miR-625-5p suppresses inflammatory responses by targeting AKT2 in human bronchial epithelial cells

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Abstract. Asthma is a common chronic inflammatory airway disease; however, whether microRNAs (miRs) could be used in the treatment of asthma remains unclear. The aim of the present study was to investigate the role of miR-625-5p in the inflammatory response of human bronchial epithelial cells (HBECs). Inflammation in the HBEC line, 16HBEC, was induced using different concentrations of lipopolysaccharide (LPS), which demonstrated that 1 µg/ml LPS was an appropriate concentration for further experiments. The association between protein kinase B2 (AKT2) and miR-625-5p was verified using a luciferase reporter assay. LPS was added to 16HBECs following the administration of miR-625-5p mimics or miR-625-5p inhibitors, and cells with silenced or overexpressed AKT2 levels, miR-625-5p was expressed at a high level in LPS-activated 16HBECs. Overexpression of miR-625-5p inhibited interleukin (IL)-6 and tumor necrosis factor (TNF)-α secretion in 16HBECs. Inhibition of miR-625-5p enhanced LPS-induced IL-6 and TNF-α secretion. miR-625-5p negatively regulated the expression of AKT2 in 16HBECs. A dual-luciferase reporter assay system confirmed that miR-625-5p directly targeted the 3'untranslated region of AKT2. Transfection with a small interfering RNA against AKT2 inhibited inhibitor of κB phosphorylation. In brief, miR-625-5p may protect LPS-induced HBECs by targeting AKT2 and inhibiting the nuclear factor-κB signaling pathway. Therefore, miR-625-5p may function as an inhibitor of asthma airway inflammation in HBECs by targeting AKT2.

Introduction

Asthma is a heterogeneous disease that involves an imbalance of airway inflammation. The airway epithelium is not only the first anatomical barrier to airborne pathogens and stimuli, but it is also capable of mounting a number of immune responses (1). When an inflammatory stimulus activates epithelial cells, a series of inflammatory cascades, including innate and adaptive immune responses, are initiated (2). These important functions of airway epithelial cells make them potential targets to treat asthma.

The protein kinase B (AKT) family of kinases (AKT1, -2 and -3) has a key role in the inflammatory response. However, it is not clear which of the AKT family, if any, is a major player in asthmatic airway inflammation. In our recent experiments, it was observed that specific variants in the 3’untranslated region (UTR) of AKT2 may influence susceptibility to asthma (unpublished data); however, the details of the involvement of AKT2 activity are unknown.

MicroRNAs (miRs) are short noncoding RNAs that pair with the 3’UTR of target mRNAs to regulate gene expression through mRNA degradation or translational repression (3). An increasing body of evidence has suggested that dysregulation of the expression of miRs is correlated with various physiological and pathological processes (4), for example, in asthma (5,6). Recent studies have demonstrated the regulation of miR gene expression in bronchial epithelial cells (BECs) (6-9). The expression levels of miR-18A, -27A, -128, -155 and -181b-5p in asthmatic BECs decreased (6), while the level of miR-19a in the epithelia of patients with severe asthma increased (7,8). A functional study demonstrated that miR-19a inhibits the proliferation of severe asthmatic BECs by targeting transforming growth factor-β receptor 2 mRNA (9). miR-181b-5p affects eosinophilic inflammation by targeting the gene encoding secreted phosphoprotein 1 (8). For other miRs, including miR-18A, -27A, -128 and -155, no consensus miR binding sites were observed in the 3’UTRs of the target genes (5).

miR-625 is a multifunctional miR that has been implicated in carcinogenesis, including colorectal adenocarcinoma (10), malignant pleural mesothelioma (11) and hepatocellular cancer (12). To date, only one study has focused on the miR-625/AKT2 axis in increasing the chemosensitivity of glioma (13). In terms of its association with asthma, miR-625-5p is significantly downregulated in pediatric asthma and targets the gene encoding estrogen receptor 1 (14). This suggested a role for this miR in the pathogenesis of allergic diseases. Therefore, the present study was designed to investigate the novel characteristics of miR-625 in the potential pathogenic mechanism of asthma. It was hypothesized that
miR-625-5p may alter the inflammatory responses in human BECs (HBECs) by targeting the AKT2 signaling pathway.

Materials and methods

Cell culture. The cell lines (16HBECs and A549 cells) were purchased from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 µg/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C in 5% CO₂.

MTT assay. An MTT assay (Beyotime Institute of Biotechnology, Shanghai, China) was used to measure cell viability. The cells were seeded in 96-well plates with 1-3x10⁴ cells in a volume of 200 µl in each well and incubated for 24 h. The cells were then incubated with different concentrations (0, 0.1, 1, 10 and 100 µg/ml) of lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) for 12 h at 37°C. The supernatant was removed from the cells and added to medium with 5 µg/ml MTT for 4 h. The medium was discarded and 100 µl solubilization solution (included in MTT assay kit) was added to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm using a Multiskan Spectrum instrument (Thermo Fisher Scientific, Inc.). The cell viability was calculated using the absorbance of the treatment group to that of the control group represented the viability of the cells.

Transfection. Cells seeded in 6-well plates at a density of 1x10⁴ cells/well were transfected with an miR-625-5p mimic (5'-AGGGGGAAGUUCAUAGUCC-3'), miR-625-5p inhibitor (5'-AGGGGGAAGUUCAUAGUCC-3'), a negative control miR (5'-UUCUCCGAACGUUGACCG-3'), and an inhibitor negative control (5'-AAGAGCUUCGCAGUGAC-3'), which were obtained from Shanghai GenePharma Co., Ltd., Shanghai, China. The ratio of the absorbance of the treatment group to that of the control group represented the viability of the cells.

Western blotting. Proteins from 16HBECs were prepared routinely using radioimmunoprecipitation lysis buffer (cat no. P0013C) and phenylmethanesulfonylfluoride (cat no: STS06) kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. A bicinchoninic assay kit (Beyotime Institute of Biotechnology) was used to measure protein concentration. The thermocycling conditions were as follows: Denaturation at 95°C for 5 sec, annealing at 60°C for 34 sec min and extension at 60°C for 1 min. The primers used are presented in Table I. The 2^ΔΔCq relative quantification method was used to calculate the mean fold expression difference between the groups (15).

Table I. Quantitative polymerase chain reaction primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>CTCCCTACCCACACCATCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAAGACCCCTCCAGATAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>TTTGTCAGTGTTCTTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTAGTGGCTGCTGTTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>AGAGCTACGAGCTGCCTGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCAGTGGTGTCGCGACAGCAG</td>
</tr>
<tr>
<td>U6</td>
<td>Forward</td>
<td>CTCCGATTGGCCAGCACA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGCCTCTACGAATTTCG</td>
</tr>
</tbody>
</table>

The levels of interleukin (IL)–6 (cat. no. D6050) and tumor necrosis factor-α (TNF-α) (cat. no. DTA00D) in the supernatant released from 16HBECs were evaluated using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from cultured 16HBECs and isolated using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China). PrimeScript reagents (Takara Bio Inc., Otsu, Japan) were used to synthesize cDNA at 37°C for 15 min followed by RT inactivation at 85°C for 5 sec. RT-qPCR was completed using a MxPRO 3000 real-time PCR system and SYBR® Premix Ex Taq™ (TaKaRa NaseH Plus; cat. no. RR420) (Takara Bio Inc., Otsu, Japan). 0.8 µl primers, 0.4 µl ROX Reference Dye or Dye II 2 µl cDNA and 8 µl dH₂O were employed and the reaction mixture was made up to 20 µl. The procedure was implemented according to the manufacturer's protocols. The thermocycling conditions were as follows: Denaturation at 95°C was for 5 sec, annealing at 60°C was for 34 sec min and extension at 60°C was for 1 min. The primers used are presented in Table I. The 2^ΔΔCq relative quantification method was used to calculate the mean fold expression difference between the groups (15).

ELISA. The cells seeded in 6-well plates at a density of 2x10⁴ cells/well were cultured with the miR-625-5p mimic or miR-625-5p inhibitor with LPS (1 µg/ml) for 24 h at 37°C. The cells and the culture supernatants were collected separately.
Inc.) for 2 h at room temperature. The immunoreactive proteins on the blots were scanned by FluorChem FC3 (Protein Simple, San Jose, CA, USA).

Plasmid construction. The AKT2 3'UTR was amplified using human DNA as a template. The wild-type (WT) 3'UTR of AKT2 was inserted into the luciferase gene in the pMIR-Report vector (Promega Corporation, Madison, WI, USA; cat. no. E1330) and termed pMIR-AKT2-WT. The primers used to amplify the wild-type sequence were: 5'-CGA GCTCGGAGGGGCTTGAAGAAGACT-3' forward, and 5'-CCAAGCTTCTGGGCTTACTGGAGCTGGAC-3' reverse. pMIR-AKT2-MUT contained a mutated sequence in the 3'UTR of AKT2 inserted into the luciferase gene in the pMIR-Reporter vector. The primers used to amplify the mutant sequences were: 5'-AAGTTATATATGCGAAACCAC CCAGGCTTGTTAGCGAGCGGAG-3' (mutated site underlined) forward; 5'-GTGGTTTCGCAATATAACTTTTTACTTAG CTTTTTGTTT-3' (mutated site underlined) reverse. All constructs were verified by direct sequencing by Sangon Biotech (Sangon Biotech Co. Ltd., Shanghai, China).

Dual luciferase reporter assay. The biological software Microrna (http://www.mirbase.org/) and TargetScan release 7.2 (http://www.targetscan.org/) were used to predict as a target gene of miR-625-5p. Cells (2x10^4) were co-transfected in 96-well plates in triplicate with a firefly luciferase vector (pRL-SV40; Promega Corporation, Madison, WI, USA; cat. no. E1330) and termed pMIR-AKT2-WT. The primers used to amplify the wild-type sequence were: 5'-CGA GCTCGGAGGGGCTTGAAGAAGACT-3' forward, and 5'-CCAAGCTTCTGGGCTTACTGGAGCTGGAC-3' reverse. pMIR-AKT2-MUT contained a mutated sequence in the 3'UTR of AKT2 inserted into the luciferase gene in the pMIR-Reporter vector. The primers used to amplify the mutant sequences were: 5'-AAGTTATATATGCGAAACCAC CCAGGCTTGTTAGCGAGCGGAG-3' (mutated site underlined) forward; 5'-GTGGTTTCGCAATATAACTTTTTACTTAG CTTTTTGTTT-3' (mutated site underlined) reverse. All constructs were verified by direct sequencing by Sangon Biotech (Sangon Biotech Co. Ltd., Shanghai, China).

Statistical analysis. All experiments were repeated three times. GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the data. The data are presented as the mean ± standard deviation for normally distributed data. A Student's t-test was used to assess the difference between two groups, while one-way analysis of variance was to analyze differences among three or more groups with a post hoc Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of LPS on the viability of 16HBECS. An MTT assay was performed to determine whether LPS influenced the viability of 16HBECS following 24 h of treatment with the various LPS concentrations (0, 0.1, 1, 10 and 100 µg/ml). LPS demonstrated a dose-dependent cytotoxic effect. A total of 1 µg/ml LPS was chosen as the best stimulatory concentration for further experiments (Fig. 1A). As presented in Fig. 1B, 16HBECS treated with LPS at 1 µg/ml significantly stimulated the cells' ability to secrete inflammatory cytokines compared with the control cells (IL-6 and TNF-α; P<0.05). 16HBECS expressed a significantly increased level of miR-625-5p compared with the A549 cell line following LPS induction (P<0.05; Fig. 1C); therefore, 16HBECS were selected for further analysis.

Effect of miR-625-5p on LPS-induced cytokine expression. ELISA and RT-qPCR were used to confirm the association between miR-625-5p and inflammatory cytokine secretion. miR-625-5p expression was significantly increased in cells transfected with the miR-625-5p mimic (P<0.05; Fig. 2A). miR-625-5p had no effect on the production of IL-6 and TNF-α in control 16HBECS. However, the mRNA and protein levels of IL-6 and TNF-α were significantly decreased in miR-625-5p mimicked transfected 16HBECS following LPS induction (P<0.05; Fig. 2B-E). Transfection with the miR-625-5p inhibitor significantly decreased miR-625-5p expression in 16HBECS when compared with the inhibitor control following LPS induction (P<0.05; Fig. 3A). Transfection with the miR-625-5p inhibitor resulted in the significantly increased expression of inflammatory cytokine mRNAs (IL-6 and TNF-α) when compared with the control in LPS treated cells (P<0.05; Fig. 3B and C). As presented in Fig. 3D and E, inhibition of miR-625-5p expression significantly upregulated LPS-induced IL-6 and TNF-α secretion.

miR-625-5p targets AKT2. The AKT2 gene was predicted as a target gene of miR-625-5p using the biological software Microrna and TargetScan. A miR-625-5p binding site was predicted in the 3'UTR of AKT2 when compared with the control in LPS treated cells (P<0.05; Fig. 3B and C). As presented in Fig. 3D and E, inhibition of miR-625-5p expression significantly upregulated LPS-induced IL-6 and TNF-α secretion.
identified in the 3’UTR of AKT2 (Fig. 4A). Overexpression of miR-625-5p significantly reduced the luciferase activity in cells transfected with the pMIR-AKT2-WT vector when compared with cells transfected with the pMIR-AKT2-MUT vector (P<0.05; Fig. 4B). Western blotting was then conducted to assess the effect of miR-625-5p on the AKT2 protein level. In the miR-625-5p mimic transfected cells, the protein level of AKT2 was significantly decreased when compared with the vector only cells (P<0.05; Fig. 4C).

Effect of miR-625-5p on signaling pathways. AKT2 is involved in triggering the nuclear factor-κB (NF-κB) signaling pathway and induces inflammatory cytokines (16). To investigate whether miR-625-5p influenced the regulation of the phosphoinositol-3-kinase (PI3K)/AKT pathway, the levels of phosphorylated AKT2 were quantified. The level of phosphorylated AKT2 pretreatment with the miR-625-5p mimic was significantly decreased compared with LPS treatment alone (P<0.05; Fig. 5A). Conversely, inhibition of miR-625-5p expression significantly increased the LPS-induced phosphorylation of AKT2 (P<0.05). The phosphorylation/degradation of inhibitor of κB (IκBα) is an essential step in the NF-κB signaling pathway (17). Phosphorylation of IκBα was assessed to determine whether the NF-κB signaling pathway is involved in the protective effect of miR-625-5p. The LPS-induced phosphorylation of IκBα was significantly suppressed by miR-625-5p overexpression and significantly increased by miR-625-5p inhibition compared with LPS treatment alone (P<0.05; Fig. 5B). To further confirm whether activation of the NF-κB pathway was dependent on the phosphorylation of AKT2, NF-κB activation (i.e., the phosphorylation of IκBα) was detected in LPS-stimulated 16HBECs pretreated for 24 h with si-AKT2 to knockdown AKT2 expression. Notably, treatment with si-AKT2 significantly inhibited the LPS-stimulated phosphorylation of IκBα (P<0.05), which demonstrated that the PI3K/AKT axis may be involved in NF-κB regulation in 16HBECs (Fig. 5C).

Discussion

In the present study, it was demonstrated that miR-625-5p inhibits the secretion of inflammatory mediators in HBECs. Furthermore, AKT2 was revealed to be a direct target gene of miR-625-5p. The results indicated that miR-625-5p suppresses the airway inflammatory response by downregulating AKT2, which inhibits the NF-κB signaling pathway. Therefore, miR-625-5p may function as an inhibitor of asthma airway inflammation by suppressing the inflammatory response in HBECs by directly targeting AKT2.

Bioinformatics analysis identified that the 3’UTR of human AKT2 paired miR-625-5p binding sites. The luciferase reporter assay verified the association between miR-625-5p and AKT2. Furthermore, the interactions between AKT2 and miR-625-5p in 16HBECs using a miR-625-5p mimic and inhibitor were demonstrated.

AKT2 is involved in insulin-mediated regulation of glucose homeostasis. However, a number of studies on AKT2 have focused on its role in tumors and demonstrated that AKT2 is
essential for tumor growth, colony formation and cancer cell proliferation, including ovarian cancer (18), breast cancer (19), and hepatocellular carcinoma (20). According to these studies, an AKT isoform has a vital role in tumor growth, colony formation and cancer cell proliferation in other lung diseases, in addition to lung tumors; however, there are contradictory results concerning its functions. Previous studies have demonstrated that AKT2 is involved in tumor growth and colony formation, and inhibiting AKT2 decreased cellular motility and migration (21-24). By contrast, another study revealed that AKT2 expression may be increased in lung tumors in a tobacco-associated model; however, loss of AKT2 expression did not lead to mutant K-ras-mediated lung tumors in a genetic model (25). In acute lung injury, Vergadi et al (26) reported that depletion of AKT2 kinase activity resulted in light acid-induced lung injury and protected mice from acid-induced lung injury (27).
The results of the present study demonstrated that miR-625-5p inhibits the secretion of TNF-α and IL-6 in 16HBECs. This was in agreement with other studies (6,28,29) in which the downregulation of multiple miRs, including microRNAs-18a, 21, -27a, -128 and -155 and miR-218 also resulted in the upregulation of IL-6 levels. However, a study by Jardim et al. (30) confirmed that the expression of TNF-α was increased in asthmatic BECs, while the level of IL-6 was downregulated compared with the samples from healthy donors. This discrepancy may have been caused by the different research methods used in the two studies. Asthma is a complex disease with a variety of phenotypes (31) and there may be two possible explanations for the discrepancy between these studies. The majority of the patients selected by Jardim et al. (30) had mild asthma, whereas the present study was only an in vitro simulation that did not involve samples of asthmatic tissue. In addition, in Jardim's study, the majority of the patients with asthma were receiving medication and this may have altered the expression of certain miRs. Further study is required to determine the underlying functions of miRs in asthmatic airway inflammation, which could lead to improved diagnostic technologies and subcategorization of asthma phenotypes, ultimately leading to targeted therapies for asthma.

The results of the present study demonstrated that miR-625-5p suppresses the inflammatory response of 16HBECs by silencing AKT2, which may be involved in regulating asthma. Similarly, another study revealed that the expression of miR-625-5p is markedly reduced in dust mite-induced pediatric asthma. Therefore, it has been speculated that miR-625-5p may exert protective effects in asthma (14). Indeed, lung tissue undergoes active differentiation of cells and miR-625 may serve a role in this process. Given the dysregulation of active inflammation in the asthmatic bronchi, it would not be surprising if the inaccurate differentiation of HBECS resulted in certain pathologies of asthma. In the present study, one miR targeting a single gene was focused on. However, a gene can be targeted by a number of miRs and, conversely, an miR can regulate a number of target genes. Therefore, it would be useful to characterize other miRs that may be associated with asthma to provide further options for targeted therapy. Knowledge of the function of miR-625 in inflammation is based on cell cultures. The authors' future work will demonstrate the association between miR-625 and AKT2 in murine models, which will increase our understanding of the role of miRs in asthma.

In conclusion, miR-625-5p may protect LPS-induced 16HBEcs from inflammation by targeting AKT2 and inhibiting the NF-kB signaling pathway.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
F-HQ and XD made substantial contributions to the design of the present study. XD, Q-XZ, BW and D-DZ performed the experiments. XD analyzed the data. F-HQ and XD wrote and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.
References

The authors declare they have no competing interests.