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Original Article

Qian Five Rhinoceros Gindeng (QFRG) protects against development of immune thrombocytopenia via miR-181a inhibition of TLR-4 expression

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Abstract: Aims: Traditional Chinese medicine (TCM) has been considered to be effective auxiliary strategy for the treatment of hemocytopenia including immune thrombocytopenia. However, the molecular mechanism is still not understood. Methods: In present study, Qian Five Rhinoceros Gindeng (QFRG) mainly containing buffalo horn, rehmannia root, radix rubia, trogopterus dung and radix salviae miltiorrhizae administrated to thrombocytopenia mice induced by injection of MWReg30. MicroRNAs (miRNAs), Toll Like Receptors (TLRs) and cytokines were assayed in monocytes separated from mice peripheral blood. The relationship between miRNAs and TLRs was investigated in Mouse leukaemic monocyte macrophage cell line RAW264.7. Results: The mice with administration of QFRG had a significant increase in platelet count, and miR-181a of monocytes was markedly up-regulated in QFRG treated group. QFRG also decreased the levels of TLR4, IL-6 and TNF-α. In addition, miR-181a inhibitor reversed the effects of QFRG on platelet count, TLR4 and cytokines. Overexpression of miR-181a in lipopolysaccharide-induced showed a decrease of TLR4, IL-6 and TNF-α level. Conclusions: QFRG protects against development of immune thrombocytopenia via miR-181a inhibition of TLR-4 expression.

Keywords: Rhinoceros horn, immune thrombocytopenia, toll like receptor, traditional Chinese medicine

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder and exhibits bleeding manifestations or purpura [1]. Increased platelet destruction or decreased platelet production is the main characteristic of ITP [2]. In addition, reduced platelet level is often accompanied by digestive and urinary tracts bleeding, subconjunctival hemorrhages and intracranial hemorrhage, which can induce legs paralysis and intracranial hypertension and hence seriously threatens the lives of patients. In present, corticosteroids and immunosuppressive agents are common used to the treatment of ITP, but the response to these western medicines has not been always satisfactory, and may cause serious adverse events [3, 4].

In the past years, traditional Chinese medicine (TCM) has been considered to be effective auxiliary strategy for the treatment of chronic disease including ITP [5]. However, the molecular mechanism underlying the TCM on ITP is still unclear, which greatly limits the application and development of TCM worldwide. In present study, Qian Five Rhinoceros Gindeng (QFRG) was prepared to treat MWReg30 induced ITP in vivo. The buffalo horn, radix rehmanniae, radix rubia, trogopterus dung and radix salviae miltiorrhizae are included but not limited in the main ingredients of QFRG. Buffalo horn is a kind of medication which can be combined with other medical materials to enhance blood quality and cure haemorrhage [6]. Rehmannia root is often used in conditions like fluid exhaustion, consumptive diseases, bleedings, anemia and menstrual disorders [7, 8]. All Radix rubia, trogopterus dung and radix salviae miltiorrhizae are good at activating blood circulation [9-11].

However, no molecular mechanism has been investigated the function of QFRG on ITP. MicroRNAs (miRNAs), a new class of endoge-

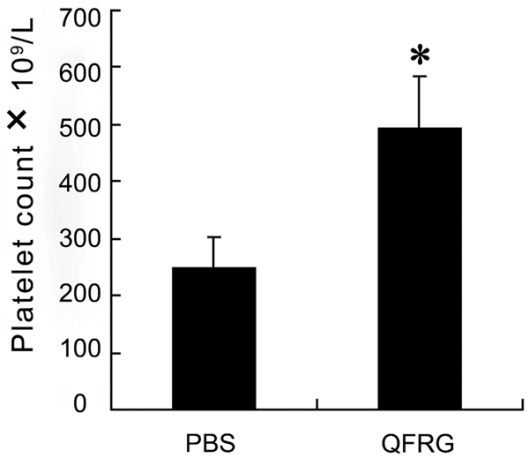

Figure 1. QFRG elevated platelet count of thrombocytopenia mice. All mice were intraperitoneally injected with 1 μg MWReg30 to establish immune thrombocytopenia. Intragastric administration of PBS (PBS group) or QFRG (QFRG group) were performed in mice for 2 week. Data are presented as the mean ± SD (n = 5), *P < 0.05.

nous and non-coding RNAs, are implicated in diverse biological pathways via binding to 3'-UTR of target genes [12-14]. A growing number of studies have demonstrated that miRNAs participate in the pathophysiology of immune disorders [15, 16]. Recently, new evidences have reported that the expression of some miRNAs in T cells, megakaryocytes and peripheral blood mononuclear cells were dysregulated in ITP patients compared with those of healthy controls [17-19], suggesting that miRNAs may be important regulatory molecules involved in the pathogenesis of ITP.

Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system [20]. TLRs are considered very sensitive to external signals and mediate inflammatory events to response against invading pathogens. In present study, we hypothesized that QFRG alleviates ITP via regulation of TLRs expression by miRNAs. In vivo and in vitro experiments were employed to examine this hypothesis.

Materials and methods

Preparation of traditional Chinese medicine

The QFRG was prepared by Preparation Center of Zhejiang Province Hospital of Traditional Chinese Medicine (Hangzhou, China). The ingredients are as following: 30 g buffalo horn, 15 g cortex moutan, 15 g radix rehmanniae, 12 g radix paeoniae rubra, 12 g radix lithospermi, 12 g radix rubia, 30 g trogopterus dung, 30 g radix salviae miltiorrhizae, 9 g radix sophorae flavescentis, 15 g radix scutellariae, 30 g cacumen platycladi, water boiling to obtain 3.70 g/ml decoction, stored at 4°C.

Thrombocytopenic mouse model

All experiments involved in animals were performed according to Ethical Principles and Guidelines for Experiments on Animals and approved by Ethics Committee of The First Hospital Affiliated to Zhejiang Chinese Medical University. Thrombocytopenia was induced in BALB/c mice (Vital River Laboratory Animal Technology Co., Ltd., China) by intraperitoneal infusion of the antiplatelet antibody MWReg30 as described previously [21]. After the establishment of thrombocytopenic mouse, 5 mice were administrated with PBS (Control group), and the remaining 10 mice were exposed to decoction of QFRG by intragastric gavage (QFRG group). 5 mice in QFRG group were intravenously injected with miR-181a inhibitor (QFRG + miR181a inhibitor group). Intragastric administration was last for 2 weeks. Blood samples were collected by retroorbital bleeding and platelet counts were measured by flow cytometry (BD Biosciences, USA).

Monocyte preparation and culture

The mononuclear cells were drew from mice peripheral blood and separated by counter flow centrifugal elutriation. Suspended monocytes (1×10^6 cells/ml) were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 20 μg/ml kanamycin, 100 U/ml streptomycin and 100 U/ml penicillin (Sigma, USA).

Microarray analysis

Total RNAs from peripheral blood monocytes of thrombocytopenia mice were isolated with MiRNEasy Mini Kit (Qiagen, Germany). The microarray analysis of miRNAs was performed by RiboBio Co. Ltd. (RIBOBIO, China) using Agilent miRNA Microarray System according to the protocols. Microarray data were analyzed

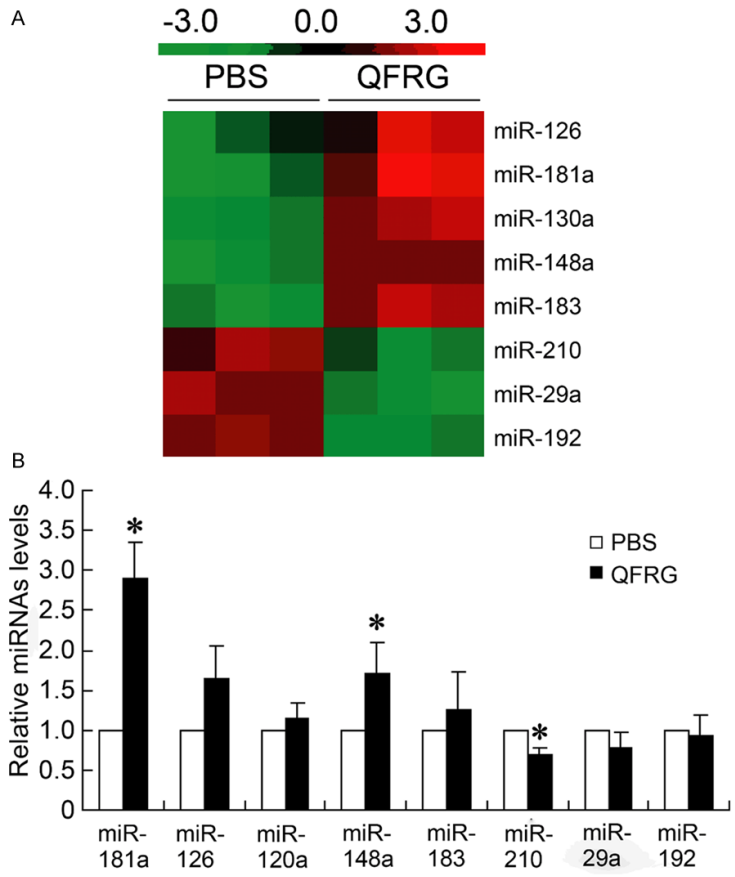

Figure 2. QFRG regulates the expression of miRNAs of peripheral blood mononuclear cell in thrombocytopenia mice. The expression of miRNAs was detected in isolated peripheral blood mononuclear cell. Data are presented as the mean ± SD (n = 5), *P < 0.05.

by GeneSpring GX v11.0 software (Agilent Technology).

Real-time PCR

The RNAs were isolated from peripheral blood monocytes or RAW264.7 cells using the MiRNEasy Mini Kit (Qiagen, Germany). TaqMan MicroRNA Reverse Trancription Kit (Applied Biosystems, USA) was used to reverse miRNA to cDNA and then quantified with TaqMan MicroRNA Assay (Applied Biosystems, USA).

TNF-α and IL-6 detection

For the measurement of cytokines, mouse leukaemic monocyte macrophage cell line (RAW264.7 cells) induced by lipopolysaccharide (LPS) were treated with or without miR-181a mimic. The concentration of TNF-α and IL-6 in peripheral blood monocytes of mice or RAW264.7 cells was detected by ELISA kits (Beyotime, China) following the manufacturer's instructions.

Down-regulation and overexpression and of miR-181a

The silence of miR-181a in thrombocytopenia mice treated with QFRG was employed by intravenous injection of miR-181a. The expression of miR-181a in RAW264.7 cells was overexpressed by transiently transfected with miR-181a mimic using the Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's instructions. MiR-181a inhibitor, miR-181a mimic and negative control were produced by RiboBio Co., Ltd. (RiboBio, China).

Macrophages culture

Mouse leukaemic monocyte macrophage cell line RAW264.7 (ATCC, USA) obtained from the First Hospital Affiliated to Zhejiang Chinese Medical University was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in 5% CO_2 humidified air. When grown to 70%-80% confluence, cells were incubated in DMEM containing lipopolysaccharide (LPS) for 24 h and transfected with miR-181a mimic.

Statistical analysis

Results were analyzed using SPSS software 18.0 and compared using one-way analysis of variance (ANOVA). Data were presented as mean ± standard deviation (SD). P < 0.05 was considered statistically significant.

Results

QFRG elevates platelet count of thrombocytopenia mice

A thrombocytopenia mice model was established by intraperitoneally injected with 1 μg MWReg30/mouse. Intragastric administration

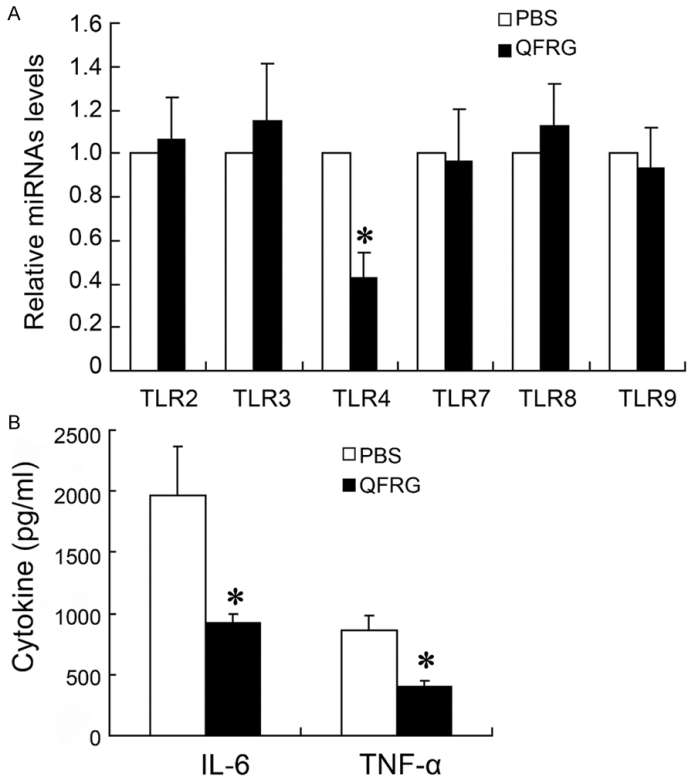

Figure 3. QFRG regulates the expression of TLRs and Cytokines of peripheral blood mononuclear cell in thrombocytopenia mice. The expression of miRNAs was detected by real-time PCR (A); the level of cytokine was measured by ELISA kits (B) in peripheral blood mononuclear cell isolated from thrombocytopenia mice. Data are presented as the mean ± SD (n = 5), *P < 0.05.

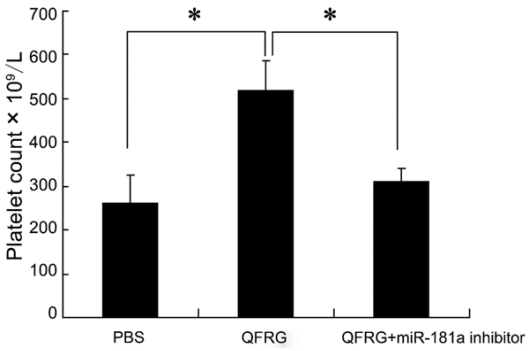

Figure 4. MiR-181a inhibitor reverses the effect of QFRG on platelet count of thrombocytopenia mice. All mice were intraperitoneally injected with 1 μg MWReg30 to establish immune thrombocytopenia; Intragastric administration of PBS (PBS group) or QFRG (QFRG group) were performed in mice for 2 week; MiR-181a inhibitor was administrated with intravenous injection; Data are presented as the mean ± SD (n = 5), *P < 0.05.

of QFRG was used to treat thrombocytopenia mice for 2 weeks. As shown in **Figure 1**, the platelet count of mice in QFRG treated group was twice as many as in PBS group, which implied that QFRG had a positive effect on treating thrombocytopenia.

QFRG regulates the expression of miRNAs of peripheral blood mononuclear cell

To investigate the mechanism of QFRG on thrombocytopenia, miRNAs were detected by microarray analysis and real-time PCR. Microarray results (**Figure 2A**) showed that QFRG administration up-regulated the expression of miR-126, miR-181a, miR-130a, miR-148a and miR-183 in isolated peripheral blood mononuclear cell, and the expression of miR-210, miR-29a and miR-192 were reduced by QFRG. The real-time PCR results (**Figure 2B**) demonstrated the similar trend of miRNAs regulation, and only the levels of miR-181a, miR-148a and miR-210 were significantly regulated by QFRG treatment. In addition, the expression of miR181a in QFRG administration group was observed almost three times as many as in control group.

QFRG regulates the expression of TLRs and Cytokines of peripheral blood mononuclear cell

The expression of TLRs was also detected by real-time PCR. As shown in **Figure 3A**, the expression of TLR4 was significantly up-regulated by QFRG treatment, but no difference was observed in level of TLR2, TLR3, TLR7, TLR8 and TLR9 between PBS and QFRG treated groups. The contents of IL-6 and TNF-α were measured by ELISA assay. As indicated in **Figure 3B**, the levels of IL-6 and TNF-α were significantly decreased in QFRG group compared with PBS group.

MiR-181a inhibitor reverses the effect of QFRG on platelet count of thrombocytopenia mice

As previously mentioned, the expression of miR-181a was markedly increased in QFRG administration group, which implied that miR-181a possibly involved in the effect of QFRG on thrombocytopenia. In vivo experiment also

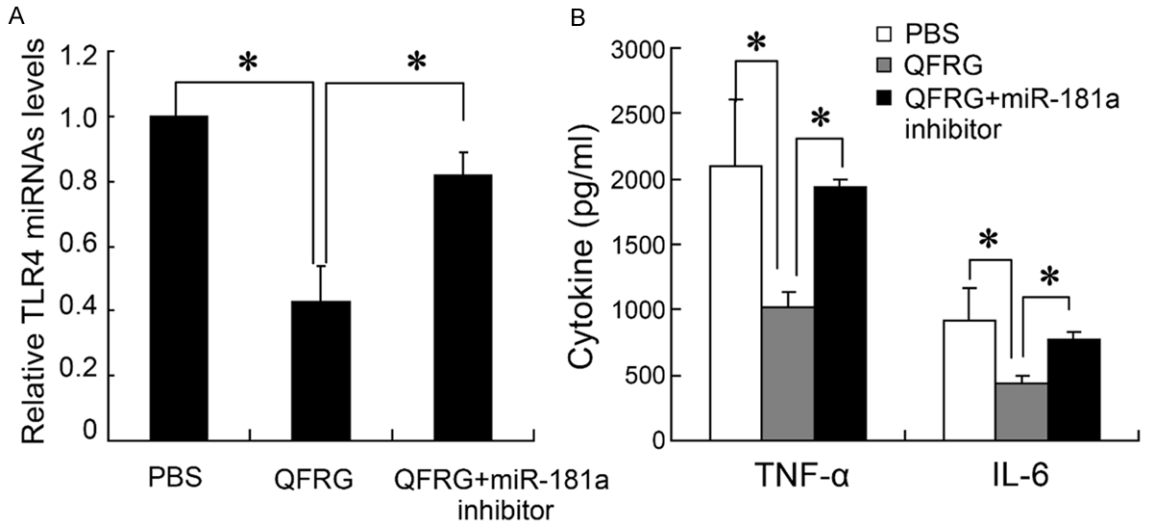

Figure 5. MiR-181a inhibitor reverses the effect of QFRG on TLR4 and cytokine levels of peripheral blood mononuclear cell in thrombocytopenia mice. All mice were intraperitoneally injected with 1 μg MWReg30 to establish immune thrombocytopenia; Intragastric administration of PBS (PBS group) or QFRG (QFRG group) were performed in mice for 2 week; MiR-181a inhibitor was administrated with intravenous injection; The relative TLR4 mRNA was detected by real-time PCR (A), and the levels of TNF-α and IL-6 were measured by ELISA kits (B) in peripheral blood mononuclear cell isolated from thrombocytopenia mice; Data are presented as the mean ± SD (n = 5), *P < 0.05.

demonstrated miR-181a inhibitor could reverse the up-regulation effect of QFRG on platelet count in mice **Figure 4**.

MiR-181a inhibitor reverses the effect of QFRG on TLR4 and cytokine levels of peripheral blood mononuclear cell

The levels of TLR4, IL-6 and TNF-α were significantly decreased in QFRG group. As expected, miR-181a inhibitor also up-regulated the expression of TLR4 mRNA and increased the levels of IL-6 and TNF-α after treatment of QFRG **Figure 5**.

The effect of miR-181a on regulation of TLR4 and cytokine expression in macrophagocytes

To further investigate the involvement of miR-181a in thrombocytopenia, in vitro experiments were performed. As shown in **Figure 6**, LPS induced an increase in levels of TLR4, TNF-α and IL-6, and which were down-regulated by miR-181a mimic in macrophagocytes.

Discussion

The major finding of this study is that QFRG markedly increased the platelet count in MWReg30-induced thrombocytopenia mice. Further study showed that down-regulation of miR-181a involved in the process of increased platelet production by QFRG via TLR4 regulation. In vitro experiments confirmed miR-181a takes part in the regulation of TLR4, TNF-α and IL-6 expression.

ITP is an autoimmune disease characterized by a decreased platelet count due to autoantibodies mediating platelet destruction and insufficient platelet production. Western medicine including corticosteroids and immunosuppressive agents were commonly used to control ITP [22, 23]. In addition, it is very popular to treat chronic disease using TCM in china because of its oral administration and lack of adverse events. The combination of various Chinese medical herbs is more effective and has less adverse events. There are eleven ingredients in QFRG in present study, and most of them have been widely used to regulate blood circulation. In vivo experiments confirmed that QFRG increased the platelet count of thrombocytopenia mice to twice, which implied an effective role in alleviating thrombocytopenia. Previous studies reported the essential components of TCM had good effects on hemocytopenia [24]. Researches also showed that TMC containing buffalo horn and radix rubiae promoted the curative effects of western medicine treatment on idiopathic thrombocytopenic purpura [25].

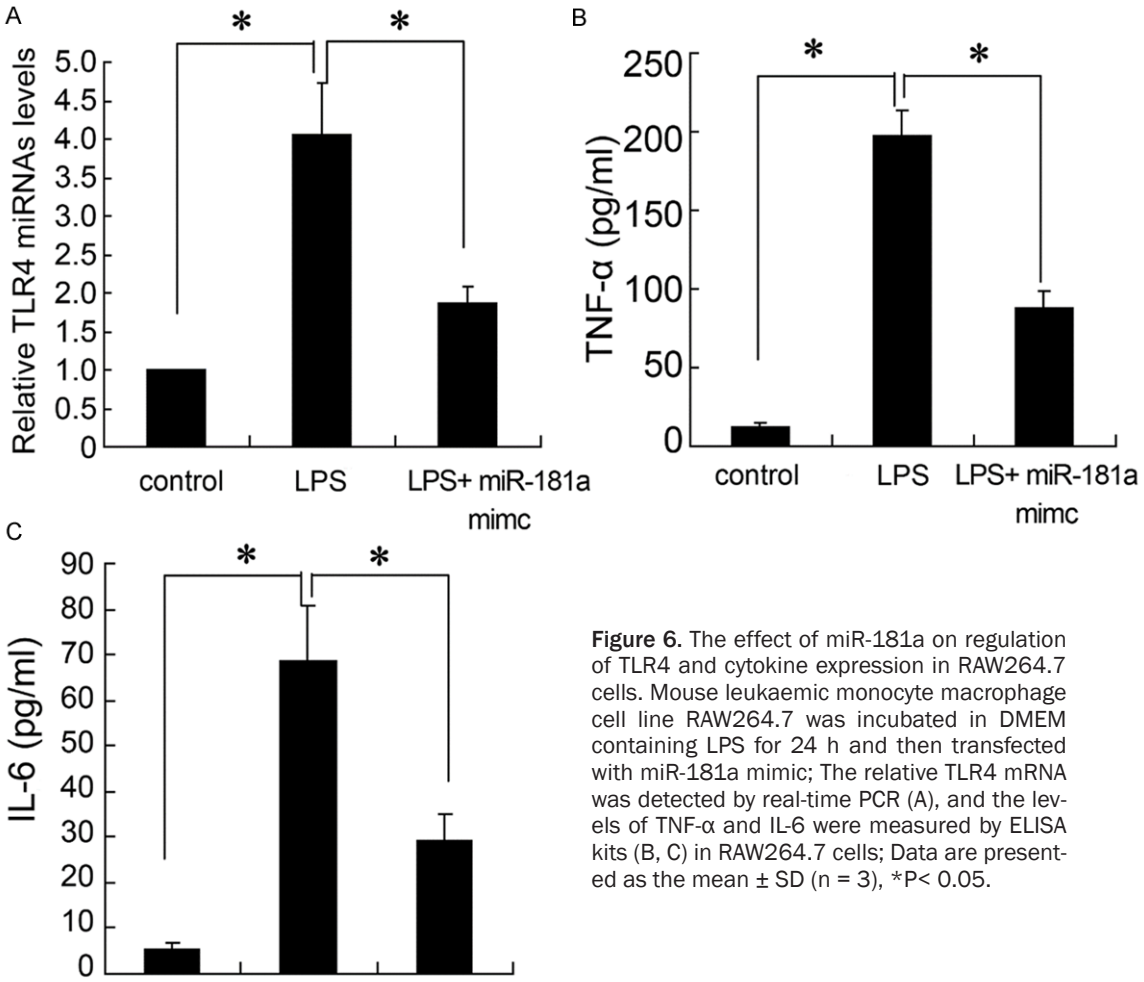

Figure 6. The effect of miR-181a on regulation of TLR4 and cytokine expression in RAW264.7 cells. Mouse leukaemic monocyte macrophage cell line RAW264.7 was incubated in DMEM containing LPS for 24 h and then transfected with miR-181a mimic; The relative TLR4 mRNA was detected by real-time PCR (A), and the levels of TNF-α and IL-6 were measured by ELISA kits (B, C) in RAW264.7 cells; Data are presented as the mean ± SD (n = 3), *P< 0.05.

Integrated traditional Chinese and Western medicine in treatment of ITP is a critical topic in future research.

However, the molecular mechanism of TMC on treatment of ITP is very complex and has not been understood, which limited the popularization in the whole world. In present study, microarray technology and real-time PCR were employed to expression of miRNAs, the results showed that miR-181a, miR-126, miR-130a, miR-148a and miR-183 levels were up-regulated, and the expression of miR-29a and miR-192 was down-regulated in peripheral blood mononuclear cells isolated from thrombocytopenia mice administrated with QFRG. Real-time PCR data found miR-181a was more significantly elevated in experimental group. Therefore, we hypothesized miR-181a involved in the process of alleviating ITP by QFRG. Infected with miR-181a inhibitor in mice treated with QFRG, the platelet count was significantly decreased, which confirmed our hypothesis. Peng et al [26] demonstrated that miR-181a level in primary Sjögren syndrome patients were profoundly elevated compared with healthy individuals. Zhu et al [27] also found miR-181a had a significant change in chronic lymphocytic leukemia patients. These data indicated a physiological effect of miR-181a on autoimmune disease.

Our further experiments demonstrated that miR-181a is a key regulator in inflammatory response, which may contribute to the effects of QFRG on increasing platelet count of ITP. Inflammatory stimuli, such as TLRs, IL-6 and TNF-α are well known to be involved in the pathogenesis of immune disorders [28, 29]. TLRs are critical receptors that transmit danger signals to the innate immune system and that mediate inflammatory events that can eventually recruit and activate cells of the adaptive immune system to respond against invading

pathogens [30]. In present study, TLR2, TLR3, TLR4 TLR7, TLR8 and TLR9 expression of peripheral blood mononuclear cells in mice were detected by real-time PCR, only increased TLR4 was observed in QFRG treated group. As Aslam et al demonstrated [20] that TLR4 expressed by platelet plays a role in the modulation of LPS-induced thrombocytopenia and TNF-α production. In addition, we also found that TLR4 was up-regulated by miR-181a inhibitor in peripheral blood mononuclear cells of thrombocytopenia mice administrated with QFRG. TNF-α and IL-6 had a similar trend with TLR4 in monocytes. The results above implied that miR-181a takes part in the regulation of TLR4 and inflammatory cytokines, which is one of molecular mechanism underlying the effect of QFRG on ITP. MiR-181a mimic was also employed to overexpress the miR-181a, the data of in vitro experiments showed that the increase of TLR4, TNF-α and IL-6 level induced by LPS was reversed by miR-181a mimic, and further confirmed miR-181a participated in the regulation of immune disorder.

In conclusion, our study revealed that QFRG increased the platelet count of MWReg30-induced thrombocytopenia mice, and miR-181a was involved in the regulation of TLR4 and inflammatory cytokines, which is one of molecular mechanism underlying the treatment of QFRG on ITP. The present findings provide a theoretical basis for wider application of TCM on immune disorders.

Disclosure of conflict of interest

None.

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