MicroRNA-217 regulates interstitial pneumonia via IL-6

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ABSTRACT

The aim of this study was to investigate the expression of miRNA-217 in pulmonary macrophages, blood mononuclear cells and serum in patients with interstitial pneumonia, and the role of miRNA-217 in interstitial pneumonia. Blood and bronchoalveolar lavage fluid specimens from 29 patients with interstitial pneumonia and 23 healthy people (controls) in the same period were collected. Lung macrophages were isolated from the bronchoalveolar lavage fluid and were cultured. The expression of miRNA-217 and IL-6 mRNA in lung macrophages, blood mononuclear cells and serum samples were detected by quantitative real-time PCR (qRT-PCR). The IL-6 protein expression levels in lung macrophages, blood mononuclear cells and serum samples were detected by Western Blot or enzyme-linked immunosorbent assay, whereas IL-6 is a direct target gene of miRNA-217 was analyzed by double-luciferase reporter assay. The results showed that IL-6 was up-regulated, whereas miRNA-217 was down-regulated in lung macrophages, blood mononuclear cells and serum samples in patients with interstitial pneumonia (P < 0.05). IL-6 was suggested to be the target gene of miRNA-217 by double-luciferase reporter assay. We speculate that the upregulation of IL-6 expression in lung macrophages, blood mononuclear cells and serum in patients with interstitial pneumonia may be related to the down-regulation of miRNA-217 expression, which may mediate interstitial pneumonia through IL-6.

Introduction

Interstitial pneumonia (IP) is a diffuse pulmonary interstitial disease without evident causes. The pathological features of IP are diffuse alveolitis and alveolar structure disorders, which eventually lead to pulmonary interstitial fibrosis [1,2]. The main pathological manifestations are destruction of the alveoli wall, widened alveolar septa, pulmonary inflammatory cell infiltration, fibroblast proliferation, connective tissue hyperplasia, necrosis and occurrence of fibrosis [3,4]. Currently, the etiology is still not clear.

Human immunity is closely related to IP; the clinical symptoms of IP may be manifested when immune resistance is reduced by pathogen invasion or cell-mediated hypersensitivity increases [5]. The development of pneumonia generally has mononuclear-macrophage involvement. Mononuclear cells in the blood have a strong phagocytosis and are involved in the immune response [6]. Macrophages, derived from the blood mononuclear cells, have many immune-related functions. The activated monocytes and macrophages can generate and release a variety of cytokines, interferons and interleukins participating in the host defense. They also produce some growth factors promoting the growth of endothelial and smooth muscle cells. Mononuclear cells around the inflammation can undergo cell division and surround foreign bodies [7,8]. It can be argued that during the onset of IP, macrophages and cytokines play an important role in the course of the disease.

Interleukin-6 (IL-6), which was used an endpoint in this study, is one of the important factors in the immune response. Lymphokines, produced by activated monocytes and tissue macrophage cells, can promote the growth and differentiatation of B cell precursors into antibody-producing cells; in synergy with colony-stimulating factor, they can promote the growth and
MicroRNA-217 is closely related to inflammation and fibrosis. For example, miR-217 is involved in the protection of dopamine receptor D2 against fibrosis of proximal tubule cells [13]. miR-217 can affect the inflammatory response and fibrosis of glomerular mesangial cells through the Sirt1/HIF-1α signaling pathway [14]. miR-217 inhibits pancreatic ductal carcinoma by targeting KRAS [15]. miR-217 expression gradually increases along with age in the endothelial cells to inhibit the expression of SirT1 and induce early cell apoptosis [16]. However, the role and mechanism of miRNA-217 in IP has not been reported.

In this study, we used quantitative real-time PCR (qRT-PCR), Western Blot, bioinformatics prediction, enzyme-linked immunosorbent assay (ELISA) and double luciferase reporter assay to detect the expression of IL-6 and miRNA-217 in pulmonary macrophages, monocytes and serum in patients with IP. We also examined the association between IL-6 expression and miRNA-217 in pulmonary macrophages, monocytes and serum in patients with IP. We also examined the association between IL-6 and miRNA-217 in IP has not been reported. Our results showed that the expression of miRNA-217 in IP was significantly higher than in healthy controls. We also found that the expression of IL-6 was positively correlated with the expression of miRNA-217 in IP. These findings suggest that miRNA-217 may play a role in the pathogenesis of IP.

Reagents and instruments

The following agents were used: miRcute miRNA Isolation Kit (TIANGEN, Beijing, China), miRcute miRNA cDNA First Strand Synthesis Kit (Tiangen, Beijing, China), miRcute miRNA Fluorescence Quantitative Detection Kit (FP401, Tiangen, Beijing, China), SuperReal PreMix (SYBR Green, EP204, Tiangen, Beijing, China), TIANScriptIIcDNA First Strand Synthesis Kit (KR107, Tiangen, Beijing, China), IL-6 primary antibody (ab6672, rabbit anti-human, abcam, USA), Trizol reagent (10606ES60, Yeasen Biology, Shanghai, China), secondary antibody (ab6721, goat anti-rabbit, abcam, USA), BCA protein concentration assay kit (RTP7102, Real-Times Technologies, Beijing, China), serum RNA Extraction kit miRNAseasy Serum/Plasma Kit (50) (UL217184, Jianlun Biology, Guangzhou, China), IL-6 ELISA kit (ab178013, abcam, USA), image lab version 3.0 software, cell preservation solution (Ruikang company, Beijing, China) and ECL substrate kit (Ab65623, abcam, USA). The qRT-PCR was performed on PCR-iQ5 (Bio-Rad). All plasmids/agomiR in the experiments were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

Sampling

Peripheral venous blood and bronchoalveolar lavage fluid specimens were collected from 29 patients with IP and 23 healthy people. Lung macrophages were isolated from the bronchoalveolar lavage fluid and were cultured. Serum was obtained by gradient centrifugation. Blood mononuclear cells were collected through centrifugation. The re-suspended cells were seeded and cultured in a cell plate under the condition of 5% CO2 37°C for 2 h, and the adherent cells were mononuclear cells.

qRT-PCR detection of IL-6 mRNA

Total RNA was extracted from samples by the Trizol method. RNA purity was assessed by the absorbance ratio at 260 and 280 nm (1.8 ≤ A260/A280 ≤ 2.1). The template cDNA was prepared from samples of 1 μg of RNA and stored at −20°C. The primers were as follows: IL-6, Forward: 5’-GGACTGGAGAAAACACC-3’; Reverse: 5’-GCAAGTCTCCTCATGATCC-3’; GAPDH, Forward: 5’-GGGAACTGCGGCGTGAT-3’; Reverse: 5’-AAAGTGGAGATTGGGT-3’. The real-time PCR reaction system (20 μL) included: 10 μL of qRT-PCR Mix, 0.5 μL of forward primers, 0.5 μL of reverse primers, 2 μL of cDNA and 7 μL of ddH2O. The reaction conditions were: pre-denaturation at 95°C for 30 s;
denaturation at 95°C for 5 s; annealing at 57°C for 30 s; extension at 72°C for 30 s, a total of 45 cycles. The results were calculated using the 2-ΔΔCt method, and the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal reference.

**Western Blot detection of the protein expression of IL-6 in lung macrophages and blood mononuclear cells**

The protein was extracted and the protein concentration was determined by BCA protein concentration assay kit. Proteins (20 μg per lane) were then electrophoresed through a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, UK) for 2 h at 100 V under ice bath. The membrane was blocked with 5% skim milk in tris-buffered saline and tween-20 (TBST) at room temperature for 1 h. The blots were stained with IL-6 primary antibody (abcam, USA, ab6672, 1:1000) and β-actin (abcam, USA, 1:5000) at 4°C overnight and secondary antibodies (Abcam, USA, ab6721, 1:3000) for 1 h at room temperature. The membrane was placed in electrogenerated chemiluminescence (ECL) luminescent solution, and the image was analyzed by image lab software version 3.0 (Bio-Rad Laboratories). The relative content of the target protein was calculated by the ration of the gray value of the target protein band to the internal reference β-actin band.

**ELISA detection of protein expression of IL-6 in serum**

The isolated serum samples and the standard sample were separately added to the test wells according to the instruction of the ELISA kit. Briefly, 50 μL of standard samples were added to the wells. The 10 μL of serum sample was mixed with 40 μL of dilution and was added to the wells. Horseradish peroxidase (HRP) labelled detection antibody (100 μL) was added to all wells, except for the blank well. Then the plate was sealed with a sealing membrane and incubated for 1 h. After washing for five times, the substrate A, B (50 μL of each) was added and incubated at 37°C for 15 min, then 50 μL per stop solution was added. The OD value at 450 nm wavelength was measured within 15 min.

**Bioinformatics prediction of upstream miRNAs of IL-6**

Bioinformatics prediction is the basis for many studies on miRNA function. In order to further explore the regulatory mechanism of IL-6 upstream miRNAs in IP, after a literature review, we used the following software or web tools to carry out the targeted gene prediction: miRanda (http://www.microma.org/microma/home.do), TargetScan (www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and PICTA (http://pictar.mdc-berlin.de/). The findings revealed miR-217 could be one of the genes regulating IL-6.

**qRT-PCR detection of MiRNA-217**

The primers used for qRT-PCR were: forward: 5'-GCTTCGCAAGCATAATCTAAAT-3', reverse: 5'-CGCTTCACGAATTTGGCTCAT-3'. The primers for miR-217 were: forward: 5'-CGCTCTACTGCATCAGGAACTGA-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'. The reaction conditions were: pre-denaturation at 95°C for 3 min; denaturation at 95°C for 12 s; annealing at 62°C for 40 s; extension at 72°C for 20 s, a total of 40 cycles. The results were calculated using the 2-ΔΔCt method, and the ratio was calculated using U6 as the internal reference.

**Double luciferase reporter assay**

The normal and mutated sequence of miR-217 that can bind with the 3'-UTR region of the IL-6 gene were synthesized, and the digestion sites of Spe-1 and HindII were added at both ends. The two DNA fragments were cloned into PMIR-REPORT luciferase reporter plasmid. The group that was cloned with the mutated DNA fragment served as the control group. The plasmid containing 3'-UTR (0.8 μg) and mutant 3'-UTR DNA sequences (0.8 μg) were transfected into 293T cells, respectively. Then, the miR-217 mimics (100 nmol/L) were transfected and the cells were lysed after 24 h of culture. The fluorescence values were measured using a GloMax 20/20 luminometer. Renilla fluorescent activity was used as the internal reference, and the procedures were carried out according to the instructions of the dual luciferase reporter kit.

**Statistical analysis**

All data were processed with SPSS18.0 statistical software package. The data were expressed as mean
values with standard deviation (±SD), and the data were tested by one-way analysis of variance (ANOVA). The LSD and SNK were used to analyze the variance. When the variance was different, Tamhane’s T2 or Dunnett’s T3 multiple comparison tests were used. 

\( P < 0.05 \) was considered as statistically significant.

### Results and discussion

#### Expression of IL-6 mRNA in samples

The mRNA expression in the samples was detected by qRT-PCR. Comparing with the controls, the patients with IP showed up-regulated IL-6 mRNA expression in the lung macrophages (Figure 1(A)), blood mononuclear cells (Figure 1(B)) and serum samples (Figure 1(C)), and the increases were of statistical significance \( P < 0.05 \). This disparity between the control group and the IP patient group suggests that IL-6 could play a regulatory role in the development of IP.

#### IL-6 protein expression in lung macrophages and blood mononuclear cells

The IL-6 protein expression in lung macrophages and blood mononuclear cells was detected by Western Blotting technique. Compared with the control group, the patients with IP showed up-regulated IL-6 protein expressions in the lung macrophages (Figure 2(A)) and blood mononuclear cells (Figure 2(B)), and the increases were of statistical significance \( P < 0.05 \). This indicated that the changes in IL-6 protein expression in pulmonary macrophages and blood mononuclear cells were consistent with the changes in the IL-6 mRNA expression (both were upregulated). This result suggests that IL-6 may play a regulatory role in the disease, both at the transcriptional level and at the level of protein function.

#### IL-6 protein expression in serum

The protein expression of IL-6 in serum was detected by ELISA. The expression of IL-6 protein in the serum of patients with IP was higher than that of the control group, and the difference was statistically significant \( P < 0.05 \) (Figure 2(C)). This shows that the protein expression of IL-6 in the serum was also up-regulated as was the mRNA expression in patients with IP.

#### Expression of miRNA-217 in samples

The expression of miRNA-217 was detected by qRT-PCR. The expression of miRNA-217 in the samples from patients with IP was significantly lower \( P < 0.05 \) than that in the control group (Figure 3(A)-3(C)). In combination with our prediction on the relationship between IL-6 and miRNA-217, the results suggest that miRNA-217 may play a regulatory role in the pathogenesis of the disease and is likely to be mediated by the transcription of its target gene IL-6.

#### Double luciferase reporter gene test

The binding sequence of miR-217 with mutant or wild-type 3’-UTR of IL-6 is shown in Figure 4(A). The results from the double luciferase reporter assay showed that the fluorescence values significantly decreased after co-transfection with miR-217 mimics and pMIR-REPORT luciferase reporter plasmids \( P < 0.05 \), while the fluorescence values of the mutant group were not statistically different \( P > 0.05 \) (Figure 4(B)). This result indicates that miRNA-217 could bind to the 3’-UTR region of IL-6 mRNA and regulate its expression.

#### Comparative analysis

In this study, we observed the expression of miRNA-217 in lung macrophages, blood mononuclear cells
and serum in patients with IP and also observed the mRNA and protein expression of the downstream target gene IL-6 of miRNA-217 in the specimens. The regulation mechanism of miRNA-217 in IP through the downstream gene IL-6 was discussed. To the best of our knowledge, this is the first study to report the role and mechanism of miRNA-217 in IP.

The most prominent difficulty of IP prevention and treatment lies in the difficulty of early diagnosis—the lack of disease-specific diagnostic markers. It is urgent...
to study the specific biological markers of IP and establish a new rapid, sensitive and efficient method for IP diagnosis and identification. Because the development of IP is often very closely related to the immune response, immune-related factors, whether as biomarkers or treatment targets, have been important areas of IP research [17].

Monocyte-macrophages are derived from bone marrow stem cells: the pre-mononuclear cells differentiate into mononuclear cells, and then enter into the blood and tissues, where they undergo morphological changes and transform into macrophages, playing an important component of the immune system. Exogenous pathogens could be cleared by mononuclear-macrophage directly [18]. The immune cell involves cells of macrophages, T lymph cells and NK cells [19]. Pathogens cause a wide range of activation of mononuclear macrophages in vivo that leads to increases of TNF-α and IL-6 in blood [20]. IL-6 is a widely studied inflammatory cytokine. IL-6 in inflammation can induce the production of C-reactive protein and fibrinogen and the development of thrombosis [21]. Excessive IL-6 binding to IL-6 receptors can cause inflammation such as rheumatoid arthritis and Crohn’s disease [22]. For rheumatoid arthritis, IL-6 can stimulate T lymphocytes, B lymphocytes and other inflammatory mediators and can promote the differentiation and maturation of B lymphocytes, hence increasing the effect of IL-1β and TNF-α. In inflammatory responses, IL-6 has a chemotactic effect on other inflammatory cells, such as neutrophils, mononuclear macrophages [23], suggesting that IL-6 plays an important role in inflammatory responses. In this study, we also found that IL-6 mRNA and protein expression were up-regulated in lung macrophages, monocytes and serum in patients with IP, which was consistent with the early lung injury and inflammation characteristics. IL-6 plays an important role in the inflammatory signalling pathway and may serve as a key to the diagnosis and treatment of IP.

The miRNAs are a class of non-coding small RNA molecules (length range 18–22 nt) that are prevalent in eukaryotes and regulate protein expression at the mRNA level [24–26]. As one type of miRNA, miRNA-217 plays an important role in inflammation and fibrosis [14,27]. The expression of IL-6 can be regulated by miRNAs. For example, it has been reported that miRNA-365 has a negative regulatory effect on IL-6 expression in HEK293 and HELA cells [12]. Thus, we suppose that miRNA-217 may be an upstream miRNA that regulates IL-6 in IP. The results of our experiments are consistent with this hypothesis: first, miR-217 down-regulation and IL-6 up-regulation in lung macrophages and blood mononuclear cells of patients with IP were found, indicating that the immune system may be regulated by down-regulation of miR-217, hence increasing the expression of IL-6 and inducing the immune response. In the serum, miR-217 was also down-regulated and IL-6 was up-regulated, suggesting that the levels of miR-217 and IL-6 observed in the serum may also reflect the inflammatory response of lung injury and tissue damage in patients with IP. We also found that in the double luciferase reporter assay, the overexpression of miR-217 reduced the fluorescence intensity of IL-6 luciferase reporter plasmids, confirming that IL-6 is a direct target gene for miR-217.

Conclusions

The results from this study suggest that, in the lung injury of patients with IP, decreased expression of miR-217 in lung macrophages and blood may up-regulate the targeted IL-6 expression. Thus, miR-217 may play an important role in the occurrence and development of IP through regulating IL-6. miRNA-217 may serve as a diagnostic marker or prognosis marker for IP. Further studies are warranted to verify this.

Disclosure statement

No potential conflict of interest was reported by the authors.

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