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ORIGINAL PAPER

Gene expression analysis of antimony resistance in *Leishmania tropica* using quantitative real-time PCR focused on genes involved in trypanothione metabolism and drug transport

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Abstract

Pentavalent antimonials remain the treatment of choice for all the clinical forms of leishmaniasis. The increasing rates of antimony resistance are becoming a serious health problem in treatment of anthroponotic cutaneous leishmaniasis (ACL). Accordingly, unraveling molecular markers is crucial for improving medication strategies and monitoring of drug-resistant parasites. Different studies have suggested the importance of genes involved in trypanothione metabolism and drug transport. In this regard, present study was designed to investigate the RNA expression level of five genes including γ-GCS, ODC, TRYR (involved in trypanothione metabolism), AQP1 (acts in drug uptake) and MRPA (involved in sequestration of drug) in sensitive and resistant *Leishmania tropica* isolates. Seven antimony-resistant and seven antimony-sensitive *L. tropica* clinical isolates were collected from ACL patients. Drug sensitivity test was performed on the samples as well as reference strains; afterwards, gene expression analysis was performed on clinical isolates by quantitative real-time PCR. The results revealed that the average expression level of AQP1 gene was decreased (0.47-fold) in resistant isolates compared to sensitive ones whereas MRPA (2.45), γ-GCS (2.1) and TRYR (1.97) was upregulated in resistant isolates. The average expression of ODC (1.24-fold) gene was not different significantly between sensitive and resistant isolates. Our findings suggest that AQP1, MRPA, GSH1 and TRYR can be considered as potential molecular markers for screening of antimony resistance in some *L. tropica* clinical isolates.

Keywords Natural antimony resistance · *Leishmania tropica* · Potential molecular marker · Gene expression · Quantitative real-time PCR

Introduction

The protozoan parasite *Leishmania* is responsible for a broad spectrum of diseases collectively known as leishmaniasis which affects 12–15 million people worldwide [25]. The most distributed clinical form of leishmaniasis is cutaneous leishmaniasis (CL) and nearly 90% occurs in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia and Sudan in addition to Syria [2]. Old World CL is an endemic parasitic disease that is distributed in more than half provinces of Iran; and occurs in two forms, zoonotic CL (ZCL) and anthroponotic CL (ACL) [22, 31].

Pentavalent antimonial compounds have been the first line treatment for leishmaniasis although their clinical value has been threatened by the emergence of acquired drug resistance [3]. In recent years, unresponsive to meglumine antimoniate (Glucantime®) in ACL patients has been reported in

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Iran, which has been related to the emergence of *L. tropica* species resistant to antimonials [12, 14].

Drug resistance is partially understood in in vitro-induced resistant parasites and suggested that resistance is an interaction between uptake, efflux and sequestration [5]. There is solid evidence that pentavalent antimonial (SbV) reduced to the active trivalent form (SbIII) inside both the intracellular parasite and the host macrophage [29, 30]. In *Leishmania*, reducing agents such as trypanothione are considered to play a key role in the reduction of antimonials [34]. In laboratory-induced cell lines, elevated levels of trypanothione seems to be related to overexpression of enzymes implicated in the synthesis of glutathione (gamma glutamyl cysteine synthetase, γ-GCS) and polyamines (ornithine decarboxylase, ODC) [11, 13]. Also, alteration in the expression of trypanothione reductase (TRYR) which is involved in the reduction of trypanothione and maintenance of the cellular redox could be associated with drug resistance. Moreover, decreased SbIII uptake in resistant strains seems to be mediated by the lower expression of aquaglyceroporin (AQP1) [21]. In addition, overexpression of multi-drug resistance protein A (MRPA) which sequestered Sb–thiol complexes has been shown to confer resistance [9].

Recently, several features of drug resistance have been determined in antimonial-resistant field isolates and proposed the diversity of mechanisms not only in the isolates of either same species, but also in different species from same country [4]. Considering the increasing of clinical drug resistance, the monitoring of antimonial resistance is fundamental issue in the anthroponotic focal region of CL. Thus, detection of molecular markers seems crucial for perceiving of clinical resistant patients and mapping the prevalence of resistance in endemic areas.

In our earlier study, the alteration in RNA level of AQP1 and MRPA was reported in just one resistant *L. tropica* isolate using cDNA-AFLP [16]. In present study, we aimed to investigate the RNA expression level of MRPA, AQP1 involved in drug transport and also γ-GCS, ODC and TRYR implicated in trypanothione metabolism, among antimony sensitive and resistant clinical isolates of *L. tropica* in order to identify appropriate marker for monitoring of drug resistance in endemic area.

Materials and methods

Clinical isolates

This cross-sectional study was conducted on 14 samples collected from skin lesions of ACL patients with positive smear skin lesions referred to the Parasitology Department of Imam Reza Hospital, Mashhad (endemic city for ACL, located at north east of Iran), during August 2013 to April 2016. The study was done under the principles of the Ethics Research Committee of the School of Public Health, Tehran University of Medical Sciences and through willingness of patients to participate.

Seven clinical isolates, obtained from ACL patients who responded to antimony therapy (Glucantime) and did not relapse within a 6-month follow-up period, were designated as sensitive; whereas seven isolates from ACL patients who did not respond to at least three systemic treatment courses of Glucantime® and still had parasites were designated as antimony resistant [12].

Clinical samples were collected by sterile lancet from the internal border of skin lesions. The serosity was transferred to RPMI-1640 medium (Gibco, Life technologies GmbH, Germany) supplemented with 15% fetal bovine serum (Gibco/BRL), 100 U/ml penicillin, 100 µg/ml streptomycin and incubated at 26 ± 1 °C. All the clinical isolates were characterized as *L. tropica* using ITS1 (Internal transcribed spacer1) PCR-RFLP [26]. Moreover, *L. tropica* clinical strains MHOM/IR/10/175 (S1) and MHOM/IR/10/827 (R1) were used in all experiments as antimony sensitive and resistant reference strains, respectively [12, 16, 27].

In vitro assay for drug sensitivity

The clinical isolates were analyzed for in vitro dug susceptibility as described previously [16]. Briefly, the mouse macrophage cell line J774A.1 (5×10^3 cells/well) were seeded in RPMI with 10% FBS in eight well chamber slides (Nunc, NY, USA). Macrophages were infected with the stationary-stage promastigotes at a parasite-to-macrophage ratio of 10:1 and then were incubated for 4 h at 37 °C. After washing, the cells were incubated for 72 h in the presence of serial dilution of Glucantime® (Sanofi-Aventis, Paris, France). Pentavalent antimony concentrations used for the isolates were 2.5, 5, 10, 20, 40, 60 and 80 µg/ml besides the control well without drug was included in each experiment. Having stained with Giemsa, the number of amastigotes per cell was counted in 100 macrophages. IC_{50} were calculated using the values for the number of amastigotes/macrophage, and defined as the effective dose of Glucantime® that reduces the survival of *L. tropica* by 50%.

RNA extraction and cDNA synthesis

Total RNA was extracted from 10^8 promastigotes of sensitive and resistant samples during the log-phase using Tripure reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. The quantity and quality of RNA were evaluated using nanodrop (ND-1000, Thermo Scientific Fisher, US) and gel electrophoresis. To avoid any genomic contamination RNA was treated with DNase I enzyme (Fermentas, Burlington, Canada) as described

by the manufacturer. Complementary DNA (cDNA) was synthesized using 1 µg RNA, 2.5 µmol/µl oligo-dT (Roche, Mannheim, Germany) and 60 µmol/µl random hexamer (Roche, Mannheim, Germany) incubated 10 min at 65 °C followed by addition of 10 mM of dNTP mix (Roche, Mannheim, Germany) 40 U/µl RNase inhibitor (Roche, Mannheim, Germany), 4 µl of 5× reverse transcriptase (RT) buffer (Roche, Mannheim, Germany) and 20 U/µl reverse transcriptase enzyme (Roche, Mannheim, Germany) and then incubated at 25 °C for 10 min followed by incubation at 50 °C for 60 min. The integrity of cDNA was checked with alpha-tubulin primers (Table 1) as housekeeping gene. The PCR condition was an initial denaturing step of 94 °C for 5 min and 35 repetitions of denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s, and extension at 72 °C for 45 s with a final extension of 72 °C for 5 min.

Quantitative real-time PCR analysis

Real-time PCR was conducted to confirm the differences in RNA expression level of target genes among sensitive and resistant *L. tropica* isolates. Specific primers were designed by Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/) (Table 1). Two independent RNA preparations were used for each real-time PCR experiments. PCR was performed in 20 µl reactions containing 1 µl cDNA target, 100 nM forward and reverse primers and 1× SYBR® Premix Ex Taq™ II (Takara, Tokyo, Japan). Experiments were carried out in duplicate for target genes and alpha-tubulin as a housekeeping gene using a Real-Time System (Applied Biosystem Instrument [ABI] step one, Foster City, CA, USA). The PCR condition was as follows: activation at 95 °C for 3 min, amplification at 95 °C for 10 s, 60 °C for 32 s for 40 cycles followed by a melt curve analysis using temperature increments of 0.2 °C every 30 s to ascertain amplification of the expected product.

Analysis of gene expression

The data are presented as the fold change in the target gene expression in the *L. tropica* field isolates normalized to the internal control gene (α-tubulin) and relative to the sensitive reference isolate S1 using the $2^{-\Delta\Delta C_t}$ method as previously described [19].

Statistical method

All experiments were conducted at least two times and the results are expressed as the mean ± standard deviations (SDs). In vitro drug susceptibility assay was presented as IC_{50} (50% inhibitory concentration), which was determined using nonlinear regression analysis with the GraphPad prism software version 6.04. The significance of differences for in vitro test results as well as expression levels in sensitive and resistant isolates was determined by Student's *t* test and the level of significance acceptable was 95% ($P < 0.05$).

Results

Drug susceptibility of *L. tropica* clinical isolates

The drug sensitivity profile of *L. tropica* isolates to Glucantime® was determined in murine macrophage cell line (J774A.1). The results showed that IC_{50} values of SbV in resistant isolates ranged from 14.61 ± 2.54 to 49.54 ± 9.65; whereas these values in sensitive isolates were 3.24 ± 0.46 to 10.41 ± 1.42 (Table 2). The average of IC_{50} values of resistant isolates was 4.92 times higher than that of sensitive stains which is statistically significant ($P < 0.0001$).

Table 1 Primer sequences of target genes used in quantitative real-time PCR

Target genes	Name	Primers	PCR product (bp)
Alpha-tubulin (housekeeping gene)	ALT F ALT R	CAGGTGGTGTCGTCTCTGAC TAGCTCGTCAGCACGAAGTG	119
Multi-drug resistance related protein (MRPA)	MRPA F MRPA R	TGGGACTTGTGCTGACACTC CTGCGAAACTGTGAAGCACT	146
Aquaglyceroporin (AQP1)	AQP1 F AQP1 R	AATCTTCGCCACATACCCTAAT ACAAACAACAGTAGTCCAATAGCC	159
Gamma-glutamylcysteine synthetase (γ-GCS)	GSH1 F GSH1 R	AGCCCTGCTTTTACACCAAG CTTGCCGTTGAAGATCTCGT	173
Ornithine decarboxylase (ODC)	ODC F ODC R	GATGGTGCGCCCTTACTTT TTGGCGAAGATGATGTCGT	162
Trypanothione reductase (TRYR)	TRYR F TRYR R	CATCAACGAGAGCTACAAGAGC GAGGATGTACTCCGTGTCGAG	157

Table 2 The clinical outcome and drug susceptibility result of *L. tropica* isolates to meglumine antimoniate (Glucantime®)

ID number	Species	Clinical outcome	IC50 (µg/ml)
Sensitive isolates			
S1[a]	*L. tropica*	Healing	6.54 ± 0.73
S2	*L. tropica*	Healing	5.19 ± 0.53
S3	*L. tropica*	Healing	3.62 ± 0.47
S4	*L. tropica*	Healing	3.24 ± 0.46
S5	*L. tropica*	Healing	8.74 ± 1.48
S6	*L. tropica*	Healing	7.11 ± 1.02
S7	*L. tropica*	Healing	7.33 ± 0.93
S8	*L. tropica*	Healing	10.41 ± 1.42
Resistant isolates			
R1[b]	*L. tropica*	Non-healing	49.54 ± 9.65
R2	*L. tropica*	Non-healing	35.02 ± 6.35
R3	*L. tropica*	Non-healing	40.52 ± 7.02
R4	*L. tropica*	Non-healing	30.77 ± 6.47
R5	*L. tropica*	Non-healing	18.73 ± 3.52
R6	*L. tropica*	Non-healing	14.61 ± 2.54
R7	*L. tropica*	Non-healing	44.16 ± 7.95
R8	*L. tropica*	Non-healing	23.53 ± 3.97

[a]S1: sensitive standard isolate, MHOM/IR/10/175 (S1)

[b]R1: resistant standard isolate, MHOM/IR/10/827(R1)

Gene expression analysis of *L. tropica* clinical isolates

The relative gene expression values of five target genes in clinical isolates were reported with respect to reference sensitive isolate S1 (MHOM/IR/10/Mash-175) (Table 3).

Expression pattern of AQP1 gene

The transcript level of AQP1 was significantly decreased in resistant isolates R1 (resistant standard isolate), R2, R5, R6 and R7 by 0.42-, 0.56-, 0.13-, 0.36- and 0.094-fold, *P* value < 0.05, while it was increased in R3 (2.06-fold, *P* value < 0.05) as compared to reference isolate S1. In remaining resistant isolates (R4 and R8) it did not change significantly. On the other hand, sensitive isolates S3 and S6 exhibited slight downregulation of AQp1 transcript level (0.77- and 0.68-fold, *P* value < 0.05) while isolates S5, S7 and S8 displayed significant upregulation (2.57-, 2.87- and 2.42-fold); and the remaining isolates exhibited no significant change in transcript level (Fig. 1a). Moreover, the average expressions of AQP1 in resistant and sensitive isolates were 0.74 and 1.57, respectively, and a significant downregulation

Table 3 Relative expression pattern of target genes in sensitive and resistant *L. tropica* isolates

Isolates	AQP1	MRPA	ODC	TRYR	GSH
[a]S1	1	1	1	1	1
S2	1.31	1.119	2.239	1.35	1.09
S3	0.77	0.74	0.43	2.38	0.93
S4	1.012	0.667	1.32	0.56	0.2
S5	2.57	3.19	1.34	0.99	1.17
S6	0.684	1.409	0.86	2.27	2.32
S7	2.87	0.649	4.88	1.97	1.12
S8	2.42	2.776	1.29	0.74	0.57
[b]R1	0.42	4.154	1.12	2.99	1.31
R2	0.56	2.26	1.322	3.91	3.06
R3	2.063	0.8	3.54	1.13	2.62
R4	1.214	7.584	5.43	3.81	1.03
R5	0.13	1.352	1.42	1.18	2.38
R6	0.36	1.57	0.27	5.58	4.47
R7	0.094	6.72	1.55	2.47	1.21
R8	1.11	3.8	1.97	1.15	1.66

Blocks highlighted in black designated as significant upregulation. Blocks highlighted in grey designated as significant downregulation. Clear block designated as not significant difference

S sensitive isolate, *R* resistant isolate

[a]S1: sensitive standard isolate [MHOM/IR/10/Mash-175]

[b]R1: resistant standard isolate [MHOM/IR/10/Mash-827]

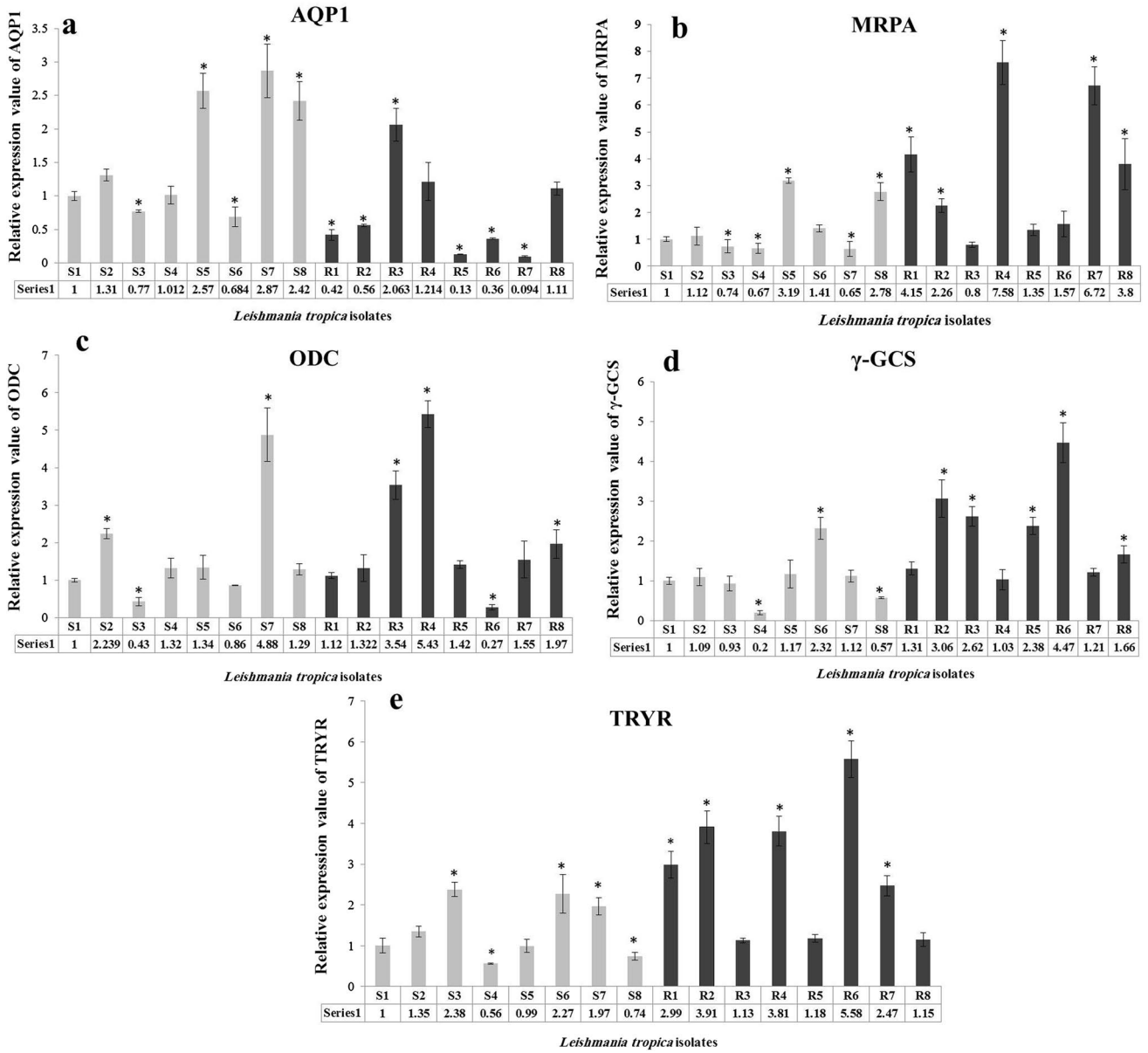

Fig. 1 Relative mRNA expression patterns of target genes (**a** AQP1, **b** MRPA, **c** ODC, **d** γ-GCS and **e** TRYR) in sensitive and resistant *L. tropica* isolates. The expression of alpha-tubulin was used to normalize the data. The values are the mean ± SD of two independent experiments. *Significantly different ($P < 0.05$)

(0.47-fold) was observed in resistant isolates compared to sensitive ones ($P < 0.05$) (Fig. 2).

Expression pattern of MRPA gene

The expression of MRPA was significantly up-regulated in resistant isolates R1, resistant standard isolate (4.15-fold), R2 (2.26-fold), R4 (7.58-fold), R7 (6.72-fold) and R8 (3.8-fold) while it was downregulated slightly in R3 (0.8-fold, P value < 0.05) as compared to reference isolate S1 and in other resistant isolates it did not reveal significant differences. In sensitive isolates S5 and S8, the transcript level of MRPA was increased (3.19- and 2.77-fold, P value < 0.05) while in isolates S3, S4 and S7 it was significantly decreased (0.74-, 0.66- and 0.64-fold). The expression of other sensitive isolates was comparable to S1 ($P > 0.05$) (Fig. 1b). Additionally, the average expressions of MRPA were significantly higher (2.45-fold) in resistant isolates compared to sensitive ones ($P < 0.05$) (Fig. 2).

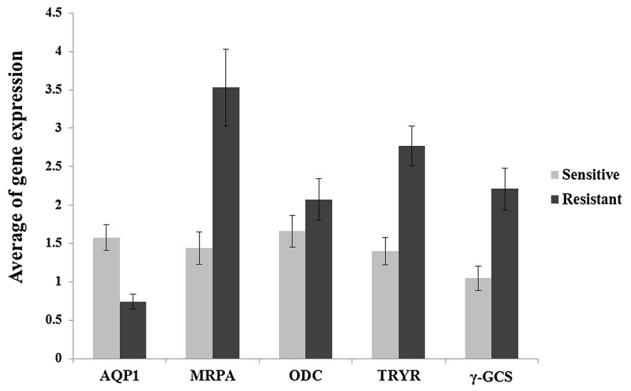

Fig. 2 The average expressions of target genes in resistant and sensitive isolates. The values are the mean ± SD of two independent experiments. Apart from ODC gene, the expressions of others were significantly different between two groups ($P < 0.05$)

Expression pattern of ODC gene

The high expression of ODC was observed in resistant isolates R3, R4 and R8 (3.54-, 5.43- and 1.97-fold, *P* value < 0.05), whereas the resistant isolate R6 displayed 0.27-fold decrease in RNA expression and other isolates and resistant standard isolate exhibited no significant change as compared to reference isolate S1. The RNA level of ODC was increased in sensitive isolates S2 and S7 (2.23- and 4.88-fold) and decreased in isolate S3 (0.43-fold); in the remaining sensitive isolates, it displayed normal expression comparable to reference strain S1 (Fig. 1c). The average expressions of ODC were 1.24-fold higher in resistant isolates compared to sensitive ones which was not statistically significant ($P > 0.05$) (Fig. 2).

Expression pattern of γ-GCS gene

γ-GCS gene was upregulated in resistant isolates (R2, R3, R5, R6 and R8) when compared to sensitive field isolate S1 (3.06-, 2.62-, 2.38-, 4.47- and 1.66-fold, respectively) (*P* value < 0.05); and in other resistant isolates as well as resistant standard isolate (R1) did not change significantly. Also, it was upregulated in sensitive isolates S6 (2.32-fold) and downregulated in S2 and S8 (0.2- and 0.57-fold) and in the remaining sensitive isolates no significant change in transcript level was observed (Fig. 1d). Additionally, a significant upregulation (2.1-fold) in the average expression level of GSH1 was observed in resistant isolates compared to sensitive ones ($P < 0.05$) (Fig. 2).

Expression pattern of TRYR gene

As shown in Fig. 4, resistant isolates R1 (resistant standard isolate), R2, R4, R6 and R8 showed significantly increased transcript level of ACP (2.99-, 3.91-, 3.81-, 5.58- and 2.47-fold) while in other resistant isolates, the transcript levels were unaltered when compared to S1. The sensitive isolates S3, S6 and S7 displayed 2.38-, 2.27- and 1.97-fold increase in transcript level, whereas isolates S4 and S8 exhibited 0.56- and 0.74-fold decrease; the expression levels in the remaining susceptible isolates were similar to that of S1 isolate (Fig. 1e). Also the average expression level of TRYR was significantly increased by 1.97-fold in resistant isolates in comparison with sensitive ones (Fig. 2).

Discussion

Pentavalent antimonial compounds are the mainstay therapy for leishmaniasis; nevertheless, their efficacies have been threatened by emergence of drug-resistant parasites in endemic regions. Recent studied have proposed that drug resistance in clinical isolates is multifactorial and different pathways might simultaneously operate in this phenomena, therefore elucidation of molecular markers as a surveillance tools for identification of resistant parasites has become as a major obstacle in endemic region [4]. In this regard, the RNA expression pattern of genes implicated in trypanothione metabolism and drug transport were investigated in clinical resistant and sensitive *L. tropica* isolates to determine potential biomarkers. Our finding revealed that all the isolates displayed disregulation in transcript level of at least one target gene.

One of the target genes was analyzed in the clinical isolates is AQP1; these protein families are membrane channels that mediate SbIII uptake and accumulation in *Leishmania* [10]. It has been reported that downregulation of AQP1 could be one of the mechanisms of antimonial resistance [7, 21] and several studies demonstrated that overexpression of AQP1 in antimony-resistant field isolate significantly increased SbIII uptake and susceptibility to antimony [10, 20]; and also, downregulation of AQP1 expression was observed in clinical isolates of *L. donovani* [28]. Additionally, in our earlier study decreased in transcript level of AQP1 was reported in one *L. tropica* clinical isolate using cDNA-AFLP [16]. Likewise, in current study, the average expression of AQP1 was downregulated (0.47-fold) in resistant isolates compared to sensitive ones. The decrease in RNA expression was displayed in majority of resistant isolates (five out of eight isolates) also a significant upregulation was observed in three sensitive isolates; however, in contrast, in one resistant isolate it was upregulated slightly and in two sensitive isolates were downregulated. These exceptions were also reported in former study was conducted on *L. donovani* field isolates through which downregulation of AQP1 was determined in seven isolates while in two resistant isolate it was overexpressed [20].

Also a few examples of upregulation of AQP1 RNA in *L. donovani* and *L. guyanensis* field isolates were reported in other studies [15, 33], suggesting the involvement of posttranslational modification in this scenario; besides it has been justified that mutation in AQP1 gene that produce abnormal protein might promote this upregulation [15].

MRPA as a member of ATP-binding cassette transporter can play vital role in antimony resistance by sequestration of metal–thiol conjugates [18, 24]. The correlation of MRPA to drug resistance was confirmed via gene transfection in mutant and natural antimony-resistant isolates [8, 24]. Moreover, elevated transcript level of MRPA was reported in *L. donovani* resistant isolates [28] and one resistant *L. tropica* clinical isolate [16]. Our study also in accordance with former studies indicated that the average expression level of MRPA was 2.45-fold higher in resistant *L. tropica* isolates compared to the sensitive ones. Indeed five out of eight resistant isolates revealed a significant upregulation in expression level of MRPA and in this line three sensitive isolates displayed a downregulation. Conversely, one resistant isolate exhibited slight downregulation in RNA level while two sensitive isolates showed upregulation. Nevertheless in agreement with our findings, a study by Kumar et al. [17] revealed that MRPA was overexpressed not only in a majority (60%) of resistant isolates, but also in one sensitive isolate. Furthermore, a significant downregulation was also reported in 40% of resistant isolates. Although the average expression of MRPA was significantly higher in resistant isolates, these discrepancies in a few isolates suggested that multiplicity of mechanism might be involved.

The other gene investigated is γ-GCS gene encoding the enzyme involved in the synthesis of glutathione, a precursor of trypanothione. The trypanothione is a major reduced thiol in *Leishmania* that is implicated in reduction of antimonial drugs as well as detoxification of oxidative stress induced by antimony [23]. Elevated thiol levels have been reported in antimony-resistant *Leishmania* isolates which might be associated with overexpression of γ-GCS [11]. In this context, overexpression of γ-GCS has been reported in some *L. donovani* and *L. guyanensis* clinical isolates [17, 32, 33]. These results are in harmony with our findings in which average RNA expression level of γ-GCS gene was 2.1-fold higher in resistant compared to sensitive *L. tropica* isolates. In current study, the expression of γ-GCS was upregulated in five of eight resistant isolates and downregulated in two sensitive isolate. However, one sensitive isolate presented upregulation in transcript level proposed the existence of different drug resistance mechanisms in the different isolates of same species. Moreover, although in some isolates of aforementioned species upregulation of γ-GCS is discriminative, in *L. braziliensis* clinical isolates no significant alteration was observed [1].

Ornithine decarboxylase (ODC) coding the enzyme that catalyzes the rate-limiting step of the synthesis of spermidine, the polyamine that moiety of trypanothione [11]. It was reported that the upregulation of trypanothione in resistant isolates associated with overexpression of ODC [13]. Furthermore, elevated transcript expression of ODC was observed in some *L. braziliensis* and *L. donovani* clinical isolates [1, 28]. In the current study, the average RNA expression of ODC was 1.24-fold higher in resistant isolates compared to sensitive ones, which was not statistically significant, suggested that this gene was not as discriminative in *L. tropica* clinical isolates.

The other vital enzyme of the trypanothione metabolism is trypanothione reductase (TRYR), involved in the reduction of the disulfide form of trypanothione leading to maintaining the cellular redox condition [11]. It has been demonstrated that increase in thiol levels in resistant isolates was accompanied by amplification of TR suggesting the possible role of this gene in antimony resistance [23]. In this context, increased transcript level of TR in resistant clinical isolates of *L. braziliensis* and *L. donovani* was also reported [1, 28]. Consistent with former studies, present study revealed the average expression level of TRYR was 1.97-fold higher in resistant isolates; the transcript level was elevated in five of eight resistant isolates and declined in two of seven sensitive isolates. On the other hand, three sensitive isolates exhibited upregulation in the expression of TRYR. In harmony with our findings, Adaui et al. [1] stated that although upregulation of TRYR was observed in the antimony-resistant isolates of *L. braziliensis*, an inconsistency was detected in some of the sensitive isolates in which TRYR expression were increased.

In the current study, the average expression levels of MRPA, γ-GCS and TRYR genes were elevated while level of AQP1 was decreased in resistant groups compared to sensitive ones. Besides, some disparities were detected in expression level among individual clinical isolates originated from the same area. Consistent with our results, such findings were observed in various clinical isolates of different species obtained from same endemic areas [1, 15]. The consensuses of findings support the idea that distinct genes might be involved in natural antimony resistance of even each isolate of same species; furthermore, the lack of a significant gene expression pattern in some isolates provides solid evidence of involvement of other genes or pathways.

Overall, our results propose that AQP1, MRPA, GSH1 and TRYR genes could be designated as the potential markers for monitoring and prognosis of emergence of resistance in endemic areas. Additionally, variety of gene association exhibited in clinical isolates supports the complexity of natural drug resistance. Further study on a large scale is needed to confirm our findings for developing drug resistance monitoring assay.

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Compliance with ethical standards

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

Ethical approval The study was approved by the Ethics Research Committee of the School of Public Health, Tehran University of Medical Sciences.

Informed consent Informed consent was obtained from all individual participants included in the study.

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