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Aquaglyceroporin1 gene expression in antimony resistance and susceptible *Leishmania major* isolates

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ABSTRACT

Background & objectives: The mechanism of antimony resistance in *Leishmania* has been studied extensively, in connection with decreased influx and/or increased eflux of the drug. Aquaporin 1 (AQP1) protein has been shown to mediate the uptake of trivalent antimony. This study was aimed to find the expression level of *AQP1* gene in resistant versus non-resistant clinical isolates of *Leishmania major* in Iranian patients.

Methods: Clinical isolates were obtained from 16 considered patients referred to Navab Safavi Clinical Center, Isfahan, Iran from October 2014 to December 2015. After diagnosis of cutaneous leishmaniasis using microscopic observation, biopsy was performed from lesion(s) of each patient and stored inside RNAlater solution at –20°C. Written informed consent was obtained from all the patients to participate in the study before recording their information and sampling based on Helsinki declaration. Each patient was treated with Glucantime and followed for three months. All sensitive and resistance isolates were considered and compared with *AQP1* gene expression using real time PCR that was analyzed with delta-delta Ct.

Results: Out of 16 clinical isolates, four patients were resistant and 12 were non-resistant. The *AQP1* gene expression in resistant isolates was significantly higher than the one in response failure isolates ($p = 0.001$).

Interpretation & conclusion: The significant over expression (0.5 fold) of *AQP1* gene in resistant versus non-resistant isolates suggests different mechanism of drug resistance such as mutations. Mutations may change the physiological function of the Aquaporin 1 protein that might affect its expression level.

Key words Antimonial drugs; *AQP1;* drug resistance; leishmaniasis

INTRODUCTION

The protozoan parasites belonging to the genus *Leishmania* are the etiological agents of a complex disease, leishmaniasis[1]. Different clinical manifestations have been showed for this tropical disease ranging from self-healing cutaneous (CL), mucocutaneous (MCL), to a lethal visceral (VL) form[2]. The major species causing cutaneous leishmaniasis in Old World include *Leishmania major* and *L. tropica*[3]. More than 70% of the global CL cases occurs in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru[4]. One of the best control strategies of leishmaniasis is chemotherapy[2]. The first line drugs are pentavalent antimony [Sb(V)], a prodrug-containing compounds, such as sodium stibogluconate (SSG) (Pentostam) and N-methyl-glucamine (Glucantime)[5] which convert to trivalent antimony [Sb(III)] as an active drug that is more toxic[6]. Unfortunately, therapeutic aspects of antimony are now challenged because of clinical resistance to this drug in many parts of the world. Drug resistance is probably an interaction between uptake, efflux, and sequestration[7], mutation or down-regulation of an uptake system. Some studies suggest that loss of Aquaporin 1 (AQP1) allele that has been reported to cause an increase in resistance to SbV may be a mechanism leading to downregulation of an uptake system[8]. The AQP1s are bidirectional membrane channels involved in transportation of small uncharged neutral solutes such as glycerol, urea and water. Also, Biyani *et al*[9] showed that disruption of AQP1 alleles in *L. major* makes these parasites to be more resistant to antimonials. Molecular features of AQP1 for analysis of its effect on the resistance to antimonials have been evaluated by various studies[9–10]. An insight into the molecular mechanism of drug resistance is required for the development of efficient strategies to monitor the emer-

gence and spreading of leishmaniasis and drug resistance in endemic countries. This study was aimed to find the expression level of *AQP1* gene in antimony resistance and susceptible clinical isolates of *L. major* samples in patients with CL.

MATERIAL & METHODS

Sampling

Clinical isolates were obtained from 19 patients referred to Navab Safavi Clinical Center, Isfahan, Iran from October 2014 to December 2015. The diagnosis of CL was performed by microscopic observation of Giemsa-stained slides. Written informed consent was obtained from each patient to participate in this study before recording the information (about demographics and treatment followed *etc*) and sampling based on Helsinki declaration. Each patient with discontinuous treatment was excluded from the study. In total, 16 patients were considered for this study. Biopsy was performed from the edges of lesion skin and was transferred into RNAlater solution (Ambion, Inc., Austin, TX) for storing at –20 °C till next experiments. Patient characteristics and clinical data were compiled for each patient. The included patients were followed after treatment with Glucantime for three months for categorizing them as either drug sensitive or drug resistance. Also, this study was approved by the Ethics Committee (36023) of the Shahid Sadoughi University of Medical Sciences, Iran.

RNA extraction and cDNA synthesis

Total RNA from all the clinical samples was isolated from tissue biopsy using the RNeasy plus mini kit (Qiagen, Germany), followed by treatment using RNase free DNase (Thermo Fisher Scientific, USA) for elimination of any possibility of genomic DNA based on the manufacturer's instructions. The RNA quality and quantity were analyzed using 1% agarose gel electrophoresis and spectrophotometer (Eppendorf BioPhotometer plus, Eppendorf, Germany), respectively[11]. Then, cDNA synthesis was performed using high capacity cDNA reverse transcription Kit (Applied Biosystems, USA) with Oligo dT and Random Hexamer primers based on manufacturer's instructions.

AQP1 expression in Leishmania major

For evaluation of *Lm*AQP1 expression, real time PCR was performed using step one ABI real time PCR and SYBR Green PCR master mix (Applied Biosystems, USA). The specific primer used for amplification of *Lm*AQP1 was designed with PRIMER3 software, *viz.* AQP1-F 5'-AGTGTGGAGCGAGGTGTTCAA-3' and AQP1-R 5'-CCGAGAGTATGCGAGGTGACAA-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization with specific primers of GAPDH, *i.e.* F 5' CCGAGAGTATGCGAGG TGACAA-3' and R 5' GCCCCACTCGTTGTCATA CCA-3' as endogenous control. Before starting the main experiment, the amplification was done with master mix PCR (Amplicon). Real time PCR was done in triplicate in 20 µl volume using SYBR Green master mix (Applied Biosystems, USA). The real time PCR program, was run using the following thermal profile: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10s, and annealing and extension at 60°C for 20s. The specificity of real time PCR reaction was verified by melting curves analysis.

Statistical analysis

The comparison of expression level of *Lm*AQP1 mRNA was analyzed using (ΔΔ) Ct method. Analyzing of other characters was done with independent t-test in the statistical package for the social sciences version 20 (SPSS, Version 16.0; SPSS Inc, Chicago, IL). Shapiro-Wilk test was used to verify that the data were normally distributed. Pearson's and Spearman's rank correlation coefficients were used to evaluate the correlation between normalized expression of *Lm*AQP1 with age of patients using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA, USA). A $p \leq 0.05$ was considered significant.

RESULTS

The mean size of the lesions in all patients was $6.85 \pm 5.9 \times 4.39 \pm 2.9$ mm^2. The mean size of lesion in patients with no response to drug was $4.7 \pm 3.1 \times 4.7 \pm 3.5$ cm^2 and the one in drug sensitive was $8.1 \pm 6.7 \times 4.25 \pm 2.7$ cm^2. The number of lesions in resistant cases varied from minimum 1 (2 cases) to maximum 16 (1 case), and in susceptible patients from minimum 1 (5 cases) to maximum 6 (1 case).

*Lm*AQP1 gene expression was studied for all the isolates obtained from patients with CL (19 samples; Fig. 1). Out of these 19 patients, four isolates failed to respond the drug, 12 were drug sensitive and three of them discontinued treatment. All the four isolates that had not shown any response to Glucantime had an up regulation of *Lm*AQP1. One of these cases, *i.e.* a 10 yr old child named B12 showed 2.75 fold more gene expression than the other cases (Fig. 1). He was referred to

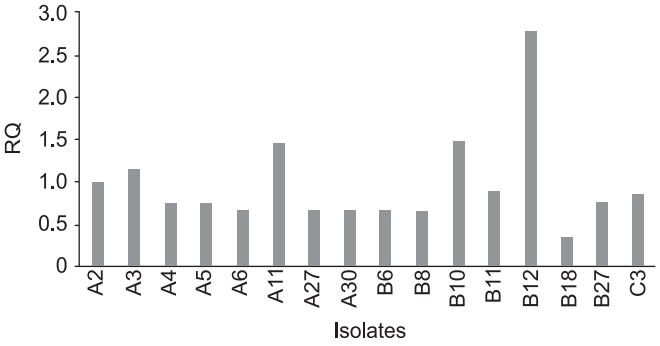

Fig. 1: Relative gene expression (RQ) of *Lm*AQP1 in clinical isolates of *L. major* by real-time PCR. Isolates A3, A11, B10 and B11 showed failure response; isolates A2, A4, A5, A6, A27, A30, B6, B8, B18, B27 and C3 were drug sensitive.

the Health Centre about 20 days after onset of disease with three lesions and his clinical feature showed allergy symptoms. The lowest gene expression was observed in B6 isolate obtained from a 16 yr-old male with three lesions that was referred after one month of disease. He responded to the treatment and cured with in a month. Statistical analysis showed that the expression level of *Lm*AQP1 was significantly different in resistant versus susceptible patients ($p = 0.001$; Fig. 2a).

Moreover, the stratification analysis for gender showed that the expression level of *AQP1* gene was not significantly different between female and male patients ($p = 0.28$; Fig. 2b). Also, the analysis did not reveal any correlation between age of patients and *AQP1* gene expression ($p = 0.28$, r = 0.26; Fig. 2c). The delta delta CT method, showed that the expression level of *AQP1* mRNA in resistant patients was 0.5 fold higher than that in non-resistant patients.

DISCUSSION

One of the major clinical concerns for treatment of different infectious diseases is drug resistance. Treatment failure of pentavalent antimonials, the recommended first-line drug for treatment of leishmaniasis has been reported in several countries. For instance, Sundar *et al*[12] showed that due to acquired resistance, over 60% of patients with VL did not respond to treatment with pentavalent antimony drugs in India. However the underlying mechanism of resistance has not been fully determined yet and remained a subject of intensive investigation [13]. The trivalent antimonials (SbIII) are formed of pentavalent antimony (SbV) after the action of metalloid reductase inside the macrophages. One of the proposed mechanisms for clinical/laboratory resistant strains development is enzymes overexpression in thiol biosynthetic path way[14–15]. In addition, upregulation and overexpression of ABC transporter resulting in sequestration of the Sb-III-thiol conjugate[16–17] and low expression of AQP1 involved in uptake of antimonials especially SbIII[7–8, 18] are considering to have a role in resistance development. Other *AQP* genes of *Leishmania* genome, including AQPα, AQPβ, AQPγ and AQPδ are less likely involved in drug uptake[19]. AQP1 is an important transporter involved in accumulation of SbIII within *Leishmania* cells and modulate drug sensitivity when expressed at increased levels[18–20]. It accumulates in flagellar pocket having critical role in movement and osmotic gradient. Consequently, it plays an important role in the cellular volume regulation of the parasite following an osmotic stress[21].

It has been previously reported that *Lm*AQP1

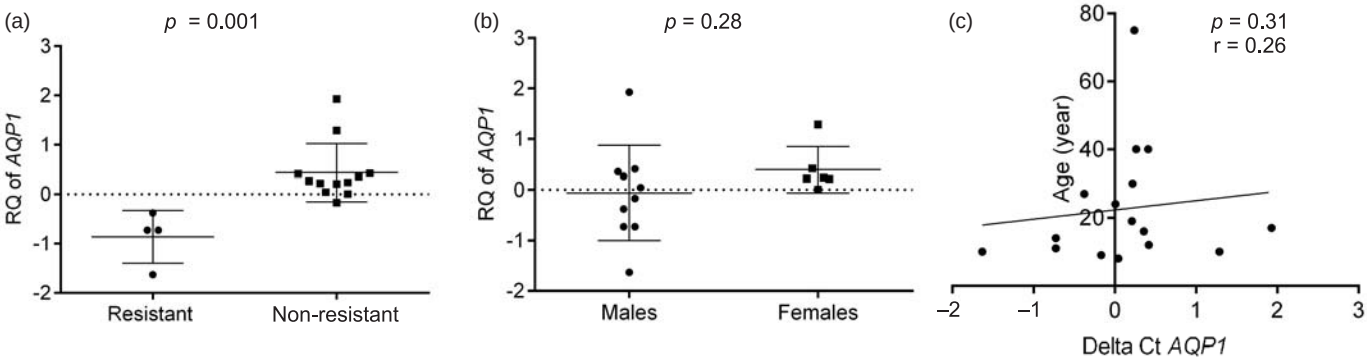

Fig. 2: (a) The expression level (RQ) of *AQP1* showed a significant difference between resistant and non-resistant patients; (b) In case of gender stratification, no any significant difference was observed between males and females; and (c) No any significant correlation was observed between age of patients and *AQP1* gene expression (Spearman correlation coefficient test).

overexpression (10 folds) could induce resistance in *L. major* strains[7]. Some other studies within *L. major* isolates are indicative of correlation between *AQP1* overexpression and susceptibility to antimony[7–8]. On the other hand, down regulation of AQP1 leads to reduced drug uptake that was seen in *L. donovani*[22]. Interestingly, our findings showed over-expression of *Lm*AQP1 in resistant strains in comparison to the sensitive isolates. These results confirm the other study on a small subset of Indian clinical isolates that showed overexpression of *Lm*AQP1 in some groups of resistant strains[18]. This phenomenon might be due to variation in the genotypes of the isolates. On the other hand, existing evidences suggest that occurrence of some special mutations could affect the gene expression level or efficiency of *Lm*AQP1. Specifically, mutation of Glu152 and Arg230, located at C-loop of *Lm*AQP1 could affect its expression[23–24]. It has been shown that mutations play an important role in changing phenotypes and pathogenicity[25–26]. Also, the number of alleles with or without mutation could affect gene expression[18]. In our study, *Lm*AQP1 in two isolates, A24 and B6 showed attenuated expression compared to the other isolates, and since these patients discontinued the treatment, they were excluded from the final analysis.

In total 16 isolates were investigated in this study among which 12 isolates were susceptible to drug response and four isolates were drug resistant. Among the drug resistant patients, three were male and one was female. While among the susceptible isolates, six each were male and female; however, statistical analysis did not show any significant difference between sex and susceptibility to drug response. The age of the patients also did not show any statistical correlation with drug response. The lesion number was different in each group ranging from 1 to 16. The maximum number of the lesions (16 lesions) was seen in a 10 yr-old male child who was referred to Health Centre after 20 days from the onset of disease. During three months follow-up period, no differences in number and size of the lesions was observed. Out of 16 patients, seven cases referred to the Health Centre had just one lesion while other patients had more than one lesion. Statistical analysis showed that there is no significant difference between number of lesions and type of responsiveness to the drug. The mean size of the lesions in all the patients with CL was $6.85 \pm 5.9 \times 4.39 \pm 2.9$ mm^2. The mean size of the lesions in patients with failure in drug response was $4.7 \pm 3.1 \times 4.7 \pm 3.5$ mm^2 and in patients with drug sensitive was $8.1 \pm 6.7 \times 4.25 \pm 2.7$ mm^2. No significant difference was observed between these two characteristics.

Some studies have reported the effect of some sex hormones on the expression of *AQP1* gene[27–28]. The gender stratification analysis and the expression of this gene did not show any significant difference. Moreover, the results revealed that the expression of *AQP1* gene was not affected by the patients age.

This study showed that *LmAQP1* gene expression was elevated in all the isolates, but it was higher in isolates with drug failure response, which has not been reported in elsewhere[20].

CONCLUSION

Leishmaniasis is one the important protozoan disease that manifests in various forms. One of the most common forms of the disease is CL. Treatment is considered as the best way to control leishmaniasis. The first line of drug for leishmaniasis treatment is antimonials but there are so many reported cases of resistance or failure in response to antimonials. The actual mechanisms involved in failure response are not clear yet, but one of the well-known causes is the transmembrane protein named *Lm*AQP1. The results of the study revealed that all sensitive and failure response isolates showed *Lm*AQP1 gene expression, but significant over expression of AQP1 gene in resistant versus non-resistant isolates suggests different mechanism of drug resistance resulting in either efflux system activation or sequestration of the drug. Further studies are required to find the actual reason behind the resistance against antimonials.

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Conflict of interest

The authors declare that they have no conflict of interest.

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