MicroRNA-223 affects IL-6 secretion in mast cells via the IGF1R/PI3K signaling pathway

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Abstract. In this study, we aimed to assess the effects of microRNA-223 (miR-223) on interleukin-6 (IL-6) secretion in mast cells and determine the underlying molecular mechanisms. Mast cells (P815) were transfected with miR-223 lentiviral vector and miR-223 inhibitor. miR-223 expression was then evaluated using reverse transcription-quantitative PCR (RT-qPCR). IL-6 levels in the supernatant were analyzed using enzyme-linked immunosorbent assay. The signaling pathways in mast cells with downregulated miR-223 were initially evaluated by gene chip. Downregulation of miR-223 and its target gene was tested using a luciferase reporter assay. The expression of phosphate-AKT (p-AKT) and its target protein insulin-like growth factor-1 receptor (IGF1R) was assessed by western blot analysis. Phosphatidylinositol 3-kinase (PI3K)-inhibitor (LY294002) and insulin-like growth factor-1 (IGF1) were used to determine the effect of miR-223 on IL-6 secretion in mast cells. The results showed that microRNA-223 reduced IL-6 concentration in the mast cells. The gene chip results predicted an induction of the PI3K-AKT signaling pathway in the mast cells. Luciferase reporter assay confirmed *IGF1R* gene to be a target of miR-223. The p-AKT and IGF1R levels increased following miR-223 downregulation in mast cells. In addition, the specific PI3K-inhibitor LY294002 decreased IL-6 secretion. Incubation with IGF1 resulted in the induction of IL-6 secretion in miR-223-expressing mast cells. In conclusion, it was shown that miR-223 reduces IL-6 secretion in mast cells by inhibiting the IGF1R/PI3K signaling pathway.

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Introduction

Allergic inflammation is accompanied by the coordinated expression of a number of genes and proteins that initiate, sustain, and propagate immune responses and tissue remodeling (1). Mast cells are immune cells of hematopoietic origin that contribute to host defense mechanisms through the receptor-mediated release of inflammatory mediators (2), as well as the inflammatory reactions associated with allergic disorders such as asthma and anaphylaxis (3,4). IgE-mediated degranulation and cytokine production by mast cells were shown to be reduced by piperine through the inhibition of Lyn, p38, extracellular signal-regulated kinase (ERK), and Ras phosphorylation (5). FcεRI-MCs release TNF-α and interleukin-6 (IL-6), which trigger anaphylaxis and mediate the symptoms and tissue effects of chronic atopic disorders (6). Mast cells were shown to be increased in the asthmatic airways, followed by IL-6 release (7).

IL-6 is a pleiotropic cytokine involved in the regulation of inflammatory and immunological responses, acute phase protein production, and hematopoiesis (8). Serum sIL-6R levels are increased in asthma patients (9), indicating the likely involvement of IL-6 in the pathogenesis of allergic inflammation. Pre-incubation of mast cells with IL-6 can significantly upregulate the IgE-mediated histamine release (10). Such observations indicated that IL-6 has a close relationship with mast cell-mediated inflammation. Regulation of inflammatory responses is ensured by coordinated control of the gene expression in participating immune system and tissue cells. A class of short single-stranded RNA molecules termed microRNAs (miRNAs or miRs) have been demonstrated to be involved in the regulation of inflammatory responses (11).

miRNAs constitute a large family of small non-coding RNAs that have emerged as key post-transcriptional regulators in a wide variety of organisms (12). Previous findings demonstrated the associations of miRNAs with many types of inflammatory state. Upregulation of miRNA-221 has been detected in lung biopsy specimens in an OVA-induced murine asthma model, whereas the inhibition of miR-221 reduced airway inflammation (13).

miR-223 was first identified bioinformatically and subsequently characterized in the hematopoietic system, where it is specifically expressed in the myeloid compartment (14,15). miR-223 is fairly specific to the hematopoietic lineage, where

it limits inflammation and prevents collateral damage during infection (16). Previous studies have reported that miR-223 can regulate neutrophil activity and inflammation (17). The downregulation of miR-223 was shown to promote IL-6 and IL-1β expression in macrophages (18). It is clear that miR-223 is important in inhibiting the development of inflammatory cells; however, miR-223 function in mast cells remains unclear. The present study aimed to examine the relationship between miR-223 and inflammation in mast cells, and determine the underlying molecular mechanisms.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). LY294002 and Cell Counting Kit-8 (CCK-8) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The mouse IL-6 immunoassay kit was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Specific monoclonal antibodies against AKT (#9272) and phosphorylated-AKT (p-AKT; #4060) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Specific polyclonal antibodies against insulin-like growth factor-1 receptor (IGF1R; sc-713) and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The secondary antibody, goat anti-rabbit IgG-HRP (sc-2004) was also obtained from Santa Cruz Biotechnology, Inc.

Cell culture and transfection. Mast cells purchased from the Shanghai Institute of Cell Library (Shanghai, China) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37˚C in 5% CO_2. The cells were transfected with miR-223 lentiviral vector or miR-223 inhibitor using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) followed by 48 h of culture. Supernatants and cells were then rinsed with ice-cold PBS and cells were collected for subsequent analysis.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from mast cells using TRIzol reagent (Invitrogen Life Technologies). Total RNA (200 ng) from each specimen was used for primer-specific reverse transcription (RT). RT was performed using the TaqMan MicroRNA Reverse Transcription kit, according to the manufacturer's instructions (Applied Biosystems Life Technologies, Foster City, CA, USA). The cDNA samples were used for quantitative PCR, according the TaqMan MicroRNA assay kit manufacturer instructions (Applied Biosystems Life Technologies). Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA). IL-6 amounts were measured using ELISA kits from R&D Systems, Inc., according to the manufacturer's instructions. IL-6 levels were expressed as a ratio of IL-6 amounts to cell number.

Microarray analysis. To identify the possible changes in the signaling pathways following miR-223 expression, the cells were divided into the blank control and miR-223 knockdown groups and subjected to microarray analysis (Kangchen Bio-tech Inc., Shanghai, China), as previously described (19).

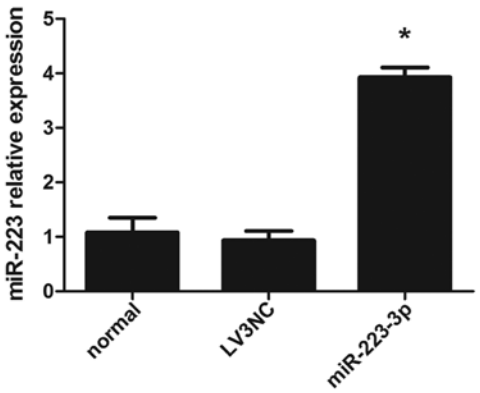

Figure 1. Expression of microRNA-223 (miR-223) in mast cells. After the transfection of miR-223 expression of lentiviral vectors in mast (P815) cells, the relative level of miR-223 was higher after transfection with lentiviral vector into mast cells, compared with the negative control and blank control group, ($p<0.05$). Data are mean ± SD (n=6) of three independent experiments. Analysis was performed using the Student's t-test, $^{*}p<0.05$.

Luciferase reporter assay. Mast cells were seeded in 24-well tissue culture plates the day prior to transfection. miR-223-3p mimics (mimics NC as control group) and IGFR-1-3'UTR vector in luciferase reporter vector were transiently co-transfected into cells. After 48 h, the cells were lysed and luciferase activity was detected with the Dual Luciferase Reporter Assay kit (Promega Corp., Madison, WI, USA) to validate the role of miRNA target gene in inhibiting translation.

Western blotting. Total protein (50 μg) from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature (20˚C) and probed with the corresponding primary antibodies overnight at 4˚C. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature (20˚C) for 2 h. Proteins were detected using the enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA). Bands were captured by the Image Lab system (Bio-Rad) and their densities were analyzed after normalization by GAPDH levels.

Statistical analysis. Statistical analysis was performed using the SPSS software (SPSS 20.0; Chicago, IL, USA). Data were presented as mean ± standard deviation (SD) from at least three independent experiments. The Student's t-test was used to analyze the differences between two groups. $P<0.05$ was considered statistically significant.

Results

Overexpression of miR-223 in mast cells. To assess the effect of miR-223 on IL-6 expression in mast cells, the cells were transfected with miR-223 lentiviral vector and miR-223 expression was detected by RT-qPCR. As expected, miR-223 levels were higher following transfection with lentiviral vector into mast cells, compared with the negative control and blank control group (Fig. 1).

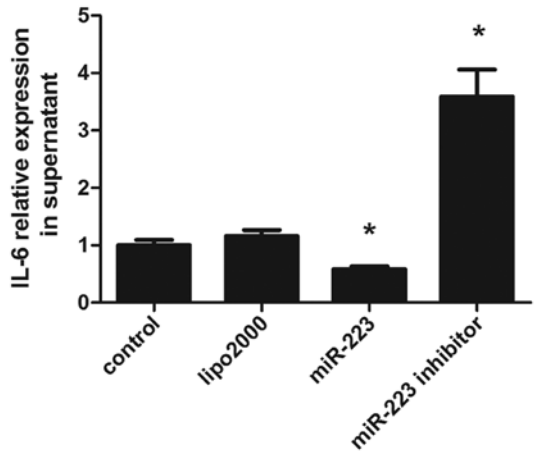

Figure 2. Effects of microRNA-223 (miR-223) on IL-6 expression. miR-223 inhibited IL-6 secretion in the miR-223 overexpression group (compared with the control group, $p<0.05$), while the downregulation of miR-223 increased IL-6 concentrations (compared with the control group, $p<0.05$) Data are mean ± SD (n=6) of three independent experiments. Analysis was performed using the Student's t-test, *$p<0.05$.

Overexpression of miR-223 inhibits IL-6 secretion. IL-6 is a multifunctional cytokine produced by a wide variety of cells and plays vital roles in immunological responses, hematopoiesis, host defense, and acute phase reaction (20). The involvement of miR-223 has been demonstrated in various types of cancer, inflammatory diseases, autoimmune diseases and other pathological processes (21). Following the transfection of miR-223 lentiviral vector into mast cells, the cells were cultured for 48 h, and the supernatants were collected for IL-6 detection by ELISA. Overexpression of miR-223 suppressed IL-6 secretion, while miR-223 downregulation resulted in increased IL-6 levels (Fig. 2).

Changes in signaling pathways in mast cells differentially expressing miR-223. After transfection of miR-223 lentiviral vector into mast cells, miR-223 markedly altered the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway as assessed by gene chip, bioinformatics analysis and pathway analysis. Conversely, this pathway was activated by miR-223 downregulation. These findings indicate that the main function of miR-223 may be associated with cytokine secretion in mast cells. According to the gene chip and pathway analysis, miR-223 regulated IL-6 secretion via IGF1R/PI3K signaling in mast cells (Fig. 3).

Luciferase reporter assay and western blot analysis confirm IGF1R as a target gene of miR-223. According to our gene chip data and bioinformatics analysis, IGF1R was a target gene of miR-223 in mast cells. To assess whether miR-223 directly affects IGF1R, luciferase reporter technology was used. As shown in Fig. 4A, IGF1R was confirmed as a target gene of miR-223 in mast cells. In addition, IGF1R levels were detected in experimental and control groups by western blotting. The results showed reduced IGF1R expression in cells overexpressing miR-223, whereas the downregulation of miR-223 produced the opposite effects (Fig. 4B).

IGF-1 promotes IL-6 secretion following the upregulation of miR-223 in mast cells. Luciferase reporter gene and western blot analysis confirmed IGF1R as a target gene of miR-223.

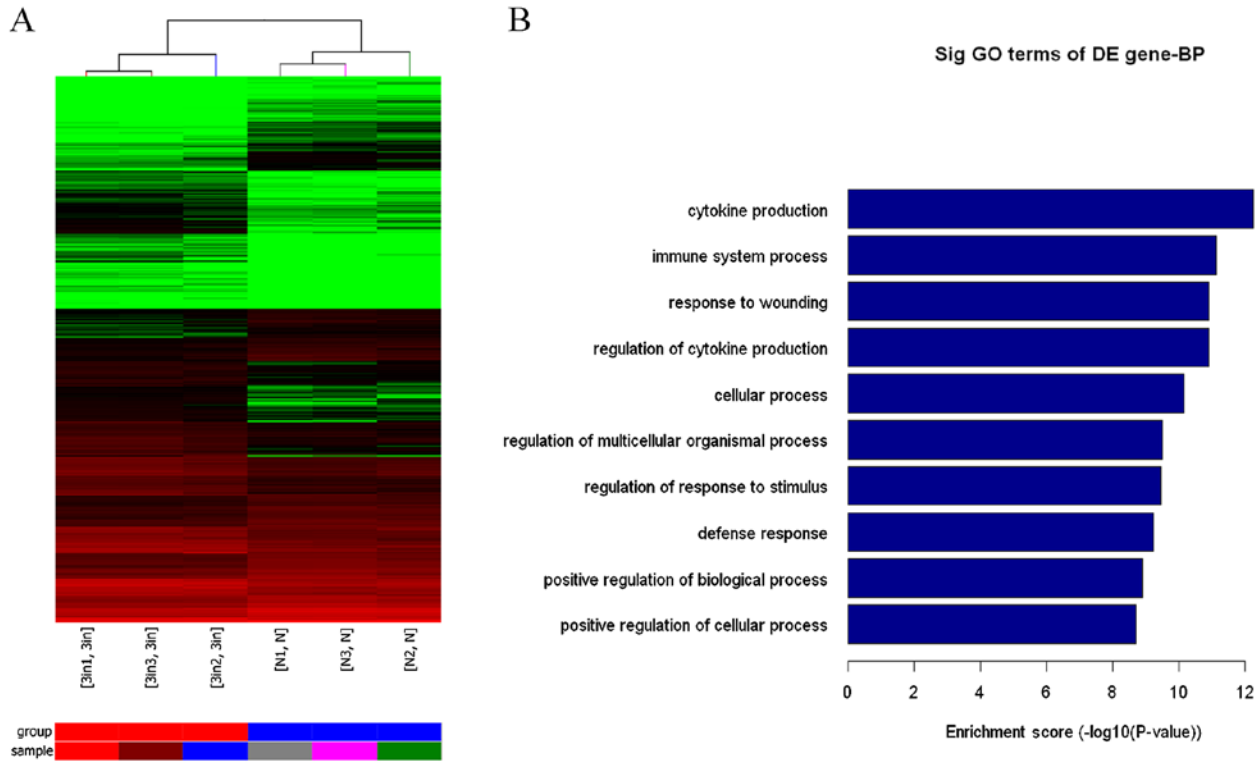

Figure 3. (A) Heat map of differentially expressed genes between microRNA-223 (miR-223) and blank control groups ($p<0.05$, n=3). (B) Gene ontology analysis showed that the main differences were associated with cytokine production ($p<0.05$, n=3).

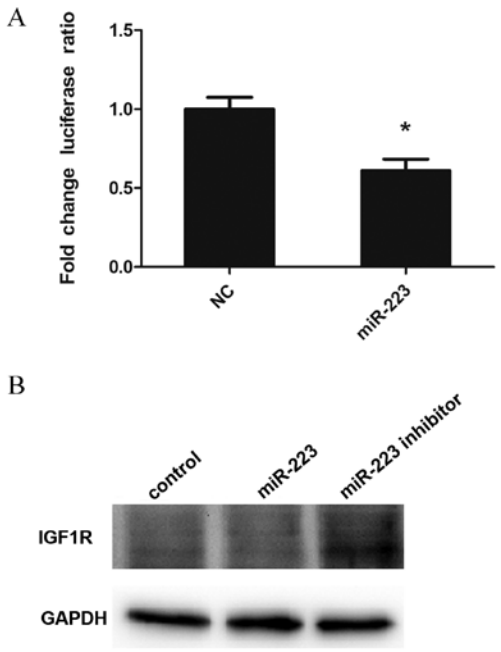

Figure 4. MicroRNA-223 (miR-223) reduces insulin-like growth factor-1 receptor (IGF1R) protein expression in mast cells. Luciferase reporter gene confirms IGF1R as a target gene of miR-223 in mast cells. (A) Compared with the control group (NC), the luciferase ratio was significantly reduced in the miR-223 group ($^*p<0.05$, n=4). (B) IGF1R protein levels in mast cells trnsfected with miR-223 lentiviral vector or miR-223 inhibitor were assessed by western blotting. Data are representative of three separate experiments.

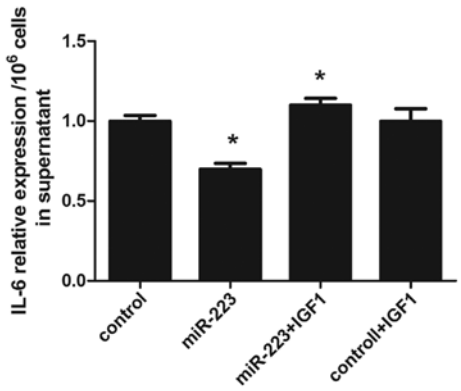

Figure 5. Effects of insulin-like growth factor-1 (IGF1) on IL-6 expression. Mast cells ($1x10^5$ cells/ml) were incubated with IGF-1 for 36 h, and IL-6 secretion in supernatants was detected. IL-6 expression was reduced in the miR-223 group (compared with the control group, $p<0.05$). When mast cells transfected with microRNA-223 (miR-223) were incubated with IGF1, IL-6 concentrations increased (compared with the miR-223 group, $p<0.05$). Data are mean ± SD (n=6) of three independent experiments. Analysis was performed using the Student's t-test, $^*p<0.05$.

Thus, mast cells were transfected with insulin-like growth factor-1 (IGF1), and IL-6 secretion was subsequently assessed using ELISA. It was found that incubation with IGF-1 reversed the IL-6 concentration decrease caused by miR-223 in mast cells (Fig. 5).

miR-223 inhibits the PI3K-AKT signaling pathway in mast cells. Since miR-223 suppressed IGF1R expression, we assessed whether the IGF1R-mediated downstream signaling

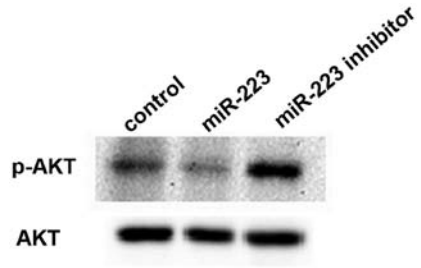

Figure 6. MicroRNA-223 (miR-223) affects the function of mast cells through the phatidylinositol 3-kinase (PI3K)-AKT signaling pathway. p-AKT protein levels in mast cells transfected with miR-223 lentiviral vector or miR-223 inhibitor as assessed by western blotting. Data are representative of three separate experiments.

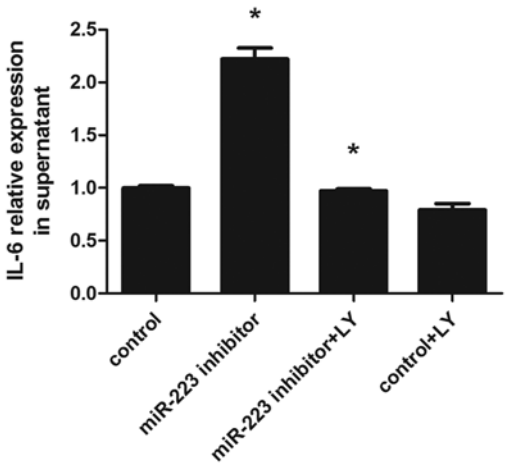

Figure 7. Effects of LY294002 on IL-6 secretion. Mast cells ($1x10^5$ cells/ml) were incubated with LY294002 (20 μM) 30 min prior to treatment with 500 ng/ml LPS for 6 h, and supernatants collected. In normal and normal+LY group, LY294002 reduces IL-6 secretion ($p<0.05$). Compared with the microRNA-223 (miR-223) inhibitor group, IL-6 expression was reduced in the miR-223 inhibitor+LY294002 group ($p<0.05$). However, IL-6 secretion in the normal and miR-223 inhibitor+LY groups showed no statistically significant difference. Data are mean ± SD (n=6) of three independent experiments. Analysis was performed using the Student's t-test, $^*p<0.05$.

pathway was also affected by miR-223. Gene chip and pathway analysis predicted that PI3K-AKT signaling was negatively regulated by miR-223 in mast cells. The expression levels of AKT, an essential protein kinase in the PI3K-AKT pathway downstream of IGF1R, and its phosphorylated form p-AKT were evaluated by western blotting. We found that total AKT protein levels were similar in the three groups. However, phosphorylated AKT protein levels were reduced after the downregulation of miR-223 and increased after the miR-223 upregulation compared with the control group (Fig. 6).

Specific PI3K-inhibitor LY294002 decreases IL-6 concentration. PI3K activity is essential for the differentiation of mast cells, as well as their long-term survival and function (22). PI3K inhibitors, including wortmannin and LY294002, have been widely reported to inhibit antigen-mediated degranulation and cytokine production in rodent and human mast cells (23-25). To investigate whether the PI3K-AKT signaling pathway plays a role in IL-6 secretion, the specific PI3K-inhibitor LY294002 was used to treat control cells and those with silenced miR-223:

cells were incubated with LY294002 for 30 min prior to treatment with LPS for 6 h. Subsequently, IL-6 secretion in the supernatants was detected by ELISA. As shown in Fig. 7, LY294002 blocked the PI3K-AKT signaling pathway and reversed IL-6 induction by suppressing miR-223 in mast cells.

Discussion

It has been demonstrated that miR-223 targets leukemia fusion protein, providing the evidence for a link between epigenetic silencing of a miRNA locus and the differentiation block of myeloid precursors (26). In the present study, miR-223 was identified as an important modulator of IL-6 secretion in mast cells. Previous studies have reported the vital role of mast cells in inflammatory reactions. MC-derived IL-10 was also shown to reduce B-cell responses and antibody production (27). miR-155 expression enhances FcεRI degranulation and the release of TNFα, IL-6, and IL-13 in relation to the activity of the PI3K/AKT pathway in mast cells (28). *Sporothrix schenckii* yeasts were shown to induce TNF-α and IL-6 release, and activate the ERK signaling pathway in mast cells (29).

In the present study, miR-223 was involved in the regulation of cytokine secretion in mast cells. The relevant role of miR-223 in the process was testified by the overexpression and knockdown experiments. Upregulation of miR-223 in mast cells resulted in reduced IL-6 secretion, while its downregulation increased IL-6 expression. From the perspective of inflammatory responses, miRNAs have recently been shown to be expressed in immune cells and to target proteins involved in inflammation regulation, consequently affecting the magnitude of the response (30). For instance, miR-146a is involved in the regulation of endothelial cell inflammation via the modulation of Nox4 expression in a diabetic atherothrombosis model (31). In addition, miR-155 was shown to contribute to regulating inflammation of allergic airway by modulating TH2 responses through the transcription factor PU.1 (32).

Based on previous studies, an increased number of molecular mechanisms of the miR-223 effect in mast cells have been revealed (33,34,37). Gene chip and bioinformatics analysis predicted IGF1R as a target gene of miR-223, and that the PI3K-AKT signaling pathway was decreased in mast cells. Subsequently IGF1R was confirmed as a miR-223 target using luciferase assay reporter. Overexpression of miR-223 in mast cells resulted in decreased IGF1R and p-AKT protein levels compared to control cells, while silencing miR-223 in mast cells increased IGF1R and p-AKT protein expression. It is now recognised that miRNAs exert their function of fine modulators of gene expression by controlling translational efficiency of a large number of target genes (35). It has been shown that miR-223 targets NLRP3, and can prevent its early translation in the myeloid lineage (36). In addition, miR-223 targets FBXW7/hCdc4 expression at the post-transcriptional level and appears to regulate cell apoptosis, proliferation, and invasion in gastric cancer (37).

The results of the present study showed that miR-223 may regulate IL-6 levels via IGF1R/PI3K signaling in mast cells. Similarly, the PI3K inhibitor LY294002 blocked AKT phosphorylation and reversed the induction of IL-6 secretion caused by miR-223 knockdown in mast cells. When mast cells were incubated with IGF1, IL-6 levels significantly increased compared with the control cells. The PI3K-AKT signaling pathway has been previously demonstrated to have a close relationship with inflammation. This association has been well studied in various tumors and inflammatory diseases. Overexpression of miR-223 during the dextran sulfate sodium (DSS)-induced mouse model of colitis-associated tumor growth inhibited AKT phosphorylation and IGF-1R expression (38). The upregulated expression of WNT5a in PCOS was shown to increase inflammation and oxidative stress predominantly via the PI3K/AKT signaling pathway (39). B-type natriuretic peptide post-conditioning also significantly inhibited a TNF-α and IL-6 level increase through the PI3K/AKT signaling pathway (40).

In conclusion, miR-223 decreased IL-6 secretion in mast cells. Additionally, IGF1R, as a target gene of miR-223, and the PI3K signaling pathway, are involved in the regulation of mast cells by miR-223.

Acknowledgements

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