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Original Article

TGF-β Targeted by miR-27a Modulates Anti-Parasite Responses of Immune System

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Abstract

Background: Immune cells and their secreted cytokines are known as the first barrier against pathogens. *Leishmania major* as an intracellular protozoan produces anti-inflammatory cytokines that lead to proliferation and survival of the parasite in the macrophages. miRNAs are small non-coding RNA molecules that regulate mRNAs expression. We aimed to investigate the relationship between the expression of TGF-β and a bioinformatically candidate miRNA, in leishmaniasis as a model of TGF-β overexpression.

Methods: The miRNAs that target TGF-β -3´UTR were predicted and scored by bioinformatic tools. After cloning of TGF-β-3'UTR in psi-CHECK ™- 2 vector, targeting validation was confirmed using Luciferase assay. After miRNA mimic transfection, the expression of miR-27a, TGF-β, as well as Nitric Oxide concentration was evaluated.

Results: miR-27a received the highest score for targeting TGF-β in bioinformatic predictions. Luciferase assay confirmed that miR-27a is targeting TGF-β-3'UTR, since miR-27a transfection decreased the luciferase activity. After miRNA transfection, TGF-β expression and Nitric Oxide concentration were declined in *L. major* infected macrophages.

Conclusion: Bioinformatic prediction, luciferase assay, and miRNA transfection results showed that miR-27a targets TGF-β. Since miRNA and cytokine-base therapies are developing in infectious diseases, finding and validating miRNAs targeting regulatory cytokines can be a novel strategy for controlling and treating leishmaniasis.

Introduction

The innate immune response is the primary defense line against microbial pathogens (1). Macrophages are innate immune cells and phagocyte foreign agents and secrete cytokines when inflammatory stimuli stimulate them (2).

To escape from the host immune system, parasites use several mechanisms such as inhibiting inflammatory cytokines production and anti-inflammatory cytokine production (3). TGF-β, as an anti-inflammatory cytokine, produced by activated macrophages (4), suppresses the synthesis of IFNγ, TNF-α, NO synthase, inhibits Th1 response, and survives the parasite within the macrophages (5).

Furthermore, it is usually produced as a latent precursor that requires enzymatic cleavage to become active form (6). TGF-β has been secreted from infected macrophages in mice and humans (7).

Leishmania major as a protozoan parasite mainly infects macrophages. Since macrophage, as immune cells, try to eliminate the parasite, it uses a different mechanism to escape from the immune system and survive in the macrophages. One mechanism is the production of anti-inflammatory cytokines such as TGF-β in the macrophages (8).

microRNAs (miRNAs), as small non-coding RNAs, regulate gene expression (9). The role of miRNAs has been demonstrated in regulating inflammatory processes and immune responses (10, 11). Among several miRNA detection techniques, quantitative Real-time PCR (RT-qPCR) is a reliable technique and stem-loop based cDNA synthesis followed by Real-Time PCR was developed for analyzing miRNA expression (12).

Recently, manipulating miRNA populations in different types of microorganisms and cells is possible, including enhancing the effect of miRNA on the target cell by using mimic miRNA or inhibiting its function by oligonucleotide analogs (13, 14).

Lately, miRNAs could regulate some cytokines expression (15). Accordingly, the mechanism of anti-inflammatory cytokine production secreted by macrophages for parasite survival can also be manipulated by miRNAs. In this regard, it is essential to identify potential miRNA–target interactions and validate microRNA targeting. Luciferase assay as an exact and sensitive technique applies to validate miRNA targeting. If miRNA can target 3'UTR of the target gene, the luciferase gene translation level will be decrease (16). miR-27 could regulate the inflammatory response of macrophages by targeting IL-10 using Luciferase assay (17).

To the best of our knowledge, there is no previous report about the validation of miR-27a targeting TGF-β using Dual-Luciferase assay. Since *L. major* as the agent responsible for Cutaneous Leishmaniasis (CL) stimulates TGF-β production to survive in macrophages, we selected this parasite for our research. We aimed to predict and validate one of the main miRNAs targeting TGF-β using bioinformatic software and Luciferase assay, respectively and practically investigate the effect of the candidate miRNA's over-expression on inhibition of TGF-β in *L. major*-infected macrophages.

Materials and Methods

Bioinformatics study

miRWalk (18) and TargetScan (19) databases and GEO databank (20) were used to find candidate miRNAs targeting TGF-β.

Cloning of TGF-β-3'UTR in psi-CHECK™-2 vector

This study was conducted in the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran during 2019-2020. PCR reaction was accomplished to amplify mouse

TGF-β 3'UTR fragment. After gel electrophoresis, PCR fragments of mouse TGF-β-3'UTR containing *XhoI* and *NotI* restriction sites were gel purified and cloned in a T/A cloning vector (Fermentas, Lithuania, USA). T/A vector was selected since T/A is an intermediate easy-to-clone and easy-to-handle vector, which facilitates cloning a fragment in different destination vectors (21). The ligation product was then transformed into the competent *E. coli* strain TOP10 bacteria. The plasmid was extracted from recombinant bacteria using a plasmid extraction kit (Favorgen, Taiwan). The recombinant vector was digested with *XhoI* and *NotI* (Fermentas, Lithuania, USA) enzymes. Then, the target sequences were cloned into the Luciferase psi-CHECK™-2 vector (Promega, Southampton, UK). This vector enables monitoring changes in the expression of a target gene fused to a reporter gene and uses for optimizing RNA interference. In fact, decrease in luciferase activity correlates with miRNA-3'UTR interaction (22). Cloned fragments were finally confirmed using PCR and sequencing.

Dual-Luciferase assay

5×10^3 HEK293T cells/ml were seeded into 96-well plates with 10% Fetal Bovine Serum (FBS) completed Dulbecco's Modified Eagle medium (DMEM), incubated at 37 °C with 5% CO_2. After 24 h, the recombinant construct and synthetic miR-27a mimic (Bioneer, Korea) or control vector were co-transfected using Lipofectamine® 2000 (Invitrogen, USA) in a serum-free DMEM medium. After 6 h, the medium was removed and replaced with DMEM complete medium. After 48 h, the Luciferase assay was performed by a Dual-luciferase reporter assay system (Promega, Southampton, UK) according to the manufacturer's instructions and previous experiments (23).

Selection of suitable model

We were looking a model for overexpression of TGF-β. Literature study showed that *L. major*-infected macrophage is a suitable TGF- β induction model (4).

MRHO/IR/75/ER strain *of L. major* was cultured in RPMI 1640 medium (Biosera, France) supplemented by 10% FBS and incubated in a humidified atmosphere at 25 ºC (24, 25).

Macrophage infection by L. major promastigote

1×10^6 RAW 264.7 cells (24, 25) were plated into 6-well plates with DMEM complete media and incubated at 37 °C with 5% CO_2. After 24 h, the macrophages were infected with the stationary phase of *L. major* (10:1 ratio) for 24 h. Afterward, infected and uninfected RAW 264.7 cells were harvested and centrifuged at 300 g for 5 min. Pellets were re-suspended in lysis solution and stored at -80 °C.

RNA extraction, cDNA synthesis, and quantitative RT-PCR (RT-qPCR)

After RNA extraction using GeneAll® kit (GeneAll Biotechnology, Korea) and quantitation of extracted RNA, cDNA was synthesized by cDNA synthesis Kit (YEKTA TAJHIZ AZMA, Iran) following the manufacturer's brochure instruction (24, 25).

RT-qPCR was performed to analyze relatively the expression of TGF-β and miRNA in control and treatment groups using Real Q Plus 2X Master Mix Green High ROX™ (Ampliqon, Denmark). The RT-qPCR reactions were done at 95° C for 15 min followed by 40 cycles of 95° C for 20s and 60° C for 40s in a StepOnePlus™ instrument (ABI, USA). The experiments were done in triplicates. SNORD 234 and β2M gene were used as reference genes for miRNA and mRNA normalization, respectively.

Synthetic miRNA mimic transfection

RAW 264.7 cells were plated into a 24-well plate and incubated at 37° C in a humidified atmosphere with 5% CO_2. After 24 h, cells were infected with the stationary phase of *L. major*. Twenty-four hours after infection, 25 µM synthetic miR-27a mimics (Bioneer, Korea) were transfected into RAW 264.7 cells using

Lipofectamine® 2000 transfection reagent. After 6 h, the medium was changed with a fresh complete DMEM medium. Finally, cells were harvested for RNA extraction, cDNA synthesis, and real-time PCR for evaluation of TGF-β and miR-27a expression as well as housekeeping genes.

Nitric Oxide (NO) detection

NO production was detected before and after miRNA mimic transfection; using the Griess method (26). Briefly, nitrite was mixed with sulfanilamide as diazotization reagent in acidic media in each well. Then, N-naphthyl-ethylenediamine was added after 5 min and incubated in a dark place at room temperature for 10 min. After that, the absorbance of purple color produced in each sample was measured at 540 nm and NO concentration was calculated using standard curve.

Statistical Analysis

The comparison between two groups was performed using *t*-tests by GraphPad Prism version 8.0.1. *P-value* < 0.05 was considered statistically significant. All tests were performed in triplicate.

Results

Bioinformatic analysis

miR-27a was selected as it targets 3'UTR sequence of TGF-β with the highest score (Table 1).

miR-27a targets TGF-β

To determine whether miR-27a may function as a targeting 3'UTR of TGF-β gene, TGF-β 3'UTR was cloned into psi-CHECK™-2 vector, downstream of the luciferase coding sequence. HEK293T cells were transfected with both reporter construct and synthetic miR-27a mimic or control (scramble). Luciferase assay confirmed that miR-27a decreased the luciferase activity about seven-fold compared to control (Fig. 1). Furthermore, miR-27a expression was downregulated in *L. major* infected macrophages compared to the control group. After miR-27a mimic transfection, miR-27a expression significantly upregulated compared to the non-transfected group (**, P<0.01; ***, P<0.001).

Table 1: Interaction between miR-27a and TGF-β

miRNA	RefSeq UID	Binding p value	Binding site	Au	Me	N pairings	TargetScan	miRDB	miRTarBase
mmu-miR-27a-5p	NM_011577	0.87	2020,2046	0.28	-3.79	20	-	-	-
mmu-miR-27a-5p	NM_011577	0.92	1684,1707	0.4	-8.16	18	-	-	-

The expression level of TGF-β before and after miRNA mimic transfection

TGF-β expression was significantly decreased after miR-27a mimic transfection in *L. major* infected macrophages compared to non-transfected cells (*, P <0.05) (Fig. 2).

Concentration of NO before and after transfection

The measurement of NO concentration showed a significantly 2-fold decline in the mimic transfected group compared to non-transfected group (**, P<0.01) (Fig. 3).

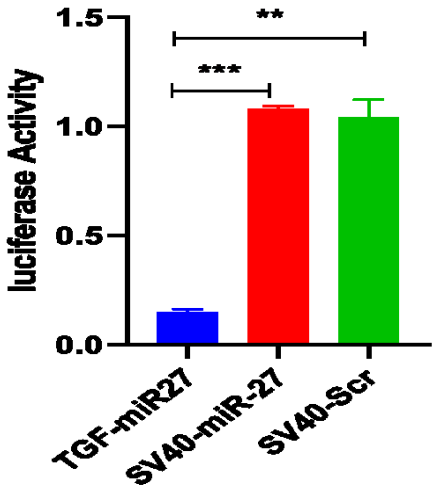

Fig.1: Dual-luciferase reporter gene assay, which showed that miR-27a targets TGF-β 3'UTR and decreases the luciferase activity (SV40 :3'UTR control vector, Scr: Scramble miRNA control). The data are representative of three independent experiments (n=3) (**, *P*<0.01; ***, *P*<0.001).

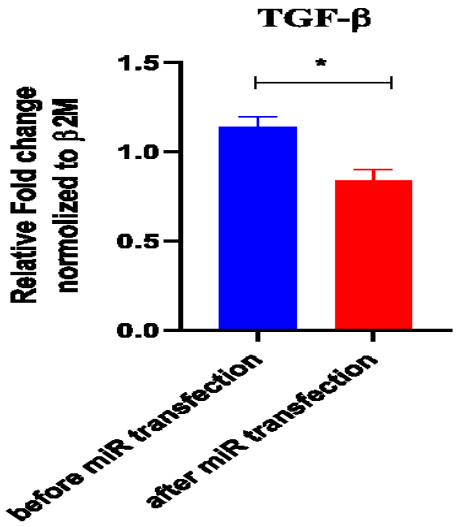

Fig. 2: TGF-β expression in L. major-infected macrophages before and after synthetic miRNA mimic transfection (25 µM). The data are representative of three independent experiments (n=3) (*, *P* <0.05)

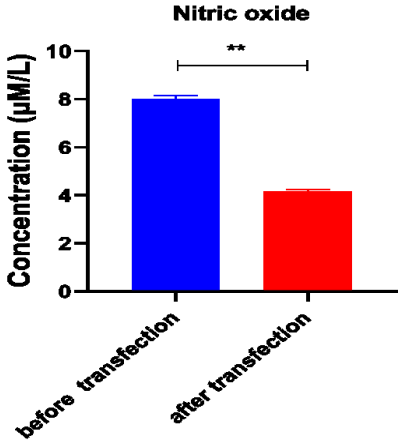

Fig. 3: NO concentration in *L. major*-infected macrophages before and after synthetic miR-27a mimic transfection (25 µM). The data are representative of three independent experiments (n=3) (**, *P*<0.01)

Discussion

Macrophages are the main residence for the replication and survival *of Leishmania,* and infection outcome depends on the interface between the macrophages and parasites (27). Immune response mediated by cytokines determines the fate of *Leishmania.* So that, TGF-β release from infected macrophages survives *Leishmania* (28). Using miRNA mimic or inhib-

itor transfection for modulation of gene expression raises new hope for treating infectious diseases (29). Our bioinformatic analysis showed that miR-27a received the highest score for targeting TGF-β-3'UTR.

Furthermore, miR-27a-TGF-β interaction was confirmed by luciferase assay. In addition, effect of miR-27a was shown inhibition of TGF-β expression in *Leishmania* infected macrophages. It is confirmed that transfection of miR-27a downregulates TGF-β expression in *L. major* infected macrophages. Interestingly, miR-27a also targets IL-10-3'UTR (luciferase assay confirmation) (17). Since IL-10 and TGF-β are anti-inflammatory cytokines and have critical role in *Leishmania* survival in macrophages (28), miR-27a was selected as the best candidate miRNA.

The information about microRNA-mRNA interaction is vital for the understanding of microRNA functions. The luciferase assay evaluates the regulation of miRNA's effect on target genes (30). Dual-Luciferase reporter assay is accomplished by serially measuring the firefly and Renilla luciferase activities and internal control to increase validity (31).

This study revealed that miR-27a decreased the luciferase activity (about 7- folds), therefore, miR-27a targets TGF-β-3'UTR. Upregulation of miR-20a was shown in *Toxoplasma gondii* human infected macrophages. An increase in apoptosis was confirmed after transfection of Locked Nucleic acid anti-miR-20a into human peripheral blood mononuclear infected cells (32). The miR-190b inhibitor decreased luciferase production in *Trypanosoma cruzi* infected cells by targeting tumor suppressor PTEN mRNA. (33). The miR-17-92 expression was increased in *Toxoplasma gondii* strain TgCtwh3 infected human macrophages. *Bim*, as a proapoptotic gene, is targeted by miR-17-92 using luciferase assay (34). Downregulation of miR-513 and upregulation of B7-H1 were reported in *Cryptosporidium parvum* infected human cholangiocytes. miR-513 target B7-H1 by reducing luciferase activity. Transfection of miR-513 precursor into H69 cells blocked T cell apoptosis in cholangiocytes (35). Malate dehydrogenase (MDH) was upregulated in the amoeboid phase of *Trichomonas vaginalis*. miR-1 mimic transfection reduced Malate dehydrogenase level since MDH mRNA has a target site for miR-1 family (36). These studies have shown that miRNAs target 3'UTR genes, and that the use of inhibitors or mimics reduces the expression of genes that may play a role in disease progression, which is consistent with our study.

The GEO data showed that, miR-27a was downregulated in *L. major* infected macrophages 24h post-infection (37). The miR-27a expression was downregulated in the liver of mice unvaccinated with *Plasmodium chabaoudi* infected RBC (38). Upregulation of miR-27a was reported in brain of *Plasmodium berghei* infected mice (39). Since, upregulation of miR-27a is related to increased expression of inflammatory cytokines, therefore, this miRNA is associated with disease pathogenesis. Role of miR-27a in pathogenesis of cerebral malaria may be different from our study. It is reported that miR-27 was upregulated in liver of infected domestic cats by *Toxoplasma gondii (T. gondii)* (40). Upregulation of miR-27 was shown in the intestines of sheep resistant to Hydatid cyst. miR-27 is involved in the inflammatory process and may play an essential role in the response of intestinal tissue to *Echinococcus granulosus* (41). The result may be indicated the inhibitory role of miR-27 in sheep intestinal infection resistant to hydatid cyst disease. Also, miR-27a upregulation was shown in BALB/c lung infected with *Schistosoma japnicom* (42). The differences between these studies may be related to the interaction between parasite and its host. In other words, miR-27a expression is different in parasite-infected cells. In addition, upregulation of miR-27a is related to pathogenesis of diseases or host resistance to infection. Therefore, miR-27 downregulation in *L. major* infected macro-

phages may indicate insignificant role in *Leishmania* pathogenesis but needs comprehensive in- vivo studies.

The result of the study showed a decrease in TGF-β expression in macrophages infected with *L. major* after miR-27a transfection. An increase in IL-12 production was shown in *T. gondii* infected macrophage transfected by miR-187 mimic. Nevertheless, after miR-187 inhibitor transfection, IL-12 production decreased (43). This study showed that miRNA mimic or inhibitor consistent with our study could target cytokines.

In our previous study, the effect of synthetic miR-340 mimic on IL-10 and TGF-β1 involved in *L. major* infected macrophages was investigated. We also used the group of miR-27a and miR-340 combination to investigate whether co-miRNA transfection can affect TGF-β1 and IL-10 expression. Interestingly, the combination of miR-340 and miR-27a had a stronger effect on the downregulation of target genes and could reduce macrophage infectivity (44). However, investigating the practical interaction of miR-27a on TGF-β1 using strong evidence techniques such as luciferase assay was one of the limitations of the project that we fulfill it in our recent project.

This study revealed that NO concentration was decreased in the transfected group. Supernatant and membrane lipophosphoglycan of *L. major* promastigotes induce NO production in J774.1A cell line (45). It is not in line with our hypothesis that NO production may be increased after transfection. Inhibition of let-7e increased nitric oxide synthase 2 (NOS2) mRNA and NO production in *L. amazonensis* infected macrophages 24 h post-infection (46). Inhibiting miR-294, miR-721 (47), miR-30e-5p, and miR-302d-3p (48) increased NOS2 expression and NO production in *Leishmania amazonensis* infected macrophages. These results can be indicated NOS2 and mentioned miRNAs interaction. Our results were not in line with mentioned research. It may be explained that miR-27a can target NO mRNA, and the mechanism of parasite limiting may be acted from another pathway instead of the NO pathway.

Recently, scientists have been looking for new drugs to treat cutaneous leishmaniasis. In the meantime, miRNA therapy has been introduced in some diseases such as hepatitis (49) passed fruitful clinical trials. On the other hand, research showed the role of miRNA mimic or inhibitor in up or downregulation of key genes in the progress of the parasitic disease, so miRNA therapy may be used to control and treat parasitic diseases in the future.

Conclusion

Using bioinformatic tools revealed that miR-27a targets 3'UTR of TFG-β, confirmed by luciferase assay. After miR-27a mimic transfection, TGF-β expression was downregulated. TGF-β is recognized as an immunosuppressive cytokine and has a significant role in persisting the *Leishmania* in macrophages; therefore, using miRNA therapy to inhibit this cytokine may control cutaneous leishmaniasis. We propose that miR-27a can also be used as an inhibitor of diseases associated with upregulation of TGF-β. However, applying animal models is essential to confirm these in-vitro results.

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Conflict of interest

The authors declare that there is no conflict of interest.

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