ORIGINAL ARTICLE

microRNA-183 down-regulates the expression of BKCaβ1 protein that is related to the severity of chronic obstructive pulmonary disease

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Abstract
Objectives: The aim of this study was to investigate the relationship between the expression of microRNA (miRNA)-183 and Ca^{2+}-activated K^+ channels β1 subunit (BKCaβ1) in the lung tissues of patients with chronic obstructive pulmonary disease (COPD).
Methods: Quantitative real-time polymerase chain reaction and Western blotting were used for detecting the expression of miRNA-183 and BKCaβ1 in the lung tissues from 45 COPD patients and 30 lung cancer patients without COPD. Possible miRNAs that target BKCaβ1 were forecasted by bioinformatics. The expression of these miRNAs in the peripheral blood of COPD patients was also examined. After transfecting vascular smooth muscle cells with pGCMV/EGFP/miR-183 plasmid, the expression of miRNA-183 and BKCaβ1 were detected by quantitative real-time polymerase chain reaction and Western blotting.
Results: The expression of BKCaβ1 in the lung tissues of COPD patients was significantly lower than control. Western blotting data showed that the expression of BKCaβ1 protein in COPD group was significantly lower than control. After transfecting the vascular smooth muscle cells with pGCMV/EGFP/miR-183 plasmid, we found that the level of BKCaβ1 mRNA was not significantly reduced by the increase of miRNA-183 level, but the expression of BKCaβ1 protein was down-regulated.
Conclusions: The present study indicated that miRNA-183 might play a role in the expression of BKCaβ1, and the expression of miRNA-183 and BKCaβ1 were possibly related with the pathogenetic pathways of COPD. Hippokratia 2014; 18 (4): 328-332.

Keywords: Chronic obstructive pulmonary disease, COPD, microRNA, BKCaβ1

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common clinical respiratory disease characterized by airflow limitation[1]. Numerous recent studies suggested that COPD is a chronic inflammatory disease that involves a variety of inflammatory mediators during the whole process of disease development[2]. The local immune response mediated by the inflammatory mediators could damage bronchial pulmonary vascular, alveolar and other structures and cause chronic inflammation of the lungs, emphysema, fibrosis and other irreversible damages. Finally, COPD patients might develop arterial hypertension and pulmonary hypertension[3-5]. The large conductance, Ca^{2+}-activated K^+ channels (BKCa), a type of calcium-activated potassium channels characterized by large conductance, is widely expressed in various tissues[6]. BKCa β1 subunit (BKCaβ1) is the major functional group for immediate regulation of BKCa channel activity, which affects systolic and diastolic function of smooth muscle[7] and further regulates the tension of the elastic bronchus and blood pressure. Because of the high prevalence of bronchial hyper-responsiveness and arterial hypertension in COPD patients, the expression pattern of BKCaβ1 in COPD lung tissue might be abnormal. In this study, we examined the expression level of BKCaβ1-related microRNA (miRNA) in peripheral blood of COPD patients and tested whether this has roles in regulating BKCaβ1 expression level.

Materials and Methods

Tissue samples

All lung tissue samples were collected from lung cancer patients and COPD patients after surgery between February 2012 and October 2013. Among them, 45 patients had COPD, 39 patients had COPD combined with lung cancer, and 6 patients had COPD lung volume reduction surgery. COPD patients with combined lung cancer were included into the experimental group, and COPD patients without lung cancer were included into the control group. The included patients had no bronchiectasis or cystic fibrosis lungs. All patients received no chemotherapy or

radiotherapy before surgeries. In addition, tissue samples were collected from areas more than 5 cm away from tumour tissues. Surgeries were performed on days when the patients had no acute respiratory infection. The classification of COPD severity was made according to the Global Initiative on Obstructive Lung Diseases proposed in 2007. Among the 45 patients with COPD, 15 had mild COPD, 22 moderate COPD, and 8 severe COPD. Forced expiratory volume in 1 second (FEV1) / forced vital capacity (FVC) values and predicted FEV1 values of all patients were less than 70% and 80%, respectively. All of the 20 patients in the control group were non-COPD lung cancer patients. The FEV1 / FVC values and predicted FEV1 values of all patients in the control group were more than 70% and 80%, respectively. COPD patients aged from 43 to 69 years, with an average age of 52 and a median age of 48. Non-COPD patients aged between 19 and 71, with an average age of 58 and a median age of 41. The study was approved by the Ethics Committee of People's Hospital of Lishui City, Zhejiang Province, and informed consent was obtained from all participants (Table 1).

Table 1: Demographic data of patients in chronic obstructive pulmonary disease group and in control group.

Items	COPD	Control
Gender (male/female)	31/14	12/8
Age (years)	52	58
FEV1/FVC (%)	62.8 ± 1.25	86.24 ± 3.10
Predicted FEV1 (%)	58.4 ± 2.36	88.03 ± 4.83

COPD: chronic obstructive pulmonary disease, FEV1: forced expiratory volume in 1 second, FVC: forced vital capacity.

Reagents

Serum RNA extraction reagent Trizol 1s and total RNA extraction reagent Trizol were purchased from Invitrogen (California, USA). Vascular smooth muscle cells (VSMCs) were purchased from the Chinese Academy Cell Bank (Shanghai, China). pGCMV/enhanced green fluorescent protein (EGFP)/miR-183 plasmid and negative control plasmid were obtained from Invitrogen (California, USA). Rabbit polyclonal anti-human BKCaβ1 antibody was purchased from Abcam, Inc. (Boston, USA). Reverse transcription system and SYBR® PrimeScript™ miRNA real-time polymerase chain reaction (RT-PCR) kit were purchased from Takara (Dalian, China). mRNA SYBR Green RT-PCR kit was obtained from KAPA BIOSYSTEMS (Boston, USA). Lipofectamine 2000 was from Invitrogen (New York, USA).

Reverse transcription

We used polyA tailing method and 1 μg total RNA for miRNA reverse-transcription reaction. RNA template (6 μl), 2 × miRNA reaction buffer mix (10 μl), 0.1% bovine serum albumin (2 μl), and miRNA PrimeScript RT enzyme mix (2 μl) were added sequentially to the cold pretreated RNAase-free eppendorf tubes on ice. The mixture was incubated for 60 min at 37 °C for polyA tailing and reverse transcription. RNAase-free water was added to reach a total volume of 100 μl. Quantitative analysis was performed on 2 μl of the mixture.

Western blotting

After sodium dodecyl sulfate polyacrylamide gel electrophoresis and polyvinylidene difluoride membrane transfer, the membrane was incubated with appropriate concentration of primary antibodies (BKCaβ1, 1:500; glyceraldehyde-3-phosphate dehydrogenase, 1: 5000) overnight at 4 °C. The membrane was washed with phosphate buffered saline with Tween® 20 for 3 times of 15 min. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, 1:5000; goat anti-rabbit, 1:1000) were added for incubation for 1 h at room temperature. After 3 times of 15 min washing, electrochemiluminescence was used for imaging.

Quantitative polymerase chain reaction (qPCR)

qPCR was performed following the SYBR Green method. The reaction system included 12.5 μl SYBR Premix Ex Taq, 1 μl PCR forward primer, 1 μl Uni-miR qPCR primer, 2 μl templates and 8.5 μl double-distilled H$_2$O, reaching a total volume of 25 μl. The amplification procedure was composed of denaturation (95 °C for 30 sec) and 40 tandem amplifying cycles (for each cycle, 95 °C for 5 sec and 60 °C for 20 sec). Relative expression level of miRNA was calculated using 2-ΔΔT method. U6 was used as the internal reference for relative quantification. Forward sequences of primers were: miR-183, ATGGCACTGGTAGAATTC; miR-200b, TAATACTGCCTGGTAATG; miR-200c, ACTGCCGGGTAATGATGGA.

Cell transfection

The transfection of human VSMCs was mediated by liposome. One day before transfection, cells in logarithmic growth phase was seeded in 24-well plates, with a density of 2×10^5 per well. The 24 wells were divided into control group (pGCMV), negative control (NC) group (EGFP), and miRNA183-VSMC group (miR-183). Transfection was performed when the cells reached 80% confluence on the next day. Plasmid (2 μg) and lipofectamine 2000 (1 μl) were individually added into 2 tubes of 50 μl medium containing Opti–MEM I medium. After 5 min standing, the mixtures in the two tubes were combined and left for standing for another 20 min. The mixture was then added to the wells for incubation for 6 h before high-glucose Dulbecco's Modified Eagle's Medium containing10% fetal bovine serum was added. At 48 h after transfection, the transfection efficiency was estimated by observing the green fluorescent protein (GFP) signal under an inverted fluorescence microscope.

Bioinformatics

Using online prediction websites, such as PicStar, miRanda and Targetscan, a list of miRNAs that potentially regulate BKCaβ1 was generated, including miR-183, miR-200b, and miR-200c, which were the top three candidates based on mirSVR score ranking, a new machine learning method for ranking microRNA target sites by a

Table 2: mirSVR scores (mirSVR is a new machine learning method for ranking microRNA target sites by a down-regulation score).

microRNAs	mirSVR scores
microRNA-183	-1.311
microRNA-200b	-1.272
microRNA-200c	-1.272
microRNA-429	-1.272
microRNA-876	-0.967

down-regulation score (Table 2).

Statistical analyses

All results were analyzed using the Statistical Package for the Social Sciences (SPSS) version 11.0 (SPSS Inc., Chicago, Illinois, USA). The data were presented as means ± standard deviation (SD). To compare the expression levels of miRNA183 and BKCaβ1 in lung tissues and VSMCs, t-test was performed. A $p < 0.05$ was considered statistically significant.

Results

The reduced levels of BKCaβ1 mRNA in lung tissues of COPD patients are related with the severity of COPD

To determine the levels of BKCaβ1 mRNA in lung tissues of COPD group and control group, qPCR was used. The results showed that BKCaβ1 expression level in COPD lung tissue was significantly lower than that in the control group ($p < 0.05$). In addition, the expression of BKCaβ1 was related with COPD severity, with a reduction of 83% in severe COPD, and a reduction of 55% in moderate COPD but
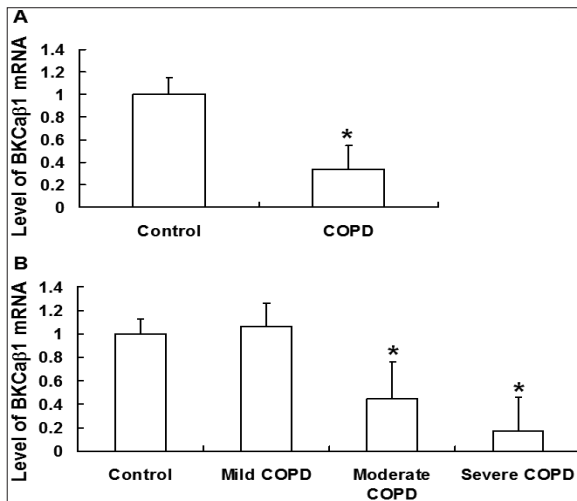

Figure 1: Ca²⁺-activated K⁺ channels β1 subunit (BKCaβ1) mRNA levels in lung tissues detected using quantitative polymerase chain reaction. **(A)** Quantification of BKCaβ1 expression in lung tissues of all chronic obstructive pulmonary disease (COPD) patients compared with those of control. Data are means ± standard deviation. *, $P < 0.05$ compared with control. **(B)** Quantification of BKCaβ1 expression in lung tissues of mild, moderate and severe COPD patients, compared with those of control. *: $p < 0.05$ compared with control.

no reduction in mild COPD ($p > 0.05$) (Figure 1).

The reduced expression of BKCaβ1 protein in lung tissues of COPD patients is also related with the severity of COPD

To quantify the expression of BKCaβ1 protein, Western blotting analysis was performed. Western blots showed that BKCaβ1 protein levels in COPD group was significantly lower than that in the control group ($p < 0.05$), which was consistent with the observation for mRNA expression. Compared with the control, BKCaβ1 protein expression was decreased by 39% in moderate COPD group and 88% in severe COPD group, but not affected in mild COPD group (Figure 2). These data suggested that the reduced expression of BKCaβ1 protein in lung tissues of COPD patients was dependent on the se-
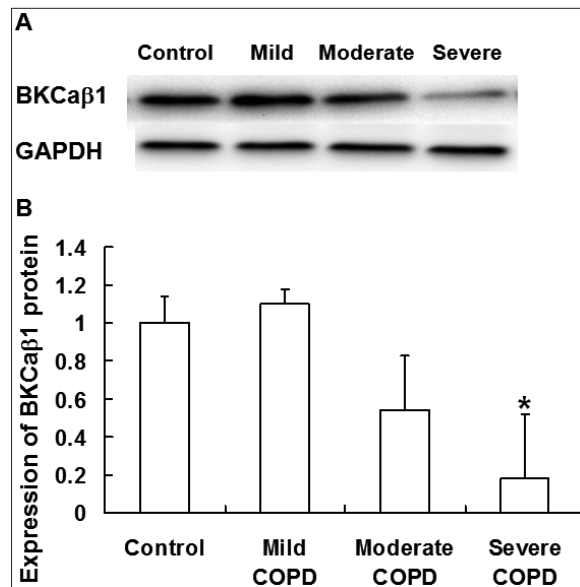

Figure 2: Ca²⁺-activated K⁺ channels β1 subunit (BKCaβ1) protein levels in lung tissues detected using Western blotting. **(A)** Western blots of BKCaβ1 proteins in mild, moderate and severe chronic obstructive pulmonary disease (COPD) patients compared with those of control. **(B)** Quantification of BKCaβ1 protein expression in mild, moderate and severe COPD patients compared with those of control. Data are means ± standard deviation. *: $p < 0.05$ compared with control.

verity of COPD.

The levels of miR-183, miR-200b and miR-200c in peripheral blood of patients with COPD were higher than those of patients in the control group, with the expression of miR-183 being the highest

To measure the levels of miR-183, miR-200b and miR-200c in peripheral blood of patients with COPD, qPCR was employed. The results showed that all three candidate miRNAs had significantly increased expression levels in COPD patients compared to the control. Among them, the level of miR-183 was the highest (Figure 3). These data indicated that the levels of miR-183, miR200b and miR200c in peripheral blood of patients with COPD
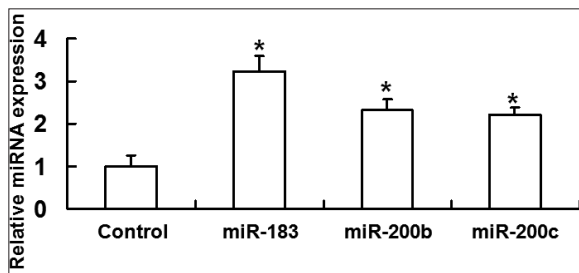
Figure 3: The levels of microRNA (miR)-183, miR-200b and miR-200c in chronic obstructive pulmonary disease patients determined using quantitative polymerase chain reaction. miRNA-183 expression in peripheral blood of chronic obstructive pulmonary disease patients is significantly increased. *: p < 0.05 compared with control.

were higher than those of patients in the control group, with the expression of miR-183 being the highest.

miR-183 reduces BKCaβ1 protein expression without altering the level of BKCaβ1 mRNA

To study the effect of miR-183 on the expression of BKCaβ1, VSMCs were transfected with constructed pGCMV/EGFP/miR-183 plasmid by liposome for qPCR and Western blotting analyses. At 48 h after transfection, strong GFP signals were widely distributed in VSMCs, with the transfection efficiency being above 60% as indicated by GFP-positive cell ratio (Figure 4A and 4B).

qPCR results showed that the level of miR-183 at 48 h after transfection was increased by 12.46 ± 0.89 fold compared with control (p < 0.05) (Figure 4C). However, the level of BKCaβ1 was not distinct from the control (p > 0.05) (Figure 4D). By contrast, Western blotting data showed that BKCaβ1 protein expression in VSMCs transfected with miR-183 was significantly decreased by 59% compared with those of controls (Figure 5). These data suggested that miR-183 reduced BKCaβ1 protein expression without altering the level of BKCaβ1 mRNA.

Discussion

COPD patients could develop peribronchial fibrosis, severe damage in lung parenchyma, and severe respiratory decompensation. Studies have shown that serum C-reactive protein, endothelin-1, and brain natriuretic peptide levels in patients with COPD were positively correlated with the severity of COPD[8-10]. Besides, hypoxia-inducible factor-1α, vascular endothelial growth factor and surfactant protein D shows abnormal expression levels in COPD lungs[11-13], which is related to hyper-responsiveness and remodeling of the airway. Further studies discovered that abnormal function of T-regulatory cells and macrophages in COPD patients might be closely related to the damage in lung tissues. BKCaβ1 can be involved in the regulation of various cellular functions. BKCaβ1 coupling with Ca^{2+} can regulate smooth muscle systolic function, activate hormone secretion, and promote syn-
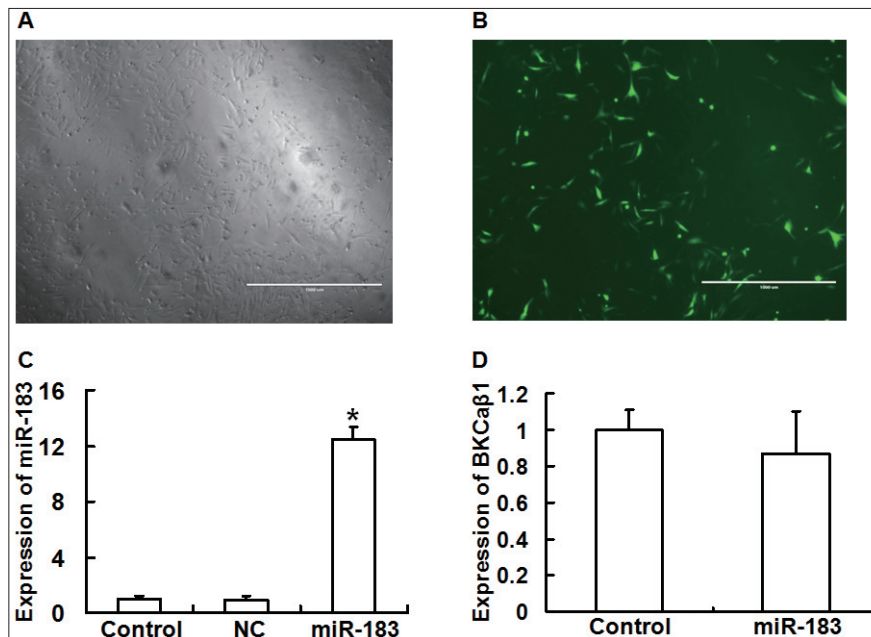

Figure 4: microRNA (miR)-183 and Ca^{2+}-activated K^+ channels β1 subunit (BKCaβ1) mRNA in cells transfected with miR-183. **(A)** Vascular smooth muscle cells before transfection with miR-183. **(B)** Vascular smooth muscle cells 48 h after transfection with miR-183. **(C)** Level of miR-183 in vascular smooth muscle cells transfected with miR-183. Data are means ± standard deviation. *: p < 0.05 compared with control and negative control. **(D)** Level of BKCaβ1 mRNA in vascular smooth muscle cells transfected with miR-183. Data are means ± standard deviation. NC: negative control.
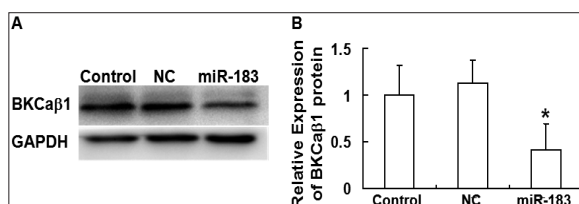

Figure 5: Effect of microRNA (miR)-183 on Ca^{2+}-activated K^+ channels β1 subunit (BKCaβ1) protein in vascular smooth muscle cells. **(A)** Western blots of BKCaβ1 protein. **(B)** Expression of BKCaβ1 protein in vascular smooth muscle cells transfected with miR-183. Data are means ± standard deviation. *: p < 0.05 compared with control and negative control (NC). GADPH: glyceraldehyde-3-phosphate dehydrogenase.

thesis and release of neurotransmitters in neuron[14]. In the development of COPD, lung inflammation leads to repeated dilatation of the trachea and the pulmonary vascular pressure is increased, but this is not quite clear and not supported by literatures. In fact, trachea dilation would lead to decreased airways resistance[15]. COPD is mainly a disease of small airways disease. Therefore, we examined the expression level of BKCaβ1 in COPD lung tissues. To study the mechanism of BKCaβ1 expression regulation, we predicted the potential upstream miRNA using bioinformatics. Furthermore, we tested the candidate miRNA expression levels in peripheral blood of COPD patients as well as the potential correlation between miRNA and BKCaβ1 *in vitro*.

The results showed that compared with the control, mRNA expression of BKCaβ1 was significantly reduced, especially in moderate and severe groups, but not in mild group. Western blotting results indicated the same trend for protein levels. Collectively, the results suggested that the BKCaβ1 expression was closely related to the developmental stage of COPD. We hypothesize that this is mainly due to the remodeling of airway and normal lung tissue damage in moderate and severe COPD patients, which lead to reduced expression of the BKCaβ1. The results predicted by multiple bioinformatics software, such as PicStar, miRanda, and Targetscan, suggested that several miRNAs such as miR-183, miR-200b, miR-200c and miR-429 might be involved in regulating BKCaβ1 expression. We detected increased expression of miR-183, miR-200b, and miR-200c in the peripheral blood of patients with COPD, but found no significant difference between different severities of COPD. Because miR-183 has the largest increase fold, we constructed eukaryotic expression plasmid with mi-R183 and transfected VSMCs *in vitro*. qPCR results suggested that the eukaryotic cell model of miR-183 overexpression was constructed successfully. Furthermore, BKCaβ1 mRNA was slightly reduced after transfection, without statistical significance. Interestingly, Western blotting results suggested that BKCaβ1 protein level was significantly reduced after transfection. Collectively, the results suggested that miR-183 might inhibit BKCaβ1 protein expression at translational level, but not through promoting the degradation of mRNA. This might be one explanation for the inconsistency between mRNA level and protein level. The BKCaβ1 mRNA and protein expression in lung tissues of COPD patients were both decreased, indicating the existence of other mechanisms that regulate mRNA expression of BKCaβ1. We speculate that it might be due to damages of airway smooth muscle and vascular smooth muscle tissues caused by immunity that down-regulates BKCaβ1. However, the exact mechanism needs further investigation.

In summary, BKCaβ1 is related with the occurrence and development of COPD. The expression level of BKCaβ1 might be correlated with the severity of the disease. Increased expression of miR-183 in COPD patients' peripheral blood might be involved in the regulation of the expression of BKCaβ1. BKCaβ1 and miR-183 could be promising biomarkers for clinical diagnosis and treatment of COPD.

Conflict of interest

All authors declare no financial or non-financial competing interests.

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