lm8–Lm18), just six isolates (Lm8, Lm-9, Lm10, Lm14, Lm16, Lm17) showed higher RQ for *JBP1* that all of them were antimonial-sensitive (Fig. 1).

Among the isolates obtained from patients with CL in the central of Iran, the RQ of *JBP1* is shown in Table 2. Statistical analysis showed that the differences in RQ of *JBP1* between the resistance and sensitive groups of the isolates from the central of the country were not significant ($p = 0.7654$).

The delta–delta CT method showed that the expression level of *JBP1* in sensitive patients from the north of Iran was 206.15 times higher than no response-antimonial ones in same region. Moreover, statistical analysis showed that the gene expression of *JBP1* gene was not significantly different between female and male patients ($p = 0.28$). Also, no correlation was reported between age and *JBP1* gene expression ($p = 0.28$).

The gene expression (RQ) of *JBP2* for all 18 isolates is shown in Fig. 2. Overall, *JBP2* gene expression was not significantly different among the resistant and sensitive isolates observed in this study ($p = 0.2470$). Lm2 showed a 33.33-fold decrease in *JBP2* expression. The mentioned isolate was obtained from a 10-year-old child that had been referred to the Health Centre of the central region of the country about 20 days after the onset of disease harboring three lesions.

Among the isolates obtained from patients with CL in central Iran, the gene expression (RQ) of *JBP2* is shown in Table 2. Statistical analysis showed that the differences in RQ of *JBP2* between the resistance and sensitive groups of the isolates from the central of the country were not significant ($p = 0.5371$).

The delta–delta CT method showed that the expression level of *JBP2* in sensitive patients from the north Iran was 2.43 times higher than no response-antimonial ones in same region ($p = 0.003$). Moreover, statistical analysis showed that the gene expression of *JBP2* was not significantly different between female and male patients ($p = 0.28$). Also, no correlation was shown between age and *JBP2* gene expression level ($p = 0.28$).

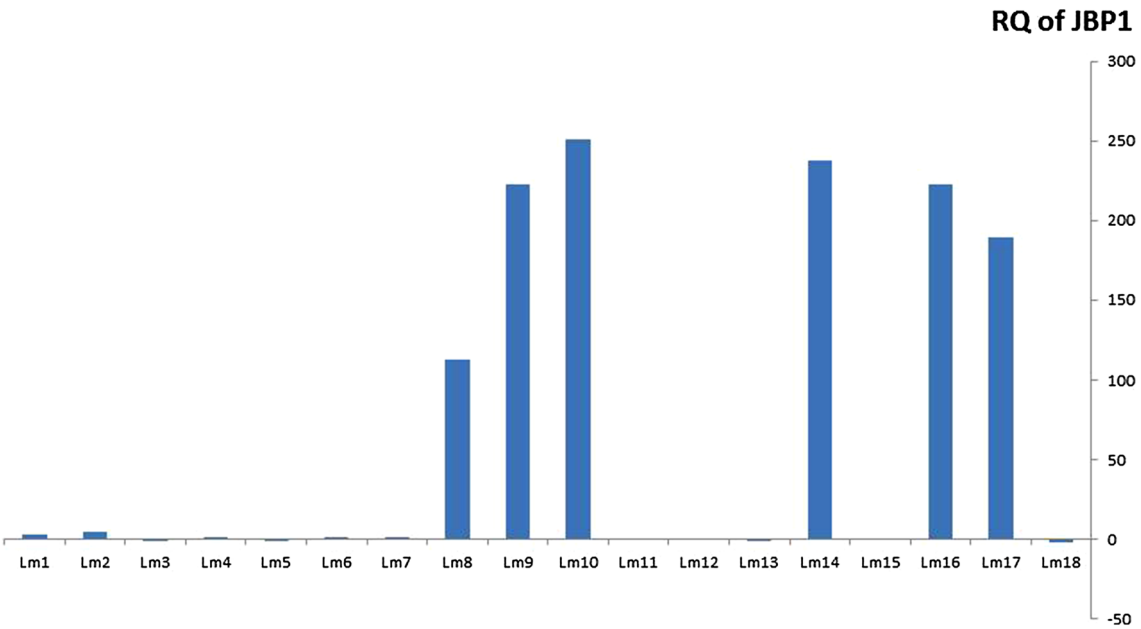

Fig. 1 The RQ of JBP1 in 12 isolates of L. major obtained from patients with cutaneous leishmaniasis (CL). The isolates of Lm2, Lm3, Lm5, Lm6, Lm11, Lm12, Lm13, Lm15

Table 2 The RQ of *JBP1* and *JBP2* in isolates obtained from patients with cutaneous leishmaniasis (CL) from the central of the country. Lm2, Lm3, Lm5, and Lm6 were the antimonial failure isolates

	Isolates						
	Lm1	Lm2	Lm3	Lm4	Lm5	Lm6	Lm7
RQ of *JBP1*	2.56	4.23	− 1.23	1	− 1.26	1.1	1.67
RQ of *JBP2*	2.48	− 33.33	1.35	1	2.48	1.06	2.55

Discussion and conclusion

In this study, the gene expression of *JBP1* and *JBP2* was analyzed among the antimonial-sensitive and no response-antimonial isolates obtained from different parts of Iran as an endemic area for ZCL, including the central and northern regions. The results showed that it is likely that *JBP1* would be related to the response to antimonial drugs. *JBP1* and *JBP2* encode JBP1 and JBP2, respectively, whose roles are different in various kinetoplastids. Cliffe et al. (2009) reported that *JBP1* and *JBP2* had no effect on viability and phenotype of *Trypanosoma brucei.* However, Ekanayake and Sabatini (2011) showed that both genes of *JBP1* and *JBP2* affected on viability in *T. cruzi. JBP1* has a critical role in *L. tarentolae* (Genest et al. 2005), and *JPB2* causes differences in gene expression in *Leishmania* (van Luenen et al. 2012). Our results showed differences in *JBP1* gene expression among the no response-antimonial isolates, too. Also, we previously showed different patterns of gene expression of *AQP1* in various no response-antimonial isolates in different parts of the country (Eslami et al. 2016). We previously showed that the gene expression of *AQP1* in no response-antimonial isolates in the central part of the country has higher expression of *AQP1* gene in comparison with the antimonial-sensitive isolates (Eslami et al. 2016), but the no response-antimonial isolates obtained from patients with CL in the north of the country showed lower *AQP1* gene expression in comparison with the sensitive ones (in press). Some other studies showed decreased gene expression of *AQP1* in clinical no response-antimonial isolates (Decuypere et al. 2005; Mandal et al. 2010; Marquis et el. 2005; Mukherjee et al. 2007). Jeddi et al. (2014) showed various gene patterns in clinical no response-antimonial isolates from Mediterranean region, resulting in different metabolic pathways (Jeddi et al. 2014). They showed that just three isolates had overexpression of *GSH1* and *TRPER,* and one isolate had overexpression of *GDH1* and *MRPA* (Jeddi et al. 2014). They also showed that no predominant gene or pathways are involved in making drug resistance. Our study showed that the clinical no response-antimonial isolates had no dominant gene for making resistance to antimonials from the central region of the

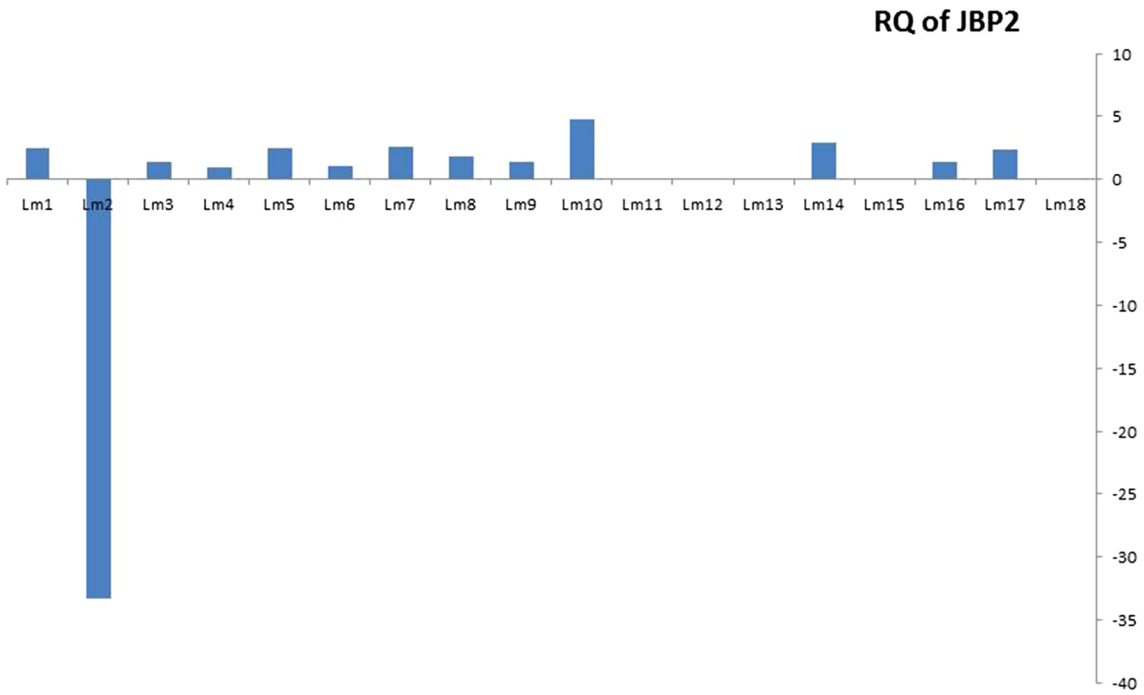

Fig. 2 The RQ of JBP2 in 12 isolates of L. major obtained from patients with cutaneous leishmaniasis (CL). The isolates of Lm2, Lm3, Lm5, Lm6, Lm11, Lm12

country. The resistance in isolates from the central seems to be multifactorial. These data are in accordance with the other studies (Adaui et al. 2011; Kumar et al. 2012). The other studies also showed genetic variation in *L. major* isolates in Iran (Eslami et al. 2014; Eslami and Salehi 2014; Eslami et al. 2011, 2012). These literatures were in agreement with our results in this study regarding the difference in gene expression in various geographical parts of the country. It seems that different *JBP1* and *JBP2* gene expressions in clinical isolates from different regions of the endemic area relate to different ways in transcription regulation. Based on our knowledge, kinetoplastida and especially *Leishmania* are the only organisms that have different ways for transcription regulation (Kramer 2012).

In this study, we showed that the sensitive isolates from the north of Iran showed the same pattern in gene expression of *JBP1* and *JBP2* in comparison with the no response-antimonial isolates. The higher gene expression of the mentioned genes corresponds to an increase in the J base synthesis. Higher synthesis of J base causes blockage of the RNA polymerase II protein (Hazelbaker and Buratowski 2012). *Leishmania* and the other trypanosomatids have unique ways for gene organization and transcription in comparison with the other eukaryotes (Campbell et al. 2003; Clayton 2002; Martinez-Calvillo et al. 2003). Based on our knowledge, three classes of nuclear RNA polymerase (RNAP) are present in eukaryotic cells, including RNAP I, II, and III. Each type of RNAP involved in different processes: RNAP I in production of 18S, 5.8S, and 28S rRNAs; RNAP II in generation of mRNAs; and RNAP III in synthesis of small essential RNAs (Lee and Young 2000; Paule and White 2000). RNAP II gene expression in *Leishmania* is the main RNAP for mRNA production in kinetoplastida that is under control of the number of base J production. Multidrug resistance (MDR) loci (Legare et al. 2001), AQP1 (Gourbal et al. 2004), HSP70 and HSP90 (Brochu et al. 2004; Vergnes et al. 2007), P299 (Choudhury et al. 2008), and ARM58 (Nühs et al. 2014) are the important molecules related to antimonial resistance in *Leishmania* spp. All of the mentioned proteins are under regulation of RNAP II. It seems that increasing the *JBP1* and *JBP2* genes expression level may increase J base synthesis in sensitive isolates in the north of Iran. Massive J base results in decreasing the *RNAP II* gene expression, following decreasing gene expression by RNAP II pathway. In this study, the isolates from the central regions of the country did not show the same events; therefore, the various pathways may involve in making no response-antimonial isolates such as findings in the other studies (Eslami et al. 2016; Kazemi-rad et al. 2013).

Overall, heterogeneous gene expressions in clinical no response-antimonial isolates showed that various genes and pathways may be involved in causing drug resistance. Therefore, it is recommended to analyze the different gene expression patterns in various geographical areas that could help for designing the program strategies for prevention and control of the disease.

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Authors' contributions statement GE conceived, designed, and drafted this manuscript. MV performed the statistical analysis. VA obtained the samples. AF drafted the manuscript. SSH and SA performed the laboratory techniques and quality control. ME reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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