The Role of *ATP-binding Cassette Transporter* Genes Expression for Treatment Failure in Cutaneous Leishmaniasis

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Abstract

Background: Leishmaniasis is one of the common diseases transmitted by sand flies in tropical and subtropical regions of the world. Currently, antimonial derivatives are the first line of treatment. Some of the members of the ATP-binding cassette (ABC) family of *Leishmania* are shown to be associated with resistance to antimonial. In this study, we evaluated *ABCI4*, *ABCG2*, *ABCC7*, and *ABCC3* gene expression in *Leishmania* isolated from patients with non-healing cutaneous leishmaniasis.

Results: Five cases were treatment failure that all of them were identified as *L. major*. All treatment failure clinical isolates were *L. major*. Gene expression analysis in treatment failure isolates showed that the ABC transported genes had a different pattern in each isolate. *ABCC7* had overexpression in all isolates. Among the treatment failure isolates, only one sample had overexpression in all ABC transporter genes under study.

Conclusions: Treatment failure has been reported for cutaneous leishmaniasis worldwide. Knowledge of the molecular mechanisms of treatment failure could solve this problem. *ABC transporter* genes are considered controversy over the mechanisms of treatment failure outcomes. In this study, we showed that ABC transporter genes could be considered one the important mechanisms.

Background

Leishmaniasis is a tropical neglected parasitic disease caused by different *Leishmania* spp., which is transmitted by *Phlebotomus* spp. Leishmaniasis clinical manifestations are three main forms of cutaneous leishmaniasis (CL), kala-azar or visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL) [1]. Leishmaniasis is endemic in more than 100 countries more than 1 billion people are at risk of leishmaniasis in endemic areas with annual estimation of 30 000 new cases of VL and more than 1 million new cases of CL annually [2]. Almost 80% of CL incidence in 2020 is reported from Afghanistan, Algeria, Brazil, Colombia, Iraq, Pakistan and the Syrian Arab Republic [2]. About 20 000 CL cases are reported annually in Iran although the real cases are higher around four or five times. Rural and urban CL is reported in Iran, but the rural type is endemic in 15 provinces [3].

Although the CL condition is self-limiting and the resulting lesions heal within a year, there are cases with diffused infection resulting in chronic ulcers [4]. Because wounds and lesions caused by leishmaniasis are more common in the upper limbs, especially in the face, it can affect a person's beauty and cause psychological and social stress in the patient [5].

No vaccine is developed against leishmaniasis, so treatment is considered the sole solution to control leishmaniasis. The first line of treatment for leishmaniasis is antimonial derivatives including meglumine antimonate (Glucantime) and sodium stibogluconate (Pentostam) used to treat the disease for more than 80 years [6]. The efficacy of antimonial is not high and resistance is reported from different parts of the world [7–9]. Recently, it was shown that a single mechanism does not lead to treatment failure and the exact mechanism(s) is not known [10]. One of the most important reported mechanisms is low

expression of the *aquaglyceroporin 1* gene (*AQP1*), resulting in reduced entry of antimony drugs into the parasite [9]. Other reported mechanisms are considered pumping the drug by ATP-binding cassette (ABC) transporters [7].

ABC transporters are one of the most prominent families of proteins with vital physiological functions. These proteins are found in various animal species, from prokaryotes to humans, and use ATP hydrolysis to exclude multiple compounds across the membranes. ABC proteins play a critical role in drug resistance through two mechanisms. The first mechanism occurs by amplifying or increasing the expression of ABC-carrying genes, which increases the transporters at the membrane level and associated with excessive drug outflow from the cells. The second mechanism is mutations in genes that alter ABC transporters' biochemical properties and affect carrier capacity [11]. In *Leishmania*, ABC protein is considered multidrug resistance protein (MRP) with a major role in metal resistance using thiol metabolism and drug efflux mechanisms [12]. P-glycoprotein A (PGPA) is a member of MRP ABC transporter family involved in drug resistance, especially antimoniate and arsenite.

The *Leishmania* genome contains 42 ABC genes ranging from *ABCA* to *ABCH* [13]. Among the available genes, a few studies have reported a link between non-response to Glucantime and the genes *ABCC3*, *ABCC7*, *ABCG2*, and *ABCI4* in *L. major* infection [7, 14]. The *ABCI4* transporter is located in the mitochondria and plasma membrane and is involved in transporting heavy metals in the parasites. The *ABCI4* gene is located on chromosome 33 [15]. The *ABCG2* transporter is located in intracellular vesicles and in the plasma membrane encoded by *ABCG2* gene located on chromosome 6. This transporter pumps the conjugated thiol-antimony compound out of the amastigote cell [13, 16, 17]. The *ABCC7* (PRP1) transporter is located in intracellular vesicles, encoded by *ABCC7* on chromosome 31. The *ABCC3* transporter (MRPA) is also situated in vesicles between the nucleus and the flagellar pocket that encodes by *ABCC3* gene located on chromosome 23. *ABCC7* and MRPA proteins are associated with tubulin vesicles and thus bind to exocytosis and endocytosis pathways [18–20]. These carrier proteins seem to play essential roles in response to the drug, therefore, the aim of the current study is to assess *ABCI4*, *ABCG2*, *ABCC7*, and *ABCC3* genes expression in *Leishmania* parasites isolated from CL lesions with treatment failure outcome after a period of standard treatment with Glucantime.

Results

The mean size of lesions in patients with treatment failure was $4.4 \pm 3 \times 3.6 \pm 1.85 \text{ cm}^2$ and $5.33 \pm 1.24 \times 6 \pm 1.44 \text{ cm}^2$ in patients with treatment response (Figure 1). The number of lesions in treatment failure cases was in the range of 1 (1 case) to 4 (1 case), and in treatment response patients ranged from a minimum of 1 (1 case) to a maximum of 3 (1 case). The samples with treatment failure outcomes were coded as TF1, TF2, TF3, TF4, and TF5 that their character is shown in Table 1.

Table 1. The gene expression of *ABCC3*, *ABCC7*, *ABCI4*, and *ABCG2* in *Leishmania* isolates obtained from patients with cutaneous leishmaniasis with treatment failure outcome.

	age	Sex	Refer after disease	ABCI4	ABCG2	ABCC3	ABCC7
TF1	8	Male	2 months	-40.92	-15.63	+1.88	+6.19
TF2	32	Male	6 weeks	-95.33	-24.96	+1.68	+6.39
TF3	29	Male	3 years	-62.46	-29.27	-1.099	+8.4
TF4	7	Female	6 months	-7.16	-1.08	+2.53	+9.72
TF5	11	Female	2 weeks	+2.12	+63.26	+36.84	+3.14

All isolates were verified as *Leishmania* using ITS1-PCR-RFLP with a fragment of about 300-350 bp after amplification (Figure 2). RFLP analysis showed 220 and 127 bp fragments identified *L. major* (Figure 3).

The *ABCC3*, *ABCC7*, *ABCI4*, and *ABCG2* genes expression in treatment failure isolates is shown in Table 1.

Discussion

In this study, we showed that *ABCC7* had overexpression in all treatment failure isolates. The gene expression pattern for *ABCI4* and *ABCG2* was the same in treatment failure isolates with low expression in all treatment isolates except TF5. *ABCC3* had overexpression in all treatment failure isolates besides TF3. In a study completed by Manzano et al. [22] strains with high *ABCI4* expression might develop resistance to antimonials, pentamidine, and amphotericin B. Given that this transporter remains unknown compared to other ABC transporters, it is expected that the expression of *ABCI4* transporter gene is different in various strains, which might explain possible reasons for the discrepancy with the results of the current study.

The *ABCI4* found in the plasma membrane and in the mitochondria should be homodimerization to be functional. It involves in responding to SbV and SbIII treatment using the efflux of metal-conjugated thiols [23]. Therefore, the overexpression of *ABCI4* results in treatment failure with antimony [15]. In our study, TF5 isolate was the only case with overexpression of *ABCI4* more than 2-fold compared with the treatment response isolate. It seems that overexpression of *ABCI4* in TF5 could be considered as one of the important mechanisms to cause treatment failure.

Perea *et al.* [17] examined the relative expression of the *ABCG2* gene in *L. major* and concluded that parasites, which overexpress *ABCG2* are resistant to antimony due to reduced SbIII accumulation and increased drug flow. Additionally, *ABCG2* could transfer thiol in the presence of SbIII. The isolates in our study showed low expression of *ABCG2* gene besides TF5. Rugani et al. [24] reported that *ABCG2* has no association with respond to treatment in *L. braziliensis* clinical isolates that confer with the results of our study. The isolates mentioned in our study had *ABCC7* over expression that may involve in treatment failure response.

ABCC3 is located in the intracellular vesicular membrane and is reported to be involved in arsenite and antimony resistance in *Leishmania* promastigotes and amastigotes [25]. Coinciding with this study, in El Fadili et al. [26] study, the expression of the *ABCC3* transporter was continuously increased with mediated by *ABCC3* that can be reversed using the butyryn sulfoximine inhibitor for glutathione biosynthesis. In a study by Jabini et al. [27], to determine the effect of combination therapy of glucantime with silymarin (*ABCC3* transmitter inhibitor) on Iranian *L. major* isolates with treatment failure pattern, it was reported that silymarin with glucantime may have beneficial effects in models of leishmaniasis mice with no response to drug. Although this study did not show a significant effect on the combined use of silymarin and glucantime, the reason for such a result could be the involvement of other factors and transmitters in response to treatment. Due to the high expression of this transmitter and the emphasis on its importance in the treatment outcomes of leishmaniasis in previous studies [19, 25, 28], in our study, MRPA was considered as one of the potential factors in causing non-response to the treatment of leishmaniasis patients. Other genes may affect the expression of genes under study and complicate resistance to treatment. For example, the increased expression of ubiquitin and amino acid permease (AAP3) genes can increase the activity of multidrug-resistance protein A or *ABCC3* [29, 30].

Various studies on different species and strains of *Leishmania* have shown that the *ABCC7* gene is overexpressed in antimony-resistant *Leishmania* strains and there is a significant cross-resistance to the antimony [18, 31]. In a study by Leprohon et al. [20], which investigated the location of ABCC transporters and their role in antimony resistance, it was concluded that *ABCC7*, *ABCC4*, and *ABCC5* were located in a tubular enclosure along the longitudinal axis of the parasite. Overexpression of at least four members, *ABCC3*, *ABCC4*, *ABCC5*, and *ABCC3* can also result in antimony resistance.

Conclusions

Treatment failure in *Leishmania* spp. represents an intrinsic feature of the parasite that is an adaptive trait. Exposure of the parasite with insufficient doses resulted in expression of ATP-binding cassette (ABC) transporters that induce occurrence of this phenotype that exhibits in the patient, although it is a multifactorial event.

In this study, the relative expression of *ABCC3*, *ABCC7*, *ABCG2*, and *ABCI4* genes in *L. major* isolates with no response to treatment changes in the relative expression of these genes may be effective as one of the effective mechanisms in the process of response to treatment in the parasite. However, two of the isolates with no response to treatment pattern had different expressions than the other isolates, so it is possible that other mechanisms were involved in this isolation.

Methods

Ethical statement

Every patient was informed about the study objective and procedure and the ones who signed an informed consent were recruited for sampling. All methods were done based on principles of the declaration of Helsinki. The study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (IR. SSU. MEDICINE. REC. 1399.194).

Study area

Varzaneh is a city in the southeast of Isfahan province (Figure 4). The city is located in the western part of Gavkhooni swamp, in longitude of 39°, 52' and latitude of 25°, 52' north, and is a well-known CL endemic area. The current population of the city is approximately 17 000.

Sampling

Leishmania isolates were collected from the edge of the lesion patients' who were referred to Health Center Laboratory of Varzaneh, Isfahan, Iran, from September 2020 to March 2021. The CL primary diagnosis was performed using direct smear and microscopic observation of *Leishmania* in Giemsa-stained slides. The collected samples were immediately transferred into RNA/later solution (Merck, Darmstadt, Germany) and stored at -20° C for further use. The patients were followed up for 3 months to check the response to treatment with Glucantime and determine non-healing cases. Non-healing cases were defined as no re-epithelialization and presence of induration in the border of the lesion.

Verification and identification

The isolates were verified and identified using an Internal Transcribed Spacer (ITS)1-PCR–RFLP [21]. The amplification was done using 400 nM primers: LITSR: 5'-CTTGGATCATTTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGCATT-3'. The reaction was conducted using thermo cycler (SimpliAmp, ABI, USA) in a 20 µl reaction solution with the amplification condition described by Eslami et al. [21]. Positive and negative controls were run in each reaction using *L. major* (MRHO/IR/75/ER) and ddH₂O, respectively. Digestion was performed using *Hae III* restriction enzyme, according to the manufacturer's instructions. The amplified fragments of about 300–350 bp detected *Leishmania* genera that were analyzed using 1.5% agarose gel electrophoresis. Digestion fragments were assessed using 3% agarose gel electrophoresis and visualized by UV light after staining with DNA Green Viewer (Pars Tous, Iran, Mashhad). The 50 bp DNA ladder (CinnaGen, Iran, Tehran) was used as the DNA molecular marker. This method can identify *L. infantum* (200, 100, and 50 bp), *L. tropica* (220 and 50 bp), and *L. major* (220 and 127 bp).

RNA extraction and cDNA synthesis

RNA extraction was conducted using the total RNA extraction kit (Vivantis, Malaysia) based on the manufacturer's instructions. The RNA quantity was estimated using Nanodrop (Thermo Fisher Scientific, USA). Then, cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) based on the manufacturer's instructions.

Gene expression analysis

The gene expression of *ABCG2*, *ABCI4*, *ABCC7*, and *ABCC3* was assessed using SYBR Green real-time PCR. The GAPDH gene [21] was considered an endogenous control. All primer pairs related to the *ABCG2*, *ABCI4*, *PRP1*, and *MRPA* were designed in this study (Table 2).

Table 2. The primer names and the sequences for gene expression of ABCG2, ABCI4, PRP1, MRPA, and GAPDH.

Primer Name	Sequence
<i>ABCI4-F</i>	5'-TGCCGTCGTCTCGCATCTCTTTTCA-3'
<i>ABCI4-R</i>	5'-ACGGCAGCAGAGCGYAGAGAAAAGT-3'
<i>ABCG2-F</i>	5'-TTCGCCGAGTTTCCCGTGCAGA-3'
<i>ABCG2-R</i>	5'-AAGCGGCTGAAAGGGCACGTCT-3'
<i>PRP1-F</i>	5'-AGGAGGGAGTGCGAAAAGGGCT-3'
<i>PRP1-R</i>	5'-TCCTCCCTCACGCTTTTCCCGA-3'
<i>MRPA-F</i>	5'-AACATCTTTTGCTCCCCACTGCCC-3'
<i>MRPA-R</i>	5'-TTGTAGAAAACGAGGGGTGACGGG-3'
GAPDH-F	5'-AGGACATTCTCGGCTTCACCAA-3'
GAPDH-R	5'-GCCCCACTCGTTGTCATACCA-3'

Amplifications were done in a total volume of 20 µl containing 2 µl cDNA, 10 µl SYBR Green I master mix, and 200 nM each of each primer using a Step One thermocycler (Applied Biosystem, USA). The thermal conditions of the reaction were 95° C for 10 min in order to the first denaturation, followed by 40 cycles of 95° C for 10 s and 60 s for 10 s. The specificity of the reaction products was confirmed by melting curve, which consisted of temperatures between 60 and 95° C with a heating rate of 0.3 °C/s. Gene expression analysis was done using $2^{-\Delta\Delta CT}$ method using follow formula:

$$\Delta\Delta CT = (CT_{\text{target gene in sample}} - CT_{\text{GAPDH in sample}}) - (CT_{\text{target gene in standard}} - CT_{\text{GAPDH in standard}})$$

Statistical analysis

The analysis was done using SPSS version 26.0 (Chicago, IL) by descriptive statistics.

Abbreviations

ABC: ATP-Binding Cassette Transporters

TF: Treatment Failure

TR: Treatment Response

CL: Cutaneous Leishmaniasis

VL: Visceral Leishmaniasis

MCL: Muco-Cutaneous Leishmaniasis

PAGA: P-glycoprotein A

AQP: Aquaglyceroporin

MRP: Multidrug Resistance Protein

ITS: Internal Transcribed Spacer

RFLP: Restriction Fragment Length Polymorphism

Declarations

- **Ethics approval and consent to participate**

Every patient was informed about the study objective and procedure and the ones who signed an informed consent were recruited for sampling. All methods were done based on principles of the declaration of Helsinki. The study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (IR. SSU. MEDICINE. REC. 1399.194).

- **Consent for publication**

Not applicable

- **Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

- **Funding**

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- **Authors' contributions**

MJB wrote the draft of the manuscript and applied the method and collected the data; GE revised the manuscript and designed the protocol and supervised the project; AK revised the manuscript and designed the methodology; AAJ supervised the methodology and revised the manuscript; MV analyzed the data statistically.

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Figures

Figure 1

The lesion of cutaneous leishmaniasis from the patients. a: TF1; b: TF2; c: TF3; d: TF4; e: TF4; f: TF5; g: TR. TF: treatment failure; TR: treatment response.

Figure 2

Agarose gel electrophoresis for detection of Leishmania analysis after amplification with specific primer pair. Lane 1: 50 DNA ladder, lane 2: negative control (ddH₂O), lanes 3 and 4: samples detected as Leishmania, lane 5: positive control with *L. major* (MRHO/IR/75/ER). The amplicon with the length of about 300-350 bp was considered *Leishmania* spp.

Figure 3

Agarose gel electrophoresis for identification after RFLP analysis. Lane 1: 50 bp DNA ladder, lanes 2 and 3: samples identified as *L. major*, lane 4: standard strain of *L. major* (MRHO/IR75/ER). The fragments with the sizes of 220 and 127 bp was considered *L. major*.

Figure 4

The geographical location of Varzaneh, Isfahan, Iran.