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Expression and significance of miR-223 in rats with pulmonary fibrosis

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Abstract. – OBJECTIVE: To explore the expression and significance of miR-223 in mice with pulmonary fibrosis.

MATERIALS AND METHODS: The rats were separated into a control group (n=15), a sham operation group (n=15), and a model group (n=45) (which was then divided into a 3-day group, a 7-day group, and a 14-day group, with 15 rats in each group). The rat model of pulmonary fibrosis was established. The rats in the model group were injected with bleomycin solution, while those in the control group and sham operation group were given the same operation and injected with the same amount of normal saline. After observing the pulmonary function indexes of the rats on the 3rd, 7th and 14th days after modeling, the rats were sacrificed by cervical dislocation, the pulmonary inflammation and fibrosis of the rats were observed, and the HYP (hydroxyproline) content and miR-223 expression level were determined. Pearson correlation analysis was employed to analyze the correlation between miR-223 and HYP.

RESULTS: The pulmonary inflammation score of the model group was significantly higher than that of the sham group and the control group, and the pulmonary inflammation of the model group significantly increased with the increase of time ($p<0.05$). The pulmonary fibrosis score in the model group was markedly higher than that in the rest two groups, and the pulmonary fibrosis in the model group elevated significantly with the passage of time ($p<0.05$). The relevant pulmonary function indexes of the model group rats were significantly lower than those of the other two groups, and the pulmonary function of the model group rats gradually decreased with time ($p<0.05$). As to the HYP, it presented notably higher content in the model group than in the remaining two groups, and its content in the model group rats increased significantly with time ($p<0.05$). The expression of miR-223 decreased with the increase of fibrosis ($p<0.05$), and the expression level of miR-223 was negatively correlated with the HYP content ($p<0.05$).

CONCLUSIONS: MiR-572 targeted CDH1 to promote cell metastasis in WT by suppressing EMT.

Key Words:
Pulmonary fibrosis, MiR-223, HYP, Rats.

Introduction

Pulmonary fibrosis is a chronic disease characterized by progressive destruction of pulmonary function and limited mobility, and its pathogenesis and effective treatment remain poorly understood[1]. Fibrosis can be caused by chronic stimuli, but most cases are classified as idiopathic and potentially life-threatening[2]. Idiopathic pulmonary fibrosis is a fatal progressive fibrosis interstitial pneumonia, which often gives rise to dyspnea and decreased pulmonary function[3,4]. Its prognosis is worse than many cancers, with a five-year survival rate of 20% to 40%[5] and a median survival of only 2 to 5 years[6].

MiRNAs are small non-coding regions of RNA with 20-22 nucleotides[7], which play a crucial role in almost every cellular process, especially in determining cell fate in the development and regulation of cell cycle[8]. miRNA has been shown[9] to be closely related to human diseases. MiR-223, a member of the miRNA family, is abnormally expressed in a variety of diseases, such as hepatocellular carcinoma, where the expression level of miR-223 is significantly reduced[10]. Neudecker et al[11] reported that miR-223 can be used as a key inhibitor to control the activity of NLRP3 inflammasome and regulate the inflammatory state of the intestine. Still, the upregulation of miR-223 is related to the degree of liver injury and liver

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cell death, and that miR-223 is expected to be a diagnostic marker for liver injury[12]. Hydroxyproline (HYP) is the most abundant post-translational modified amino acid in the human proteome, and its main component is collagen[13]. There are data confirming that the HYP assay can be used as a reliable method for quantitative fibrosis, it is related to histological data, and it is helpful for preclinical evaluation of potential treatments for pulmonary fibrosis[14]. In this study we found that miR-223 is abnormally expressed in pulmonary fibrosis, so this study was to explore the expression and significance of miR-223 in pulmonary fibrosis by establishing a rat model of pulmonary fibrosis.

Materials and Methods

Materials and Reagents

Seventy-five male SPF rats with a weight of 30 g-45 g (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), bleomycin hydrochloride for injection (Hisun Pharmaceutical Co., Ltd., Zhejiang, China, State Drug Approval Document Number: H20055883), centrifuge (ZhenNuo Biological Technology Co., Ltd., Shanghai, China), ELISA kit (Gelatins Biological Reagent Co., Ltd., Jiangxi, China), sterile injection needle (Aladdin Biochemical Technology Co., Ltd., Shanghai, China), HE stain (Fanke Biotechnology Co., Ltd., Shanghai, China), light microscope (Sanger Biotechnology Co., Ltd., Shanghai, China), quantitative Real Time-PCR instrument (Huafeng Biological Technology Co., Ltd., Guangzhou, China), TRIzol reagent (Biolab Technology Co., Ltd., Beijing, China), UV spectrophotometer (Clinx Science Instrument Co., Ltd., Shanghai, China), reverse transcriptase (Kanglang Biological Technology Co., Ltd., Shanghai, China), primer synthesis of miR-223 and internal reference (Taihe Biotechnology Co., Ltd., Beijing, China). This study was approved by Local Animal Ethics Committee.

Establishment of Model Rats

The rats were divided into a control group (n=15), a sham operation group (n=15), and a model group (n=45) (which was then divided into a 3-day group, a 7-day group, and a 14-day group, with 15 rats in each group). The rats in the model group were subjected to a transverse incision in the middle of the neck, and the fascia and muscles were longitudinally separated to expose the trachea. Sterile injection needles were used to penetrate into the space between the tracheal cartilage rings and 0.1 ml bleomycin solution (5 mg/kg) was injected. While rats in the control group and the sham group were given the same surgical procedure and injected with the same amount of saline. After modeling, they were back to normal feeding again.

Treatment and Sampling of Rats

The rats were sacrificed after the pulmonary function indexes on the 3rd, 7th, and 14th days after modeling were observed. Rat lung tissues were removed under sterile conditions, and the right tissue samples were preserved at -70°C for the detection of HYP (hydroxyproline content) and miR-223 expression. While the left lung tissues were fixed, dehydrated, and paraffin embedded.

Observation of Pulmonary Inflammation and Fibrosis

The histopathological changes of lung tissue were observed by HE staining and blind selection by pathologists. Referring to relevant methods[15], pulmonary inflammation and fibrosis were scored. 0 points: no inflammation; 1 point: mild inflammation, with inflammation range less than 20% and alveolar structure intact; 2 points: moderate inflammation, with a range from 20 to 50%; 3 points: severe inflammation, with a range greater than 50%. Fibrosis score: 0 points: no fibrosis; 1 point: mild fibrosis, with fibrosis range less than 20%; 2 points: moderate fibrosis, with fibrosis range of 20-50%; 3 points: severe fibrosis, with fibrosis range greater than 50%.

HYP Content Determination

Some spare lung tissue was put into a tube, dissolved with 0.1 ml of water, and mixed well. After heating in a water bath in boiling water for 20 min, the pH value in the tube was adjusted to 6.0-6.8. Then, an appropriate amount of activated carbon was added and mixed, and centrifuged at 3500 r/min for 10 min. After that, 0.6 ml of the obtained supernatant was added to the blank tube, the standard tube, and the measuring tube, and was then mixed. The solution was then immersed in water bath at 60°C for 15 min and centrifuged at 3500 r/min for 10 min. Finally, the supernatant was taken to measure the absorbance of each tube under 0.5 cm light path and the HYP content was calculated according to the formula. HYP content = (measured OD value-blank OD value) / (standard OD value-blank OD value) × standard content (5 μg/mL) × total hydrolysis sample volume / sample volume (mL).

Table I. Primer sequences.

	Upstream primer (5'-3')	Downstream primer (5'-3')
MiR-223	CAGAAAGCCCAATTCCATCT	GGGCAAATGGATACCATACC
GAPDH	ATGTTCGTCATGGGTGTGAA	GGTGCTAAGCAGTTGGTGGT

qPCR Detection of MiR-223 Expression Level

The total RNA in the sample was extracted by TRIzol strictly according to the operation instructions. The concentration and purity of total RNA were detected by ultraviolet spectrophotometer. RNAs with OD260/OD280 ratio between 1.8 and 2.0 were selected, and then, cRNA was synthesized by reverse transcriptase and oligonucleotides, according to the operation instructions. We did the reverse transcription reaction system (20 μL) as follows: buffer solution (4 μL), reverse transcriptase (2 μL), total RNA (2 μL), de-RNA enzyme water (12 μL); reaction conditions (42°C water bath for 1 h, 95°C water bath for 5 min). Amplification reaction was carried out by a PCR instrument with GADPH as the internal reference, and the expression level of miR-223 was detected by fluorescence quantitative PCR with miR-223 specific primers according to the operation instructions (Table I). We did the PCR reaction system (20 μL) as follows: upstream primer (0.4 μL), downstream primer (0.4 μL), Taq DNA Polymerase (0.5 μL), and ddH2O was added to make up to the reaction volume. The reaction conditions were: pre-denaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 30 s, 40 cycles in total. Three replicates were set for each experiment, and the experiment was repeated three times. The experimental results were analyzed using a relative quantitative method, and the relative expression of miR-223 was calculated by $2^{-\Delta CT}$.

Outcome Measures

Pulmonary function indexes of each group were observed, including total lung capacity (TLC) and carbon monoxide diffusion capacity (DLco). The Pearson correlation analysis was adopted to analyze the correlation between miR-223 and HYP.

Statistical Analysis

Difference verification was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). The measurement data, which was expressed as mean ± standard deviation (x±SD), were verified by the t-test. Repeated measurement of variance analysis was performed among multiple groups, and LSD-t was used to validate ANOVA. $p<0.05$ was considered to be statistically significant.

Results

Pulmonary Inflammation Scores of Rats in Each Group

The pulmonary inflammation scores in the sham operation group, the control group, and the model group were (0.13±0.08) points, (0.14±0.07) points, and (2.46±0.33) points, respectively. Overall, the pulmonary inflammation scores in the model group were significantly higher than those in the sham operation group and the control group ($p<0.05$). The inflammation scores of the model group rats on the 3rd, 7th, and 14th days were (1.57±0.24) points, (1.98±0.28) points, and (2.27±0.31) points, respectively. The results at different times exhibited that the pulmonary inflammation of rats in the model group also increased significantly with time ($p<0.05$; Figure 1).

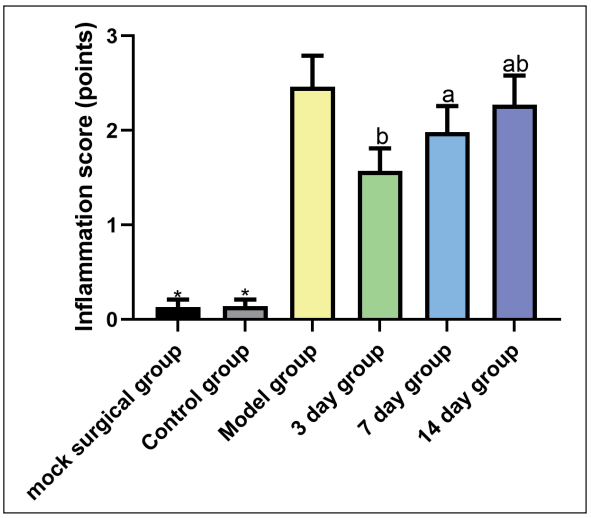

Figure 1. Pulmonary inflammation scores of rats in each group. The pulmonary inflammation score in the model group was significantly higher than that in the sham group and the control group ($p<0.05$). The results at different times showed that the pulmonary inflammation of rats in the model group increased significantly with the increase of time ($p<0.05$). Note: * indicates $p<0.05$ compared with the model group; comparison in the model group at different times: a indicates $p<0.05$ compared with the 3-day group; b indicates $p<0.05$ compared with the 7-day group.

Pulmonary Fibrosis Scores of Rats in Each Group

The scores of pulmonary fibrosis in the sham operation group, the control group, and the model group were (0.24±0.12), (0.23±0.12), and (2.55±1.32), respectively. In general, the pulmonary fibrosis score of the model group was remarkably higher than that of the sham group and the control group (p<0.05). The pulmonary fibrosis scores of the model group on day 3, 7, and 14 were (1.33±0.54), (2.04±0.68), and (2.57±0.89), respectively, indicating that pulmonary fibrosis in the model group increased significantly with time (p<0.05; Figure 2).

Changes of Pulmonary Function in Rats in Each Group

The TCL indexes of rats in the sham group, the control group and the model group were (83.58±9.24)%, (83.47±9.25)% and (63.56±7.24)%, respectively. While the DLco indexes of rats in the sham group, the control group, and the model group were (86.65±9.54)%, (86.56±9.55)% and (67.24±7.36)%, respectively. The above data revealed that the pulmonary function indexes in the model group were significantly lower than those in the rest two groups (p<0.05). The TCL indexes of the rats in the model group on day 3, 7, and 14 were (78.76±8.25)%, (65.43±7.21)% and (59.89±6.35)%, respectively, and the DLco indexes of the rats in the model group on day 3, 7, and 14 were (80.52±8.67)%, (73.26±7.64)%, and (62.68±6.48)%, respectively. The results at different times showed that the pulmonary function of rats in the model group decreased gradually with the increase of time (p<0.05; Figures 3 and 4).

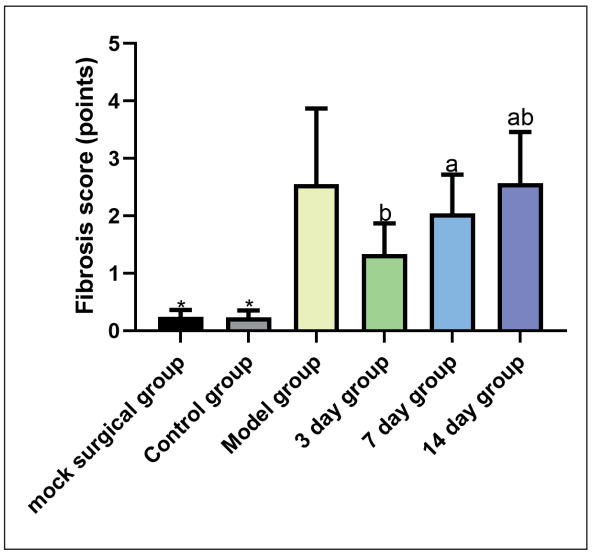

Figure 2. Pulmonary fibrosis scores of rats in each group. The pulmonary fibrosis score in the model group was significantly higher than that in the sham group and the control group (p<0.05). The results at different times exhibited that the pulmonary fibrosis in rats in the model group elevated significantly with time (p<0.05). Note: * indicates p<0.05 compared with the model group; comparison in the model group at different times: a indicates p<0.05 compared with the 3-day group; b indicates p<0.05 compared with the 7-day group.

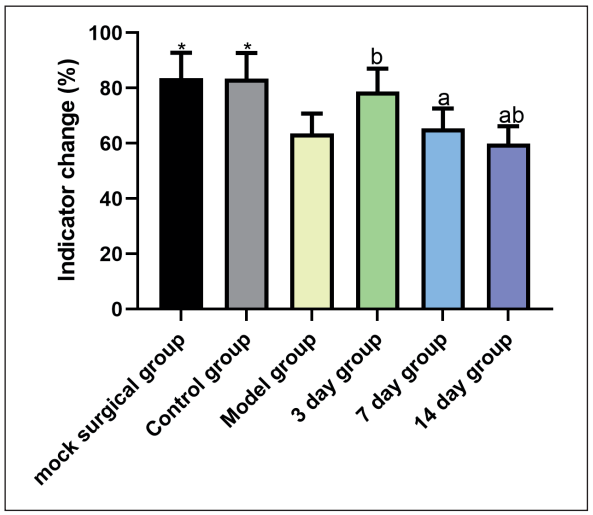

Figure 3. Changes of TCL index of rats in each group. The TCL index in the model group was notably lower than that in the sham group and the control group (p<0.05). The results at different times showed that the TCL index of rats in the model group declined significantly with the increase of time (p<0.05). Note: * indicates p<0.05 compared with the model group; comparison in the model group at different times: a indicates p<0.05 compared with the 3-day group; b indicates p<0.05 compared with the 7-day group.

HYP Content in Each Group

The HYP content in the sham operation group, the control group, and the model group was (0.42±0.04) μg/mg, (0.41±0.04) μg/mg, and (0.58±0.08) μg/mg, respectively. Overall, the HYP content in the model group was significantly higher than that in the sham operation group and the control group (p<0.05). The HYP content of rats in the model group on the 3rd, 7th, and 14th days was (0.42±0.03) μg/mg, (0.47±0.06) μg/mg, (0.55±0.07) μg/mg, indicating that the HYP content in the model group dramatically increased with time (p<0.05; Figure 5).

Expression Level of MiR-223 In Each Group

The expression levels of miR-223 in the sham group, the control group, and the model group were (1.24±0.26), (1.25±0.25), and (0.47±0.08), respectively. In general, the expression level of

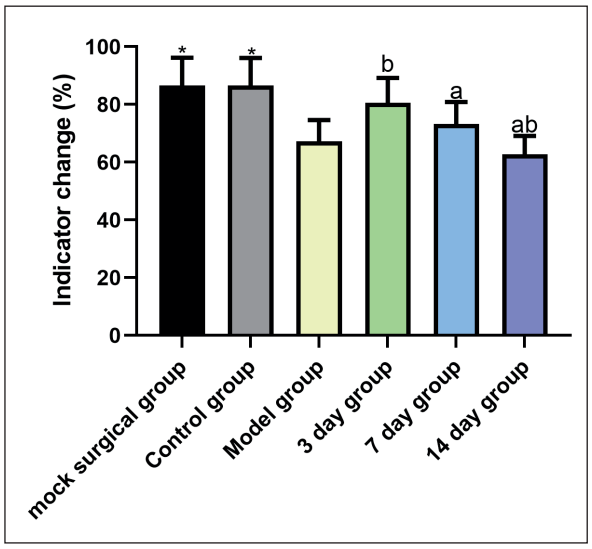

Figure 4. Changes of TCL index of rats in each group. The DLco index in the model group was notably lower than that in the sham group and the control group (p<0.05). The results at different times showed that the DLco index of rats in the model group reduced significantly with the increase of time (p<0.05). Note: * indicates p<0.05 compared with the model group; comparison in the model group at different times: a indicates p<0.05 compared with the 3-day group; b indicates p<0.05 compared with the 7-day group.

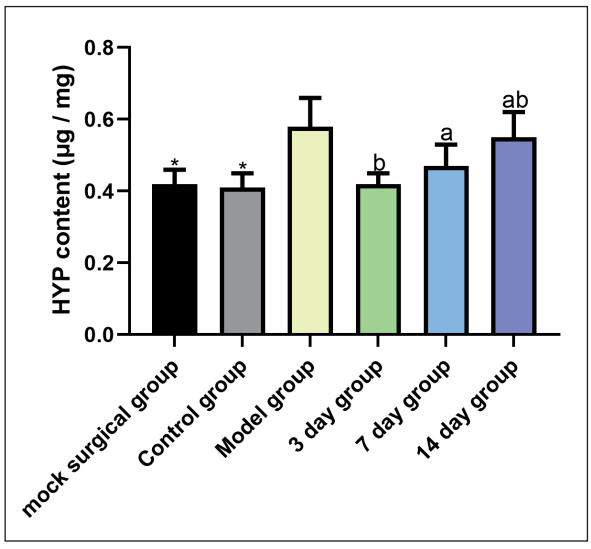

Figure 5. HYP content of rats in each group. The HYP content in the model group was noticeably higher than that in the sham group and the control group (p<0.05). The results at different times indicated that the HYP content of rats in the model group raised significantly with the passage of time (p<0.05). Note: * indicates p<0.05 compared with the model group; comparison in the model group at different times: a indicates p<0.05 compared with the 3-day group; b indicates p<0.05 compared with the 7-day group.

miR-223 in the model group was significantly lower than that in the other two groups (p<0.05). The expression levels of miR-223 in the model group on day 3, 7, and 14 were (1.06±0.23), (0.67±0.18) and (0.45±0.06), respectively, which indicated that the miR-223 expression level in the model group also decreased significantly with the passage of time (p<0.05; Figure 6).

Correlation Between MiR-223 and HYP

Pearson correlation analysis demonstrated that miR-223 was negatively correlated with HYP (r=-0.641, p<0.05; Figure 7).

Discussion

HYP is an amino acid with antioxidant properties[16], and it has been shown that abnormalities in HYP metabolism play a key role in the pathophysiology and pathogenesis of different diseases[17]. HYP is a key factor in stabilizing collagen, but its instability or abnormal activity can lead to stomach and lung diseases[18]. Pulmonary fibrosis is a progressive and fatal lung disease with histopathological characteristics, including excessive extracellular matrix deposition,

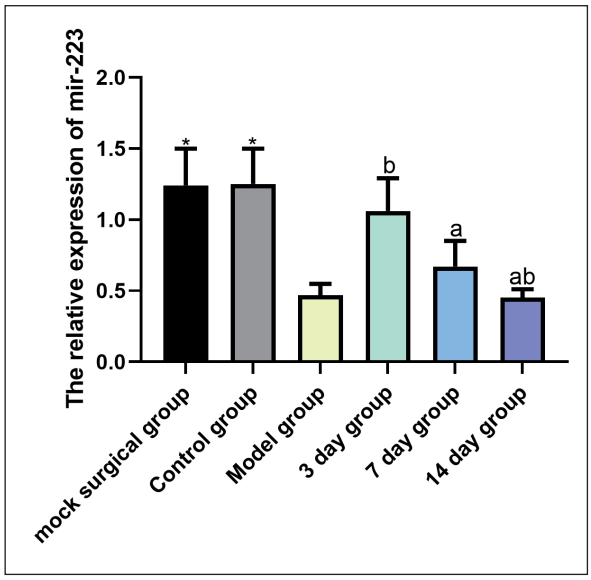

Figure 6. Expression level of miR-223 of rats in each group. The expression level of miR-223 in the model group was notably lower than that in the sham group and the control group (p<0.05). The results at different times showed that the miR-223 expression of rats in the model group dropped significantly with the increase of time (p<0.05). Note: * indicates p<0.05 compared with the model group; comparison in the model group at different times: a indicates p<0.05 compared with the 3-day group; b indicates p<0.05 compared with the 7-day group.

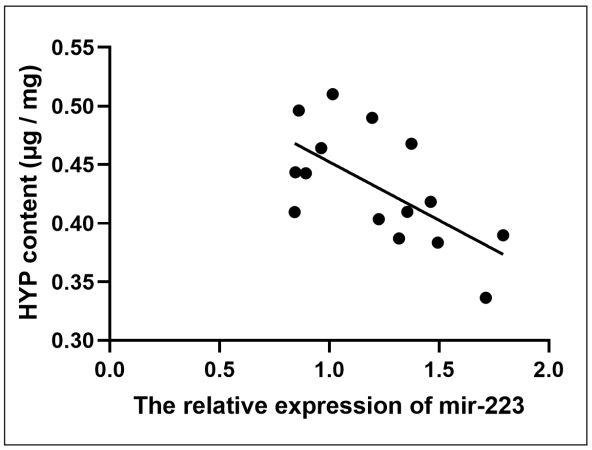

Figure 7. Correlation between miR-223 and HYP. Pearson correlation analysis demonstrated that miR-223 was negatively correlated with HYP (r=-0.641, $p<0.05$).

flaky chronic interstitial inflammation, fibroblast proliferation and alveolar collapse, which leads to progressive fibrosis and loss of pulmonary function, while amplified hydroxyproline levels are associated with collagen accumulated in alveolar space and reflect harmful changes related to fibrosis[19]. In this study, we found that the HYP presented markedly higher content in rats in the model group than normal rats, and its content raised with time in the model group. In bleomycin-induced pulmonary fibrosis, lung injury is known to be accompanied by inflammation and subsequent fibrosis[20]. Therefore, we also noticed that the pulmonary inflammation and fibrosis of the rats in the model group became more and more serious with the increase of time, and the reason behind it, as we speculated, may be the activation of HYP. Ren et al[21] showed that HYP is an important biomarker for idiopathic pulmonary fibrosis. As a member of the miRNA family that is functionally related to the occurrence and progression of various complex human diseases[22], miR-223 plays a vital role in mesenchymal stem cell-induced myocardial protection in sepsis[23]. In pancreatic cancer chemotherapy, the downregulation of miR-223 can reverse the phenotype of epithelial-mesenchymal transformation and inhibit the migration and invasion of gemcitabine-resistant cells; moreover, it is known[24] that miR-223 may be a new therapeutic target for reversing the chemotherapy resistance of PC. Cantoni et al[25] have also shown that miR-223 is effective in inhibiting cell biology in the regulation of autoimmune encephalomyelitis and multiple sclerosis, suggesting its potential new therapeutic role. MiR-223 is not only abnormally expressed in diabetes mellitus, sepsis, and other diseases, but also affects the induction of hematopoietic and inflammatory diseases, indicating that miR-223 can be a prognostic marker for the diagnosis and treatment of immune diseases[26]. Still, Ray et al[27] have pointed out that, compared with normal liver specimens, miR-223 expression in hepatocellular carcinoma liver biopsy specimens is lower, and may be bound up with the growth and migration of liver cancer cells. In the experimental model of autoimmune hepatitis, exosomes derived from bone marrow stem cells can protect liver injury, but the mechanism may be related to the regulation of NLRP3 and caspase-1 by exocrine miR-223[28]. Therefore, miR-223 plays an important role in various diseases, so in this study, we also explored the possible role of miR-223 in pulmonary fibrosis. We found that the expression of miR-223 in rats in the model group was significantly lower than that in normal rats, and with the gradual increase of time, the expression of miR-223 gradually decreased, suggesting that the expression of miR-223 in pulmonary fibrosis was reduced, and may be related to the increase of the degree of pulmonary fibrosis. MiR-223-deficient mice are prone to lung injury, and the overexpression of miR-223 can transfer from neutrophils to alveoli[29], which can help reduce pulmonary inflammation in mice and promote the remission of ventilator induced lung injury. Yuan et al[30] have found that therapeutic enhancement of miR-223 can inhibit inflammatory targets and is also considered as a potential treatment for controlling excessive innate immune response during mucosal inflammation. We speculated that the continuous decrease in miR-223 expression may be related to the increase in the severity of inflammation and fibrosis. In this study, we also made a correlation between miR-223 and HYP, and the results showed that miR-223 and HYP were negative related. Further, we hypothesized that the lack of miR-223 might be related to the initiation of HYP activity, but there are no relevant studies and the mechanism remains yet unclear at present.

In this study, we still have some shortcomings; for example, we have not explored the relationship between miR-223 and multiple related fibrosis proteins. In future studies, we will continue to explore the relevant mechanisms between miR-223 and HYP.

Conclusions

To sum up, the expression of miR-223 in rats with pulmonary fibrosis keeps decreasing and may be related to the continuous aggravation of pulmonary inflammation and fibrosis in rats. In one word, miR-223 may be used as a potential diagnostic criterion of pulmonary fibrosis.

Fund

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Conflict of Interests

The authors declare that they have no conflict of interests.

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