Original Article

MicroRNA-365 in macrophages regulates Mycobacterium tuberculosis-induced active pulmonary tuberculosis via interleukin-6

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Abstract: The present study is to investigate the relationship between microRNA (miR)-365 expression and the levels of interleukin (IL)-6 mRNA and protein in patients with active tuberculosis. From June 2011 to June 2014, 48 patients with active pulmonary tuberculosis induced by Mycobacterium tuberculosis were included in the study. In addition, 23 healthy subjects were enrolled as control. Macrophages were collected by pulmonary alveolus lavage. In addition, serum and mononuclear cells were isolated from peripheral blood. The levels of miR-365 and IL-6 in macrophages, mononuclear cells and serum were determined using quantitative real-time polymerase chain reaction. The protein expression of IL-6 in macrophages and mononuclear cells was measured using Western blotting, while that in serum was detected by enzyme-linked immunoabsorbent assay. Expression of IL-6 mRNA and protein was significantly enhanced in patients with active pulmonary tuberculosis. Increase of IL-6 protein concentration in serum was probably due to the release of IL-6 protein from mononuclear cells in the blood. In addition, miR-365 levels were significantly lowered in patients with active pulmonary tuberculosis. Up-regulated IL-6 expression in macrophages, mononuclear cells and serum in patients with active pulmonary tuberculosis is related to the down-regulation of miR-365, suggesting that miR-365 may regulate the occurrence and immune responses of active pulmonary tuberculosis via IL-6.

Keywords: MicroRNA-365, Mycobacterium tuberculosis, active pulmonary tuberculosis, interleukin-6

Introduction

Tuberculosis is a chronic infectious disease caused by Mycobacterium tuberculosis (M. tuberculosis). Pulmonary tuberculosis is the most common tuberculosis that is characterized by tuberculosis infection in the lungs. Patients with active tuberculosis carry reproductively active and toxic M. tuberculosis in their bodies [1, 2]. In addition to lesions in the lungs, M. tuberculosis affects other organs, causing pain and economic losses to the patients. Pulmonary tuberculosis is closely related to human immune. For example, the occurrence of pulmonary tuberculosis can be caused by lowered body resistance induced by M. tuberculosis or enhanced allergy mediated by cells [3]. The occurrence of pulmonary tuberculosis usually involves mononuclear phagocytes. The monocytes in the blood have strong phagocytosis, participating in immunoreactions. Lung macrophages, as a kind of immunocytes, are transformed from monocytes in the blood, exerting lots of functions. Activated monocytes and tissue macrophages can produce and release many types of cytotoxins, interferons (IFNs) and interleukins (ILs), which participate in defense mechanism of the body. In addition, factors that can promote the growth of endothelial cells and smooth muscle cells are also produced. Monocytes can divide around inflammation sites, encompassing foreign matters [4, 5].

One of the important factors in immunoreactions, IL-6, is a kind of lymphokine produced by activated monocytes and tissue macrophages. It transforms precursor B cells into cells that generate antibodies. Together with colony stimulating factors, IL-6 promotes the growth and differentiation of primitive bone marrow-derived
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cells, and enhances the lytic function of natural killer cells [6-8].

microRNA (miRNA or miR) is a kind of single-strand non-coding RNA with 21-23 bases. It binds to the 3’-untranslated region of target mRNA, the expression of which is inhibited by reduced translation or stability [9, 10]. Circulating miRNA is released into the blood by normal cells or impaired cells, participating in cell signaling and transformation of genetic information [11, 12]. miRNA stably exists in peripheral blood, and cannot be degraded by RNase. The concentration of miRNA in the blood is high, reaching a level that can be detected as biomarkers. In addition, miRNA in the plasma or serum can also be used as biomarker [13, 14].

By now, there are already some research progresses on the regulatory mechanism of IL-6 in mRNA and miRNA levels. For example, a study shows that miR-365 negatively regulates the expression of IL-6 in HEK293 and HELA cells [15]. However, there is no report on the regulatory effect of miR-365 on IL-6 in macrophages of patients with active tuberculosis. In the present study, we investigate the relationship between miR-365 expression and the levels of IL-6 mRNA and protein in patients with active tuberculosis.

Materials and methods

Patients

From June 2011 to June 2014, 48 patients with active pulmonary tuberculosis induced by M. tuberculosis were included in the study. In addition, 23 healthy subjects were enrolled as control. All the 48 patients had positive results in the test for M. tuberculosis. In addition, the patients were diagnosed to have pulmonary tuberculosis by clinical characteristics, imaging, and pathological biopsies [16]. The included patients had no complications or infections in the heart, liver and kidney, no diabetes, or no tumors.

Macrophages were collected by pulmonary alveolus lavage. The lavage was filtered and centrifuged before removing the supernatants. The sediments were then fixed and sorted. Afterwards, cell density was adjusted to 1-3 × 10⁶/ml, and cultured under 37°C for 60 min. After washing with 0.5 mol/L phosphate-buffered saline, the remaining macrophages were saved and stored in Eppendorf tubes for storage at -20°C. For serum collection, peripheral blood (10-15 ml) was obtained from the subjects and stored at 4°C for 1-2 h. Then, serum in the upper layer was subject to centrifugation at 400 × g for 10 min. The supernatants were then aliquoted into Eppendorf tubes in volumes of 100 μl for storage at -70°C. To obtain peripheral blood mononuclear cells, red blood cells were diluted by equal volumes of Hank’s solution, and then added gently on top of lymphocyte separation medium that was mixed with serum-free Iscove’s modified dubecco’s medium at a ratio of 1:1. After centrifugation at 400 × g for 30 min, the middle layer of mononuclear cells were gently transferred to a new centrifuge tube and supplemented with 5 times of volumes of D-Hank’s solution, followed by centrifugation at 300 × g for 10 min. The cells were washed with phosphate-buffered saline twice and seeded onto culture plates for incubation at 37°C in 5% CO₂ for 1-2 h. The cells that were adherent were mononuclear cells. All procedures were approved by the Ethics Committee of Qingdao University. Written informed consents were obtained from all patients or their families.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (10606ES60, Yeasen, Shanghai, China) following the manufacturer’s protocol. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Waltham, MA, USA) from 1 μg RNA and stored at -20°C. QRT-PCR was performed using TransStart Top Green qPCR SuperMix kit (TransGen Biotech. Co., Ltd., Beijing, China) on PCR-iQ5 (Bio-Rad, Hercules, CA, USA). The primers for IL-6 were 5’-GGCACTGGGAGAAAACACCC-3’ (forward) and 5’-GCAGGCTCTCATTGAATCC-3’ (reverse); the primers for GAPDH were 5’-GGGAAACTGCGGCGTGAT-3’ (forward) and GAGGAGTGGCT-3’ (reverse). PCR reaction system (25 μl) included SYBR Premix EXTaqTM (12.5 μl), upstream and down stream primers (10 μM, 0.5 μl each), sterile water (10.5 μl), and cDNA (1 μl). The PCR protocol was as follows: initial denaturation at 95°C for 30 s; 45 cycles of denaturation at 95°C for 5 s and
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The primers for miR-365 were 5'-GCGTAATGCCCCTAAATCC-3' (forward) and 5'-CTCGCTTCGGCAGCACA-3' (reverse); the primers for U6 were 5'-AACGCTTCAGAGATTCCG-3' (forward) and 5'-CTCGCTTCGGCAGCACA-3' (reverse). The PCR protocol was: initial denaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 34 s. The 2^{-ΔΔCt} method was used to calculate miR-365/U6 ratio.

Western blotting

Precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) was used for the extraction of total proteins. Protein concentrations were determined by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (20 μg) were then mixed with equal volume of 2 × sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 5 min. Afterwards, 10 μl samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit an IL-6 antibody (1:1000) and β-actin antibody (1:5000) (Abcam, Cambridge, MA, USA) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit-horseradish peroxidase conjugate antibody (1:3000; Abcam, Cambridge, MA, USA) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab (Bio-Rad, Hercules, CA, USA) software was used to acquire and analyze imaging signals. The relative content of IL-6 protein was expressed as IL-6/β-actin ratio.

Figure 1. Expression of IL-6 mRNA in patients with pulmonary tuberculosis. The levels of IL-6 mRNA in (A) macrophages in the lungs, (B) mononuclear cells in the blood, and (C) serum were examined using qRT-PCR. The data were expressed as means ± standard deviation. *P<0.05 compared with control.
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Enzyme-linked immunoabsorbent assay (ELISA)

IL-6 levels in serum were measured using a commercially available IL-6 ELISA kit (ab17-8013; Abcam, Cambridge, MA, USA) according to the manufacturer’s manual. Absorbance at 450 nm was measured using a microplate reader (model 680, Bio-Rad, Hercules, CA, USA) within 15 min after stopping the reactions.

Bioinformatics

To explore possible genes that can regulate IL-6, we used target gene prediction software such as miRanda, TargetSean, PieTar, MiRanda and BibiServ. miR-365 was among the genes that were predicted to regulate IL-6.

Dual-luciferase reporter assay

Plasmids (0.8 μg) with 3'-UTR and mutant 3'-UTR DNA sequences were transfected into 293T cells, followed by transfection with miR-365 mimics (100 nM) before incubation for 24 h. Cells were lysed for the determination of fluorescence intensity using GloMax 20/20 luminometer (Promega, Madison, WI, USA). Using Renilla fluorescence as internal reference, dual-luciferase reporter assay was performed by Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s manual.

Statistical analysis

All results were analyzed using SPSS 18.0 (IBM, Armonk, NY, USA). The data were expressed as means ± standard deviation. Multiple sets of measurement data were compared using one-way analysis of variance. In case of homogeneity of variance, LSD and SNK were used; in case of heterogeneity of variance, Tamhane’s T2 or Dunnett’s T3 were used. Differences with P<0.05 were considered statistically significant.

Results

Expression of IL-6 mRNA is enhanced in patients with active pulmonary tuberculosis

To measure the levels of IL-6 mRNA, qRT-PCR was performed. The data showed that IL-6 expression levels in macrophages, mononuclear cells and serum from patients with pulmonary tuberculosis were significantly higher than those in the controls (P<0.05) (Figure 1). The result suggests that the expression of IL-6 mRNA is enhanced in patients with active pulmonary tuberculosis.
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IL-6 protein expression is enhanced in macrophages in the lungs and mononuclear cells of the blood

To test the expression of IL-6 protein in macrophages and mononuclear cells, Western blotting was used. The data showed that the levels of IL-6 protein expression in macrophages and mononuclear cells were significantly increased compared with those in controls (P<0.05) (Figure 2A and 2B). The result indicates that IL-6 protein expression is enhanced in macrophages in the lungs and mononuclear cells in the blood.

IL-6 protein concentration is enhanced in serum, probably due to the release of IL-6 protein from mononuclear cells in the blood.

miR-365 levels are lowered in patients with active pulmonary tuberculosis

To examine the levels of miR-365, qRT-PCR was employed. The data showed that miR-365 expression levels in macrophages, mononuclear cells and serum from patients with pulmonary tuberculosis were significantly lower than those in the controls (P<0.05) (Figure 3). The result suggests that miR-365 levels are lowered in patients with active pulmonary tuberculosis.

miR-365 regulates the expression of IL-6 by binding with its 3’-UTR

To verify the link between IL-6 and miR-365, dual-luciferase reporter assay was used. The data showed that fluorescence intensity in wild-type group was significantly weaker than that in control (P<0.01), while that in mutant group was not different from that in control (P>0.05) (Figure 4). The result indicates that miR-365...
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Figure 4. Interaction between microRNA-365 and IL-6. Plasmids (0.8 μg) with 3'-UTR and mutant 3'-UTR DNA sequences were transfected into 293T cells, followed by transfection with miR-365 mimics (100 nM) before incubation for 24 h. Cells were lysed for the determination of fluorescence intensity using GloMax 20/20 luminometer (Promega, Madison, WI, USA). Then, dual-luciferase reporter assay was performed. The data were expressed as means ± standard deviation. **P<0.01 compared with control.

regulates the expression of IL-6 by binding with its 3'-UTR.

Discussion

Tuberculosis is usually closely related to human immune. Therefore, factors related to immune system are always important research topics for tuberculosis [17]. During the occurrence and development of tuberculosis, cell and humoral immune defects exist. The cells that participate in cell immune responses mainly include macrophages, T lymphocytes, and natural killer cells [18]. A study shows that cytokines that participate in the immune responses of tuberculosis include Th1 type cytokines such as IL-2 and IFN-γ, Th2 type cytokines such as IL-5 and IL-4, and pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 [19]. When the lungs are attacked by M. tuberculosis, the antigens or metabolic products of M. tuberculosis activate the production of inflammatory factors such as TNF-α and IL-2 from macrophages in the lungs. Subsequently, the inflammatory factors promote the generation of IL-6 from macrophages in the lungs and epithelial cells in bronchus. Activation of mononuclear phagocytes by cytokines and endotoxins induce the elevation of TNF-α and IL-6 in the blood [20].

IL-6 plays important roles in immune responses, inflammation, cell differentiation, coagulation, and the occurrence and development of tumors. IL-6 production in inflammation induces the production of C-reactive protein and fibrinogen, facilitating the formation of thrombus [21]. Elevated IL-6 can induce inflammatory diseases such as rheumatoid arthritis by binding to IL-6 receptors [22]. In inflammatory responses, IL-6 causes chemotaxis to other inflammatory cells such as neutral lymphocytes and mononuclear phagocytes [23]. In the present study, we detected elevated IL-6 mRNA and protein expression in serum, macrophages from the lungs and mononuclear cells from the blood. These results indicate that mononuclear cells and lymphocytes were activated by impaired cells in the lungs caused by M. tuberculosis infection, and secreted abundant IL-6 factors to generate numerous antigen immune responses.

In a previous report, abnormal miR-365 expression is found in patients with breast cancer and endometriosis [24]. In NIH3T3 cells, radiation by ultraviolet significantly increases miR-365 expression, exhibiting anti-tumor effects [25]. In addition, overexpression of tumor suppressor gene NGX6 induces reduced expression of miR-365, suggesting that miR-365 possesses oncogene activity [26]. The expression of miR-365 is down-regulated in old lung fibroblasts, indicating that miR-365 probably participates in the regulation of cell proliferation [27]. However, few studies are focused on the regulation of active pulmonary tuberculosis by miR-365. According to bioinformatics prediction, we hypothesized that miR-365 might be an upstream miRNA that regulates IL-6. In addition, miR-365 exerts its negative regulatory effect on IL-6 expression in HEK293 and HELA cells [15]. In the present study, we found that miR-365 was down-regulated and IL-6 was up-regulated in mononuclear cells in the blood of patients with active pulmonary tuberculosis. Similar trends in miR-365 and IL-6 expression were also observed in macrophages in the lungs, suggesting that the down-regulation of miR-365 may induce immune responses by enhancing the expression of IL-6. In the serum, down-regulation of miR-365 and up-regulation of IL-6 were also observed, suggesting that increased IL-6 in mononuclear cells in the blood was released into the serum. The levels of miR-365 and IL-6 in the serum reflect inflammatory responses and tissue lesions in pulmonary tuberculosis in a certain extent. The study also indicates that miR-365, IL-6 and immune system have internal regulatory relationships in active pulmonary tuberculosis.
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tuberculosis. In the future, understanding about the regulatory mechanism of miR-365 on IL-6 may also be applied in the treatment of diseases related to the immune system. However, the present study has limited sample number and great variance in patients’ residential region, health conditions and complications. Together with these, various types of factors related to pulmonary tuberculosis lead to difficulties for the study [28-30]. Despite efforts made in search for the relationship between miR-365 and active pulmonary tuberculosis, more evidences that demonstrate the direct relationship between miR-365 gene and active pulmonary tuberculosis are still needed.

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Disclosure of conflict of interest

None.

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