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Original Article

Expression Analysis of Multiple Genes May Involve in Antimony Resistance among *Leishmania major* Clinical Isolates from Fars Province, Central Iran

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Received 20 Nov 2015

Accepted 13 Apr 2016

Keywords:

Antimony resistance,
Leishmania major,
Molecular marker,
Iran

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Abstract

Background: Treatment of Cutaneous Leishmaniasis (CL) is being faced with serious difficulties in Fars Province, due to emerging of resistance against meglumine antimonite (Glucantime®). In this context, determining some biomarkers for drug sensitivity monitoring seems to be highly essential. Different studies have been carried out to decipher the genes might be involved in antimony resistant phenotype in *Leishmania* spp. Here, we selected three genes: AQP (as drug transporter), TDR-1-1 (as drug activator), and γ -GCS (inducing reduction environment) for comparative expression analysis on clinical resistant and sensitive isolates of *L. major*.

Methods: The clinical isolates of *L. major* were collected from CL patients referred to Valfajr Health Center, Shiraz from Oct 2011 to Feb 2012. The susceptibility test was performed to confirm drug sensitivity of strains in vitro as well. Then, the gene expression analysis was performed by quantitative real-time PCR using SYBR® Green.

Results: By comparison of expression level between strains, up regulation of γ -GCS gene and down regulation of AQP gene were observed in resistant strains compared to the sensitive isolates; however, down regulation of AQP was not statistically specific. Analysis of TDR-1-1 gene unexpectedly showed a high level of expression in the non-responsive cases.

Conclusion: The γ -GCS, at least, can be considered as a suitable molecular marker for screening antimony sensitivity in clinical isolates, although AQP and TDR-1-1 gene seem not to be reliable resistant markers.

Introduction

The most common form of leishmaniasis, Cutaneous Leishmaniasis (CL), has raised a major health problem in the Middle East particularly in Iran (1, 2). Due to lack of an effective vaccine, chemotherapy has remained as a main strategy for prevention and control of disease in endemic regions. Over 60 decades, the antimonial compounds (SbV) have played vital role in the treatment of all forms of leishmaniasis (3, 4). The emergence of antimony resistance, unfortunately, has caused a serious threat for control and therapy of disease in many endemic areas including Iran (5-7). In this context, the most warning report from Iran comes from Fars province, Iran where the rate of meglumine antimonite (Glucantime®) resistance has been reported as 34.9% (7). Thus, urgent need seems to be substantial to determine the molecular markers for applying the rational therapy and more accurate epidemiological studies (8, 9). Despite of insufficient understanding of drug action, many studies have been performed to decipher some genes may involve in resistance phenomenon (3, 10, 11). Most of these works have indicated that antimony resistance in *Leishmania* is a multi-factorial phenomenon and several factors such as decline of drug uptake, deterrence of drug activation, and thiol metabolism can contribute in inducing resistant phenotype (9). In different species of *Leishmania*, a membrane transporter called Aquaglyceroporin (AQP) has been identified which can act as a drug carrier into parasite cells and therefore, decreasing in expression of this gene may lead to resistant phenotype (12). Furthermore, an enzyme called thiol dependent reductase 1 (TDR-1-1) has been found that catalyzes the reduction of pentavalent antimonial SbV (pro-drug) into the active form of antimony, perhaps it is assumed that high amount of components SbIII and so a high level of gene expression might be observed in sensitive isolates than

resistant ones (13). Another possible mechanism is the concentration of thiol within *Leishmania* cells. In fact, the thiol can maintain a reducing environment to help parasite surviving against oxidative circumstance created by antimony components. The main thiol in the Trypanosomatidae family is trypanothione (TSH) that the γ -GCS gene encoding γ -glutamyl cysteine synthetase catalyzes one of the rate limiting steps in producing TSH in *Leishmania*. Then, it is thought that high levels of γ -GCS expression could be observed in resistant parasites (11, 14).

Due to increasing number of Iranian CL patients unresponsive to meglumine antimonite (Glucantime®), the first line drug, a growing demand for detection of biomarkers seems crucial for monitoring of clinical resistant patients and mapping the prevalence of resistance in endemic regions (6-8). However, only a few studies were done on gene expression analysis on *L. tropica* but not on *L. major* (15).

The objective of the present study, therefore, was to investigate the gene expression patterns of clinical isolates of *L. major* obtained from CL patients in Fars Province as a high prevalence of antimony resistance to reveal the possible and reliable molecular markers. In this sense, we aimed to compare the expression levels of these three genes, AQP, TDR-1, and γ -GCS by quantitative real-time PCR between sensitive and resistant strains of *L. major*. Indeed, these genes were opted among various other opportunities because according to the majority of other studies, these genes can play pivotal roles in fundamental steps of interaction between drug and *Leishmania* cell (11-14).

Materials and Methods

Clinical Isolates

This is an experimental study undertaken on 10 samples obtained from CL patients referring to the Valfajr Health Center in Shiraz, Iran from Oct 2011 to Feb 2012. The study was done under the principals of the Ethics Research Committee of Shiraz University of Medical Sciences and through willing of patients to participate. The samples were collected from skin lesions of patients if the following criteria were met: Pregnant, lactating, and all underlying patients such as diabetics and immunodeficiency were excluded from this study. Then, the patients received a standard protocol regime, intramuscular Glucantime® (20 mg/kg/day dose for 20 days) and followed up to three months. Successful clinical outcome was defined as complete re-epithelialization of all lesions, while failure treatment was considered when their ulcers were remained with no changes after finishing the complete treatment procedure even after three months following up. Eventually, these clinical isolates were designated as responsive (sensitive strain) and non-responsive (resistant strain) based on their response to therapy.

Parasites and culture

Promastigote forms of parasites were harvested by transformation of amastigotes aspirated from patient's ulcers. The promastigotes were grown in RPMI-1640 medium supplemented with 10% FBS, 100IU/ml of penicillin, and 100g/ml of streptomycin, and incubated at 24°C in 25cm² culture flasks (Nunc). Then the species identification was performed based on a previous study using PCR method that amplified the conserved area of the minicircle kinetoplastic with specific primers of forward LINR4 and reverse LIN17, which can specifically distinguish among different *Leishmania* spp. (7).

Reference sensitive and resistant strains

The reference strain (MRHO/IR/75/ER), sensitive to antimony, was originally bought from the Pasture Institute of Iran. In addition, a clinical isolate, which showed high rate of

resistance to Glucantime® in both clinical level and in vitro assay, was used as a reference resistant strain here called R4.

In vitro sensitivity assay

The in vitro susceptibility of these clinical strains was measured by amastigote - macrophage assay as described elsewhere (16). Briefly, 5×10^5 of J774 macrophage in log phase of growth were seeded in each 8-well chamber slides with cover slips (Nunc#177445). After 1h, the adherent macrophages were infected with late stationary phase of promastigotes at a ratio of 10:1 (promastigote/cell) for 4h. Then, the excessive promastigotes were removed and each well was replaced with 400µl medium containing different dilution of Glucantime® (15µg/ml, 30µg/ml, 45µg/ml and 60µg/m) Glucantime®. Meglumine antimonite (Glucantime®) was prepared from Sigma-Aldrich Chemical Company (011M0125V). Meanwhile, all experiments were done in triplicate and there was a negative control well without drug and for more confirmation the sensitivity of all isolates were confirmed by repeating in vitro assay using the high concentration of drug (60µg/m). Lastly, after 5 d of incubation, the slides were stained with Geimsa and parasite burdens were calculated. In fact, parasite burdens were calculated as the percentage of infected macrophages \times (mean number of amastigotes/macrophage) and compared to the parasite burdens of control wells (no drug). Based on the reduction of total parasite burdens, 50% inhibitory concentrations (IC50) were calculated for each well and IC50 of each isolate was compared with IC50 of reference (sensitive and resistant) isolates by the chi-square test (McNemar). If there was a significant difference between IC50 of reference strains and each field isolate, this clinical strain was characterized as resistant to drug by in vitro assay and vice versa (17).

Total RNA preparation

Total RNA was isolated from 10⁶ log phase promastigotes of fivesensitive and fiveresistant samples using TRIzol® reagent (Invitrogen#12183-555) according to the manufacture’s protocol. Then, RNAs were treated with DNase (CinnaGen#PR891627), quantified by NanoDrop™2000 Spectrophotometer and kept at -80°C until cDNA synthesis.

-cDNAs synthesis

cDNAs were generated using first-strand cDNA synthesis kit (Invitrogen#4368814), total RNAs (5µg/reaction) were reverse transcribed into single -strand cDNA exactly based on instruction of protocol provided in the kit.

Primers

The genomic information of *L.major* was obtained from the following site: <http://Tritryp.org> and the primers were de-

signed according to sequence of AQP, TDR-1, and γ-GCS genes by Primer3 software. In addition, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was selected as the reference gene. The reverse and forward sequences of each gene are shown in Table 1.

Real-time PCR amplification

Real-time PCR was performed by SYBR® Green PCR Master Mix (ABI# 4309155) on the ABI Prism 7000 system. Each reaction had a total volume of 20 µl, containing 2 µl of cDNA sample, 10µl SYBER green master mix, and primers at a final concentration of 200nM. PCR amplification was carried out under the following thermal conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles, each consisting of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 60 °C for 1 min.

Table1: The sequences of forward and reverse primers were used in this study for real-time PCR

Gene	Primers	Product size bp
GAPDH	Forward TAT CAA GGT CGG CAT CAA	150
	Reverse CGT GTC GTA CTT CAT CTG	
AQP	Forward TGA ACT CTC CTA CAA CCA T	200
	Reverse GAA CTC GGC AAC ATA CTC	
TDR-1-1	Forward GCA ATG GTA CAA GCA GAT	185
	Reverse GCG AGG AAG AAC TCA AAT C	
γ-GCS	Forward CGC ATA TCC TCA AGT CTC	95
	Reverse TAT CGC TAT CTG TGA ATC G	

A melt curve analysis had done using temperature between 55 °C and 95 °C to determine non-specific products and primer dimers may be formed during amplification. In addition, a no-cDNA template was included as a negative control to ascertain absence of contaminations.

Analysis of quantitative data

The cycle threshold (C_T) values for all samples were determined by the Step One System software (V1.2) and were used to evaluate their relative expressions compared with the reference gene, GAPDH, to normalize the

results. Thus, the differences in gene expressions were calculated by comparative C_T method (18). In this method, first, ΔC_T values were measured by (C_T of target gene - C_T of reference gene). Next, the relative expression levels were obtained by subtracting ΔC_T values of each sample to ΔC_T of reference sensitive samples to get ΔΔC_T. Finally, the normalized expression ratio called Relative Quantification (RQ) was calculated as 2^{-ΔΔC_T}.

Statistical analysis

Regarding to determining the specific differences between the C_T values of sensitive

and resistant strains, a Chi-square test with consideration of $P < 0.05$ was performed through SPSS program (version 16.0 Chicago, IL, USA).

Results

Comparative analysis of gene expressions of AQP, TDR-1, and γ -GCS genes were investigated in natural sensitive and resistant isolates of *L. major*.

In vitro assay

In the case of clinical outcome, we had five-responsive and five-non-responsive (non-healing) isolates after receiving a complete treatment protocol with Glucantime®. Besides, the result of susceptibility *in vitro* assay showed a strong agreement with clinical outcomes which means all five responsive isolates were sensitive *in vitro* assay as well, and all non-responsive strains were resistant to Glu-

cantime® *in vitro* susceptibility test. In this experiment, IC50 values of field isolates represented ranging from $(15.2 \pm 0.06 \mu\text{g/ml})$ to $58.18 \pm 0.11 \mu\text{g/ml}$, while the IC50 was $15.8 \pm 0.42 \mu\text{g/ml}$ for reference sensitive strain and was $58.13 \pm 0.34 \mu\text{g/ml}$ for reference resistant strain.

-Expression profiling of AQP gene

In comparison between the two groups of strains, a lower level of expression, 0.56 fold less, was observed in resistant isolates. Although some resistant samples such as R1 and R2 showed a more reduction rate in comparison with sensitive strain, a statistically significant discrepancy ($P < 0.05$) was not demonstrated between the two study groups. Fig.1 depicts the comparative gene expression level between sensitive and resistant isolates based on Relative Quantification ($2^{-\Delta\Delta\text{CT}}$).

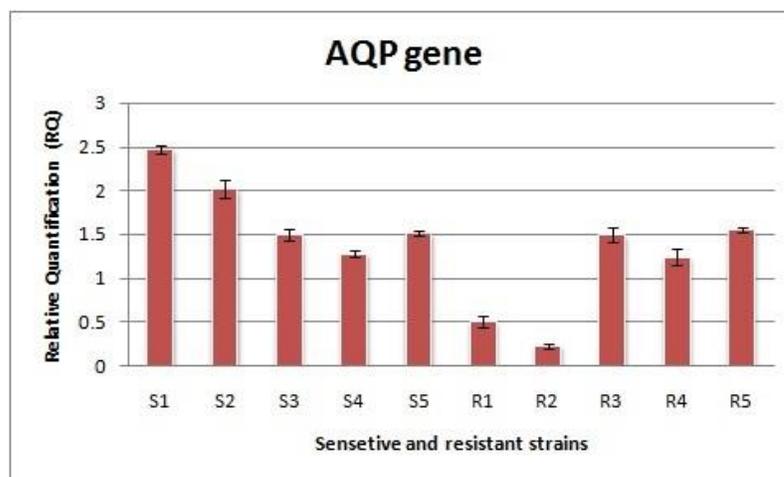


Fig. 1: the comparative expression levels of AQP gene are depicted between sensitive and resistant isolates of *L. major* by real-time PCR. The Y-axis is based on Relative Quantification (RQ) calculating as $2^{-\Delta\Delta\text{CT}}$ and the X-axis represents the name of samples: sensitive strains are shown by S (1-5) and resistant strains are shown by R (1-5)

Expression profiling of TDR-1 gene

Despite a diverse pattern of expression among different field isolates, the expression level of TDR-1 was interestingly found to be 0.87 fold in the resistant strains compared to sensitive ones which were statistically significant ($P < 0.05$) between resistant and sensitive iso-

lates. The highest level of expression belonged to a natural resistant isolate called R3 and the lowest level was noted in another unresponsive case, R5. Fig. 2 represents the relative gene expression levels between sensitive and resistant isolates.

Expression profiling of γ -GCS gene

According to comparison expression analysis of γ -GCS gene, indeed, a significant difference was clarified statically ($P < 0.05$) and this gene was expressed 20 fold more in the resistant strains in comparison with sensitive

clinical isolates. Among all resistant samples, the highest level of expression was detected in R5 strain. The comparative results for sensitive and resistant isolates are provided in Fig. 3.

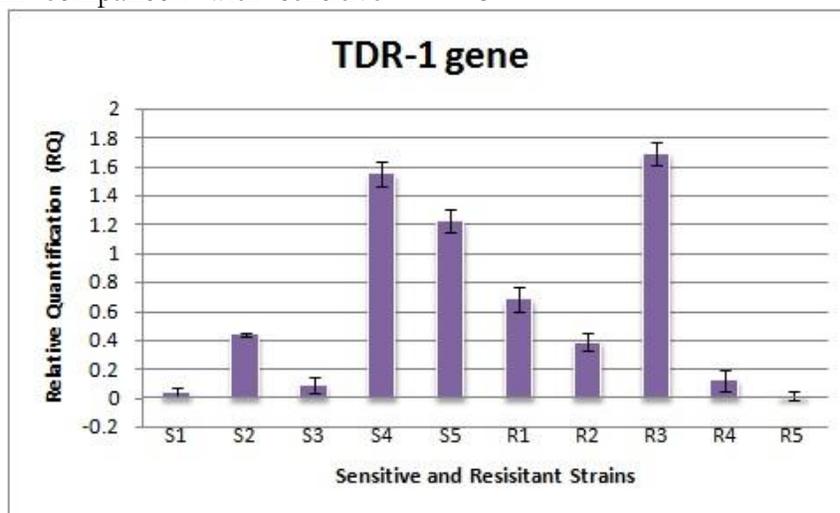


Fig. 2: this graph illustrates the comparative expression levels of TDR-1-1 gene between sensitive and resistant isolates of *L. major* based on Relative Quantification (RQ). As it can be seen, higher amount of expression are observed in the most resistant isolates compared to sensitive ones. Sensitive strains are shown by S (1-5) and resistant strains by R (1-5)

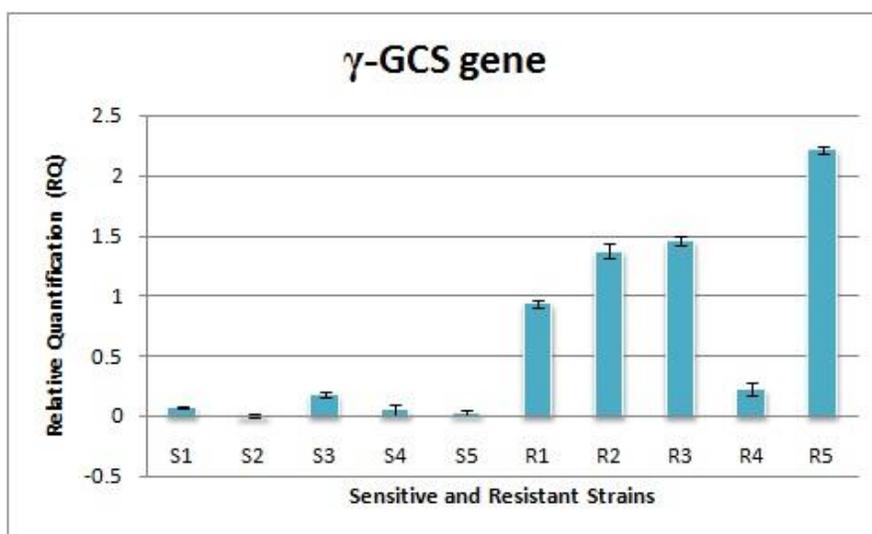


Fig. 3: this bar chart shows how the expression levels of γ -GCS gene are significantly higher in all resistant isolates of *L. major* compared to sensitive clinical strains based on calculation of Relative Quantification (RQ). The abbreviation S indicates as sensitive, S (1-5), strain and R as resistant strain, R (1-5)

Discussion

Owing to emerging of antimony resistance in Iran, an urgent action is demanded to estab-

lish a feasible and efficient monitoring approach for screening resistance in CL patients (7, 19). the present study was designed for investigating the differential gene expression profiles of clinical sensitive and resistant strains of *L. majoris* follows: (i) AQP; coding for a protein responsible for uptake of antimony; (ii) γ -GCS; coding for an enzyme involved in thiol synthesis, and (iii) TDR-1; coding for a protein catalyze the reduction of Sb(V) to Sb(III).The preliminary results on promastigotes form of parasites pronounced that the expression patterns were different between two studied groups regardless of some disparities (10, 11). For AQP gene, it is in charge of drug accumulation in *Leishmania* cells and so it assumes that lower level of expression can be predicted in resistant cells (20). In our finding, however some resistant samples showed lower amount of expression compared to sensitive isolates, there was no specific difference between two groups. Likewise, our result, in spite of down regulation of AQP in the antimony resistance strains of *L. donovani*, not consistently pattern was observed in all resistant or sensitive isolates (21). Whereas in another former study, the expression of AQP gene was decreased in all antimony resistant isolates of *L. Mexicana* (22). Furthermore, in a recent work performed on clinical resistant and sensitive isolates of *L. tropica* from Iran, obvious down regulation of AQP gene in resistant strain was reported (15).Altogether, these findings imply there are some vivid disparities between gene expression of AQP in various strains and species as well.

The TDR-1 gene was another target investigated in this research. In fact, the product of this gene plays an important role in activation of antimony components by reduction of pro-drug Sb (V) to active form Sb (III), hence, a lower expression level is being projected in resistant *Leishmania* cells (14), while, no direct correlation has been found between occurrence of resistance phenotype and gene expression (15). In contrast, a contradictory finding noticed in our research, which the ex-

pression level of this gene was 0.87 fold higher in resistant group in comparison with sensitive group. However this observation was unexpected, but in agreement with our findings, in another work the expression levels of TDR-1in field strains obtained from *L. braziliensis* in Brazil were specifically higher in non-responsive isolates than responsive ones (23).

Moreover, a study on antimony resistance on *L. donovani* field isolates showed that in resistant parasite, increased level of thiol found and this data could be related to induce a reductive environment by thiol, which can preserve the *Leishmania* cells against the oxidative situation promoted from antimony components (24). In this context, Wyllie et al. produced again some results, which could completely illustrate that elevated levels of thiol were existed in resistant isolates of *L. donovani* (25). In the present survey, we found the expression levels of γ -GCS, which has crucial role in producing thiol components in *Leishmania* cells were 20 times more in natural resistant isolates compared to the sensitive parasites. Nevertheless, on the contrary, a report from Nepal indicated that the expression level of γ -GCS gene in some resistant isolates of *L. donovani* dropped compare to sensitive strains (26).

Another issue that requires to precisely being concerned is the presence of some noticeable disparities even among strains belonged to same group particularly in the term of TDR-1gene. For instance, a dramatic difference between gene expression profiling of two resistant isolates (R3 and R4) observed even so far from the differences observed between sensitive and resistant isolates. Indeed, these kinds of outcomes achieved through working on various clinical isolates obtained from different endemic areas around the globe (11, 27). Torres et al. reported entirely mosaic patterns of expression in clinical isolates of *L. braziliensis* and *L. gnyanensis* in Brazil, like some parts of our results (28). Consequently, this fact was stated that because of multi-factorial phenomenon of antimony resistance, deci-

phering some biomarkers to distinguish resistance phenotype of clinical isolates seems to be feasible but so complex approach (30).

Conclusion

γ -GCS gene may play a key role in the induction of resistance in clinical isolates of *L. major* strains collected from Fars Province and more likely, it can be considered as suitable biomarker at least in this circumstance with no available molecular marker for identification of antimony resistance. The findings about AQP and TDR-1 genes reflect considerably the multi-factorial feature of antimony resistance in *Leishmania*. However, further comprehensive studies should be undertaken on more clinical isolates to confirm our preliminary data. We hope the future findings on this issue can provide a suitable trajectory for tracking treatment failure in CL patients in Iran.

Acknowledgments

This investigation was a part of PhD thesis project of Miss Nafiseh Ghobakhloo received financial support from the Vice Chancellor of Research Affairs, Shiraz University of Medical Sciences (Grant No. 5804). The authors declare that there is no conflict of interest.

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