Original Article

Ectopic expressed miR-203 contributes to chronic obstructive pulmonary disease via targeting TAK1 and PIK3CA

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Abstract: MiRNA is a group of powerful short non-coding RNAs that suppress the expression of protein coding genes by targeting to the 3'UTRs of mRNAs. Some researchers have detected the miRNAs expression profile in tissue and blood samples of chronic obstructive pulmonary disease (COPD) patients recently. Several disturbed miRNAs were found to be related to COPD; however, the mechanisms were still well understood. In this study, we first detected the expression of 11 candidate miRNAs in the lung samples of COPD patients, non-COPD smokers and non-smoker controls. We found that the expression of miR-181a, miR-203, miR-338, miR-1 and miR-199a was altered compared with control. Subsequently, we detected these five miRNAs expression in the blood samples of the participants. A significant higher expression of miR-203 was found in the blood samples of smokers and COPD patients. Predicted by bioinformatics tools and confirmed by luciferase assay and western blot, we demonstrated that TAK1 and PIK3CA are two direct targets of miR-203. Furthermore, we detected a lower p-IκBα and p-p65 level in the bronchial/tracheal epithelial cells from COPD patients compared with the cells from healthy controls, when stimulated by LPS. The concentration of TNF-α and IL-6 in the medium from bronchial/tracheal epithelial cells from COPD patients is also lower. Meanwhile, the miR-203 level was down-regulated significantly in the control cells, but non-significant change in the cells from COPD patients. miR-203 represses NF-κB signaling via targeting TAK1 and PI3KCA and miR-203 overexpression may contribute to the COPD initiation.

Keywords: MicroRNA, chronic obstructive pulmonary disease, NF-κB

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic airway inflammatory disorder characterized by irreversible airflow limitation. According to WHO estimates, about 65 million people have moderate to COPD and more than 3 million people died of COPD in 2005, which corresponds to 5% of all deaths globally.

COPD is always common in men; however, because of increased tobacco use among women and higher risk of exposure to indoor air pollution, the disease now affects men and women almost equally. Smoking is the most important risk factor for COPD development; meanwhile genetic predisposition and pathogen infection also contribute to the pathogenesis of COPD. COPD is further associated with a systemic inflammation which is confirmed to trigger comorbidities [1, 2].

All normal airway epithelial cells serve as the first line of defense against foreign materials and infectious agents via the secretion of cytokines. These include the action of cilia, as well as the secretion of mucus, antimicrobial proteins and peptides, and the production of a variety of immunological factors [3]. A hallmark of the pathological changes in the airways of COPD patients is altered appearance of the epithelium, including squamous cell metaplasia, goblet cell hyperplasia and reduced immunological response to pathogens [4, 5].

MicroRNA (miRNA) is a group of short non-coding RNAs that suppress the expression of protein coding genes by partial complementary
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binding, especially to the 3' untranslated regions (UTRs) of messenger RNAs (mRNAs). According to the predicted results of bioinformatics, there may be more than 60% of protein coding genes the expression of which is regulated by miRNA. In order to explore the roles of miRNAs during COPD pathogenesis, researchers detected the miRNAs profile in tissue and blood samples of COPD patients recently [6-8]. Several disturbed miRNAs were found in the clinical samples from COPD patients. However, the biological functions of these miRNAs in epithelial systems and the mechanisms of how they are related to the pathogenesis of COPD were still not well understood.

Despite the long established evidence that COPD is associated with major changes in epithelial morphology, the contributions of epigenetic factors during the pathogenesis of COPD is still not well understood. To investigate the impact of smoking on COPD pathogenesis in miRNA level, we detected the expression of 11 candidate miRNAs in the clinical lung samples from smokers with or without COPD and non-smock controls. The function of disturbed miRNAs were predicted by using online bioinformatics tools and determined by using airway epithelial cells.

Materials and methods

Clinical samples collection

This study includes three study groups: the first group consisted of 15 healthy non-smokers with no smoking history (NS); the second group consisted of 15 current smokers (≥ 10 years) without respiratory symptoms or airflow limitation(s); and 17 current smokers with COPD (≥ 10 years, GOLD stages II-IV) (Table 1). None of the subjects were using oral or inhaled corticosteroids or receiving immunosuppressive treatment and none reported any other serious illness. COPD was diagnosed according to the criteria recommended by the National Institutes of Health [9]. Lung biopsies were obtained from all participants through surgical resection for suspected or confirmed lung cancer.

Cell culture

Normal human (healthy non-smoker) bronchial/tracheal epithelial cells (NHBE; n = 2) and COPD-diseased human bronchial/tracheal epithelial cells (DHBE; n = 2) were purchased from Lonza (Walkersville, MD). Cells were grown on collagen-coated plates using 1% collagen type I (Nalgene Cukture 3D Matrix, Rochester, NY). Cells were cultured in BEBM medium supplemented with the BEGM Bulletkit. Once confluent, cells were washed with PBS and starved for 24 h in starving medium (99% BEBM medium with 1% BEBM medium supplemented with BEGM Bulletkit) prior to treatments.

Jurkat T cells (ATCC, Manassas, VA) were cultured in 90% RPMI 1640, 10% fetal bovine serum at 37°C, 5% CO₂, Lipofectamine® RNAiMAX (Life Technologies, Grand Island, NY) was used for miRNA transfection into Jurkat cells.

RNA extraction and miRNA quantification

Total RNA was extracted from tissues and cells, using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The amount of candidate miRNAs was measured by stem-loop quantitative PCR with U6 snRNA as the internal control. Each sample was measured in triplicate and the experiment was repeated at least three times.

Peripheral blood samples were collected in EDTA tubes (BD Biosciences, Franklin Lakes, NJ, USA) 250 μl whole blood mixed with 750 μl TRIzol LS (Life Technologies, Grand Island, NY, USA) and archived at -80°C until use.

For RNA extraction, 150 μl chloroform was added into each tube, and then vortexed vigorously for 15 seconds and allowed to sit at room temperature for 3 minutes. Phase separation was achieved by centrifuging the sample for 12,000 × g for 15 minutes at 4°C. After centrifugation, 400 μl of the aqueous phase was carefully transferred to a new tube. 400 μl isopropanol was added and RNA was collected by 12,000 × g for 15 minutes at 4°C. The RNA concentration was quantified by a Nanodrop nanodrop spectrometer.

Dual luciferase assay

MiRNAs mimics, inhibitors and controls were purchased from GenePharma Co, Ltd (Shanghai, China). Full length of PIK3CA 3'UTR and a 354 bp length TAK1 3'UTR containing two putative miR-203 target sites were cloned
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Table 1. Clinical information of patients

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>S</th>
<th>COPD</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Age, yr</td>
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<td>54 ± 3.9</td>
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<td>Sex, m:f</td>
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<tr>
<td>FEV1, % pred</td>
<td>104.9 ± 5.1</td>
<td>101.1 ± 2.7</td>
<td>44.7 ± 1.9*</td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>84.6 ± 1.1</td>
<td>82.7 ± 1.3</td>
<td>52.0 ± 2.1*</td>
</tr>
<tr>
<td>FVC, % pred</td>
<td>102.5 ± 5.6</td>
<td>101.7 ± 3.7</td>
<td>72.1 ± 2.7*</td>
</tr>
<tr>
<td>Pack-years</td>
<td>-</td>
<td>40.3 ± 7.2</td>
<td>42.7 ± 5.5</td>
</tr>
</tbody>
</table>

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; NS = nonsmoker; S = current smoker without COPD (≥ 10 pack-years). *P < 0.001 versus S and NS.

Reagent (Invitrogen, Carlsbad, CA, USA) and quantified by BCA method. Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 20 μg samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-PIK3CA monoclonal antibody (Abcam, Cambridge, MA, USA) at dilution of 1:300, rabbit anti-TAK1 monoclonal antibody at dilution of 1:1000, and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at dilution of 1:5000 for 1 h at 37°C. The specific protein antibody complex was detected by incubating with horseradish peroxidase conjugated goat anti-rabbit at dilution of 1:5000 or rabbit anti-mouse IgG at dilution 1:5000 for 2 h at 37°C. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control. The band density was analyzed by using Quantity One software (Bio-Rad). The experiment was done in triplicate and results were analyzed by student t-test.

Supernatant protein analysis

Sandwich enzyme-linked immunosorbent assays (ELISAs) for TNFα and IL-8 were carried out on cell culture supernatants according to the manufacturer’s instructions (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK). Supernatants were diluted with RPMI 1640 sufficiently to yield results within the linear range of the assay (dilutions were: 1:5-1:100) for ELISA analysis. All assays were done in duplicate. Plates were read by using an ELISA plate reader.

Statistical analysis

Data were analyzed by using SPSS Statistical Package version 16. Independent two group’s analyses are used t-test. Results of tissue miRNAs levels were analyzed by using Mann-Whitney U test. P < 0.05 was considered statistically significant.

Results

To explore the roles of miRNAs during COPD pathogenesis, we first detected the expression
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Figure 1. The expression of candidate miRNAs in lung tissue samples. Lung tissue samples were collected from COPD patients, current smokers and healthy controls and total RNA was extracted by using Trizol. The amount of candidate miRNAs was measured by stem-loop quantitative PCR with U6 snRNA as the internal control. Each sample was measured in triplicate and the experiment was repeated at least three times. The results were analyzed by student’s t-test and P < 0.05 was considered statically significant. *P < 0.05, **P < 0.01.

of 11 candidate miRNAs in lung samples of COPD patients, non-COPD smokers (S) and non-smoking controls (NS) by real time RT-PCR. These miRNAs were reported to have disturbed expressions in lung tissue samples like miR-1, miR-133, miR-206 [6] or in plasma samples like miR-145, miR-338 [7] or in bronchoalveolar lavage fluid samples miR-132 [8] of COPD patients; or to be related to immune disorders, like miR-17, miR-21, miR-203, miR-181a and miR-199a [10-12].

As shown in Figure 1, miR-203 is expressed higher in the S group people but lower in the COPD patients when compared with NS. Meanwhile, the expression of miR-1, and miR-199a was significantly repressed, the expression of miR-181a and miR-338 level was up-regulated in the COPD patients. We further detected these five miRNAs expression in the peripheral blood of the participants and found that only the expression of miR-203 was up-regulated in S and COPD group (Figure 2).

To unveil the biological function of disturbed miR-203 in the process of COPD and the reason why miR-203 is down-regulated in lungs but up-regulated in blood, we focused on the direct target genes the expression of which was modulated by miR-203. Firstly, we predicted the target genes of miR-203 by using online bioinformatics tool TargetScan (http://www.targetscan.org/). To our surprise, TAK1 and PIK3CA, two positive regulators of NFκB pathway, are putative targets of miR-203 (Figure 3A).

To understand whether TAK1 and PIK3CA are direct targets of miR-203, full length of PIK3CA 3’UTR and a 354 bp length TAK1 3’UTR containing two putative miR-203 target sites were cloned into pmirGLO vector downstream of firefly luciferase coding region to generate luciferase reporter vector (Figure 3B). For luciferase reporter assays, HEK293T cells were seeded in 48-well plates. MiR-203 mimic or inhibitor was transfected with one of the reporter vectors. 48 h post-
transfection, cells were harvested, lysed and assayed. As shown in Figure 3B, the relative luciferase activities repressed significantly by miR-203 transfection. Meanwhile, the relative luciferase activities were up-regulated when endogenous miR-203 was inhibited (Figure 3C and 3D). To further identify miR-203 target sites in 3'UTR of TAK1 and PIK3CA, two mutant reporter vectors with 3 nucleotides variation in each predicted miR-203 target site were constructed (Figure 3G). As shown in Figure 3F, the firefly luciferase activity was not repressed by miR-203. These results indicated that miR-203 represses firefly luciferase expression through targeting TAK1 and PIK3CA 3'UTR.

To further understand whether endogenous TAK1 and PIK3CA were modulated by miR-203, Jurkat cells were transient transfected with miR-203. 48 hours post transfection, cells were lysed and the expression of TAK1 and PIK3CA was detected by western blot. As shown in Figure 3E, the protein level of TAK1 and PIK3CA was significantly reduced by miR-203 mimic and up-regulated by miR-203 inhibitor. These results indicated that TAK1 and PIK3CA are direct targets of miR-203.

TAK1 and PIK3CA are NF-κB positive regulators and play important roles when the host is challenged by pathogens. Meanwhile, smokers are more susceptible when challenged by respiratory pathogens. Therefore, according to our previous study, we hypothesize that overexpressed miR-203 may represses anti-pathogen immune response in smokers and contributes to COPD initiation.

To confirm our hypothesis, bronchial/tracheal epithelial cells from COPD patients and healthy controls were stimulated by LPS for different times. The supernatants were collected for TNF-α and IL-6 detection, and the cells were used for protein and miRNA quantification. As exhibited in Figure 4A, the protein levels of phosphorylated IκBα and p65 were raised gradually in the control cells. Meanwhile, their expression was also up-regulated in COPD...
miR-203 expression in COPD

miR-203 expression in COPD

Figure 3. miR-203 represses TAK1 and PIK3CA expression by targeting 3'UTR. A: Predicted miR-203 target sequence in the 3' UTR of TAK1 (a) or PIK3CA (b). B: A schematic diagram for reporter vector construction. Full length of PIK3CA 3'UTR and a 354 bp length TAK1 3'UTR containing two putative miR-203 target sites were cloned into downstream of firefly luciferase coding region in pmirGLO vector to generate luciferase reporter vectors. C, D: Dual-luciferase assay. HEK293T cells were seeded in 48-well plates. 40 pmol MiR-203 mimic or inhibitor was transfected with 100 ng one of reporter vectors were transfected into HEK293T cells by lipofectamine. 48 h post-transfection, cells were harvested, lysed and assayed. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC) and analyzed by student’s t-test. *P < 0.05, **P < 0.01. E: Jurkat T cells were transfected with miR-203 mimic or inhibitor. 48 h post-transfection, cells were lysed and the protein level of TAK1 and PIK3CA were detected by western blot. The experiment was done in triplicate and the density of the bands was determined by using Quantity One software. The results were analyzed by student t-test and P < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01. F, G: Schematic diagram for mutant reporter vectors construction. Red letters represents the mutant sites. H: Dual-luciferase assay. 40 pmol MiR-203 mimic or inhibitor was transfected with 100 ng one of the mutant reporter vectors were transfected into HEK293T cells by lipofectamine. MiR-203 mimic or inhibitor was transfected with one of the reporter vectors. 48 h post-transfection, cells were lysed and assayed. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC) and analyzed by student’s t-test. *P < 0.05 was considered statistically significant.

cells, but has very low quantities. On the contrary, the expression of miR-203 was reduced 55.0% and 60.8% in the cells treated by LPS for 15 min and 30 min (Figure 4B). However, the
miR-203 expression in COPD

Expression of miR-203 was not significantly changed after LPS treatment in the cells from COPD patients (Figure 4B). Subsequently, TNF-α and IL-6 levels in the cell cultures were detected by ELISA. As shown in Figure 4C, the TNF-α level has more than 100-fold up-regulation after LPS treatment in the control cell cultures, but only 20.3 and 19.5 fold up-regulation in the COPD cell cultures. Similarly, the IL-6 level was up-regulated to 4.55 and 7.26 fold in control cell cultures, which was more significant than COPD cell cultures.

Discussion

MiRNA is a group of powerful short non-coding RNAs that suppress the expression of protein coding genes by targeting to the 3’UTRs of mRNAs. Recently, some researchers have detected the miRNAs expression profile in tissue and blood samples of COPD patients [6-8]. Several disturbed miRNAs were found to be related to COPD, however, the mechanisms were still not well understood [6-8, 13]. In this study, we first detected the expression of 11 candidate miRNAs in the lung samples of COPD patients, non-COPD smokers and non-smoker controls. We found that the expression of miR-181a, miR-203, miR-338, miR-1 and miR-199a was altered in the lung specimens of COPD patients when compared with control. Subsequently, we detected these five miRNAs expression in the blood samples of the participants. A significant higher expression of miR-203 was found in the blood samples of smokers and COPD patients. Predicted by bioinformatics tools and confirmed by luciferase assay and western blot, we demonstrated that TAK1 and PIK3CA are two direct targets of miR-203. Furthermore, we detected a higher p-IκBα and p-p65 levels in the bronchial/tracheal epithelial cells from COPD patients compared with the cells from healthy controls, when stimulated by LPS. Meanwhile, the miR-203 level was up-regulated significantly in the control cells, but non-significant change in the cells from COPD patients. Ultimately, TNF-α and IL-6 levels exhibited a significantly higher up-regulation in the control cells demonstrating a weaker anti-pathogen immune response may contribute to the COPD initiation.
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In this study, we found that overexpressed miR-203 was existed in the lung and blood samples of smokers. Subsequently, we demonstrated that miR-203 functions as an immune response inhibitor through targeting TAK1 and PIK3CA. Furthermore, higher miR-203 expression and weaker NF-κB signaling activation was detected in the COPD-diseased bronchial/tracheal epithelial cells when stimulated by LPS. These results suggesting that miR-203 overexpression may contributes to the increased susceptibility to bacterial infection of the airways in smokers [5, 14, 15]. However, the expression of miR-203 was up-regulated in the blood samples but down-regulated in the lung tissue samples of COPD patients. The up-regulated blood miR-203 level can be used as a new biomarker for COPD diagnosis, but the reason of why miR-203 is reduced in blood needs to be further examined. The lower miR-203 level in lung tissues of COPD patients may represents a lasting stimulated inflammation in the lungs which is a significant character of COPD.

Except miR-203, we found that the expression of miR-181a and miR-338 was up-regulated in the lung of COPD patients. The function of miR-181a in immune system was partially unveiled recently. There are reports indicated that miR-181a can augment the sensitivity of TCR-mediated T cell responses to peptide antigens and regulate the positive and negative selection of T cell during thymic development [16]. Meanwhile, up-regulated serum miR-181a is found to be associated with the early pathogenic process of chronic obstructive pulmonary disease in asymptomatic heavy smokers [17]. So the overexpression of miR-181a in lung may be related to stimulated immune response of COPD patients. Since there is no report about the biological function of miR-338, the role of miR-338 overexpression need to be further examined.

In conclusion, we found disturbed miR-203 was existed in the lung tissue and peripheral blood samples of smokers and COPD patients. MiR-203 functions as a NF-κB inhibitor through repression TAK1 and PIK3CA expression and its up-regulation contributes to the susceptibility to bacterial infection since miR-203 is down regulated in the blood and the lung of smokers and in the lung of COPD patients. Our results suggest that altered miR-203 level may be used as a new biomarker for COPD diagnosis and miR-203 may be a potential therapeutic target for COPD.

Disclosure of conflict of interest

None.

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