rs2910164 Polymorphism Confers a Decreased Risk for Pulmonary Hypertension by Compromising the Processing of microRNA-146a

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Key Words
rs2910164 • miR-146a • COX2 • PGI2 • Pulmonary hypertension

Abstract
Objective: To identify the association between rs2910164 polymorphism and development of pulmonary hypertension, as well as underlying molecular mechanism. Methods and Results: 281 patients diagnosed with pulmonary hypertension and 325 normal controls were recruited, and rs2910164 genotype was determined in each participant: As a result, the rs2910164 polymorphism was significantly associated with the development of pulmonary hypertension after adjusting some potential confounding factors. Additionally, lung tissue samples were obtained from 39 patients who received surgical intervention for lung cancer, and mRNA and protein expression levels of miR-146a, COX-2 and PGI 2 production were examined. Furthermore, we confirmed COX-2 is a target of miR-146a in pulmonary smooth muscle cells, and identified a differentially expressed miR-146a and COX-2 in each rs2910164 genotype group. We observed a significant association between rs2910164 polymorphism and the levels of either COX-2 or PGI 2 using real-time PCR and western blot. In conclusion, the results of this study demonstrate that the rs2910164 CC and GC genotype is associated with a decreased risk of pulmonary hypertension, which could be attributed to defective miRNA processing and compromised ability to inhibit production of COX-2 and PGI 2.

Introduction
Pulmonary arterial hypertension (PAH) is a serious and sometimes life-threatening medical disorder. PAH is characterized by the development of pulmonary vascular resistance...
that results in an elevation of right ventricle afterload and leads to right heart failure [1]. The etiopathogenesis of PAH has been associated with chronic hypoxemia disorders such as interstitial lung disease and chronic obstructive pulmonary disease [2]. A growing body of evidence suggests that the proliferation of pulmonary artery smooth muscle cells (PASMCs) plays a crucial role in the development of PAH by contributing to pulmonary vascular remodeling and elevated resistance [3]. Thus, it would be of great significance to identify the molecular mechanisms underlying the proliferation of PASMCs. This may, in turn, improve our understanding of the pathogenesis and help to identify new therapeutic targets.

Prostacyclin (prostaglandin $I_2$ or PGI$_2$), a potent vasodilator, has been proven to be clinically effective in the treatment of PAH [4]. Endogenous production of PGI$_2$ is primarily catalyzed by cyclooxygenase-2 (COX-2), which has been reported to be significantly downregulated in hypoxia-induced PAH [2]. A study by Li et al. indicated that hypoxia-induced proliferation of PASMCs and vascular remodeling were abrogated by an administration of H$_2$S, which substantially promoted production of COX-2/PGI$_2$ [3].

MicroRNAs (miRNAs) are a class of approximately 22-nucleotide non-protein coding RNAs that bind to the 3’ untranslated region (UTR) of target gene messenger RNA (mRNA) and regulate up to one third of all protein-coding genes, which can result in translational repression and mRNA degradation [5]. Studies have shown that miRNAs are functionally involved in the control of a variety of biological processes including the cell cycle, differentiation, inflammation, and proliferation [5]. Over time, data have increasingly indicated that variants in miRNA sequences may trigger disease by altering the expression or maturation of miRNAs or by interfering with miRNA interaction with mRNA [6, 7]. An rs2910164 polymorphism in miR-146a precursor has been associated with various malignancies [8-14]. Jazdewski et al. [8] reported that a C allele of rs2910164 may compromise the mature processing of miR-146a and prevent the suppression of target genes including interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis receptor-associated factor 6 (TRAF6).

Based on the above evidence, our hypothesis was that the miR-146a rs2910164 polymorphism may negatively affect the expression of the target miRNA and cause an aberrant upregulation of COX-2 which is known to protect the carrier against the development of PAH. To test this hypothesis, we assessed the inhibitory effects of different miR-146a genotypes on expression levels of COX-2 and the production of PGI$_2$ in lung tissues collected from patients with PAH compared with normal controls. We also evaluated the association between miR-146a rs2910164 polymorphisms and the risk of PAH.

**Materials and Methods**

**Patients**

A total of 281 subjects diagnosed with PAH and 325 age- and sex-matched normal controls were enrolled in our hospitals between Jan 2011 and Dec 2013. Lung tissue samples were collected from 39 patients who received surgical intervention for lung cancer in Xijing Hospital, Fourth Military Medical University. The samples were collected from regions of the pulmonary parenchyma in an area that appeared healthy and were far enough from the tumor that the sample was free of pleura or large airways (at least 2 cm from the tumor). Five milliliters of peripheral blood was obtained from all the subjects. All subjects were of Han ethnicity. The study protocol was approved by investigational review committees at Fourth Military Medical University, and a written informed consent was obtained from each participant. Interviewer-administered questionnaires were conducted to collect demographic data and information on known risk factors.

**Determination of PGI$_2$**

Production of PGI$_2$ in lung tissues was measured using an enzyme immunoassay (Four seasons biotechnology, Beijing, China) according to instructions provided by the manufacturer.
**Real-time PCR**

A miRNeasy kit (Qiagen, Valencia, CA) was used to isolate total RNA. A nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE) was used to determine the content and purity of the isolated RNA. A SuperScript III (Invitrogen, Carlsbad, CA) was used to synthesize cDNA from 1 µg of RNA per sample. A thermal cycler (MJ Research, Watertown, MA) was used to analyze COX-2 and U6. For real-time PCR, reverse transcription of the total RNA was performed at 25 °C for 5 min and at 42 °C for 30 min using a 10 µl reaction mixture of iScript II reverse transcription supermix (Bio-Rad Laboratories, Mississauga, Ontario). Expression of miRNA and mRNA was assessed by a two-step TaqMan®RT-PCR (Applied Biosystems, Carlsbad, CA). A small nuclear RNA (snRNA) known as a U6 snRNA was used as an internal control for the miRNA analysis. Expression of miRNA was standardized to the U6 snRNA level, and a $2^{-\Delta\Delta C_{\text{t}}}$ method was used to determine the relative expression levels.

**Genotyping of miR-146a rs2910164 polymorphisms**

A DNA extraction kit (Invitrogen, Carlsbad, CA) was used to isolate genomic DNA. The DNA was stored at -20°C for future use. The chromosome segment containing the rs2910164 polymorphism was PCR-amplified using the following primer set: forward, 5’-ATT TTA CAG GGC TGG GAC AG-3’ and reverse, 5’-TCT TCC AAG CTC TTC AGC AG-3’. The genotyping was performed using direct sequencing.

**Cell culture**

Human PASMCs were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and maintained in DMEM high glucose medium containing 10% FBS at 37°C with 5% CO₂ and 95% air. The cells were passaged every 2 days. miR-146a analogs (5’-ugagaacugaauuccauggguu-3’), suppressants (5’-aacccauggaauucaguucuca-3’) and anti-COX2 siRNA (5’-ccaagatagtgatcgaagacta-3’) were purchased from Ribobio (Guangzhou, China). The oligonucleotides were transfected into the PASMCs using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Measurement of cell proliferation**

Cell viability was evaluated based on the instructions enclosed with the CCK-8 solution. Briefly, 5,000 cells/well were inoculated in 96-well plates and grown to approximately 60-70% before transfection. Forty-eight hours after the transfection, the CCK-8 solution (10 μl) was diluted with FBS-free DMEM high glucose medium (100 μl) at a ratio of 1:10, added to each well and incubated at 37°C for 3 h. The absorbance (A) was determined at 450 nm using a microplate reader (Molecular Devices, LLC, Silicon Valley, CA USA). Experiments were repeated 4 times.

**Western blot analysis of protein expression**

Equal amounts of lysates were loaded onto a 12% SDS-PAGE following a heat-induced denaturation for 5 min at 100°C. The separated protein was then transferred into PVDF membranes. The membranes were then blocked with 5% BSA in TBS-T and incubated with primary antibodies against COX-2 or β-actin at 4°C overnight. The membranes were washed with TBS-T three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, signals were detected using an enhanced chemiluminescence system (Applygen Technologies, Beijing, China), and Image J 1.47 software was used to calculate integrated optical density of protein bands.

**Luciferase assay**

PASMCs were plated at a density of 5.5×10³ cells/well in a 96-well plate and incubated for 24 h. Using lipofectamine 2000, the cells were co-transfected with a luciferase plasmid containing a wild-type, mutant COX-2 3’UTR or Renilla luciferase plasmid (as a control for transfection efficiency) and control miRNA which were miR-146a mimics using lipofectamine.
2000 (Invitrogen, Carlsbad, CA). After 48 h of incubation, luciferase activity was assayed using a Steady Glo Luciferase Assay System (Promega, Madison, WI, USA).

**Apoptosis assay**

Forty-eight hours after transfection, the PASMCs were incubated with 2% H₂O₂ for 12 h. Then, the cells were harvested and resuspended in phosphate-buffered saline (PBS) followed by fixation in ethanol at room temperature overnight. The cells were next washed with PBS and resuspended in a staining solution (50 mg/ml propidium iodide, 1 mg/ml RNase A and 0.1% Triton X-100 in PBS, all purchased from Invitrogen, Carlsbad, CA). The stained cells were then analyzed for apoptosis using a FACSCanto II (BD Biosciences, San Jose, CA).

**Statistical analysis**

Statistical analysis was performed using SPSS 19.0 (IBM, Chicago, IL). Differences between the treatment groups containing more than two elements were assessed using a one-way ANOVA, followed by a Newman-Keuls multiple comparisons test. Analysis of the groups containing two elements was performed using Student’s two-tailed unpaired t-test. The results were expressed as the mean ± standard deviation. Logistic regression analysis was used to adjust the potential risk factors. A p value < 0.05 was considered statistically significant.

**Results**

Two hundred eighty-one patients diagnosed with PAH and 325 normal controls were recruited for this study. The demographic and clinicopathological characteristics of the participants, such as age, sex, height, weight, smoking index, and lung function, are described in Table 1. The genotype frequency of the rs2910164 polymorphism in the control group followed a Hardy-Weinberg equilibrium, whereas the genotype frequency in the PAH patient group did not follow the equilibrium. No difference was noted between the case and control groups in sex, age, height, weight, or smoking status. Using a chi-squared analysis, we found that the CC genotype of rs2910164 was significantly associated with a decreased risk of PAH when GG (OR=0.44, 95% CI: 0.26-0.77) and GG/GC (OR=0.47, 95% CI: 0.28-0.78) were set as the reference. It remained significant after adjusting for potential risk factors such as age, sex, height, weight, lung function and smoking status.

Lung tissue was collected from 39 participants who received a surgical intervention to treat lung cancer, and the expression of the miR-146a and the rs2910164 genotypes (GG:12, GC:16, CC:11) were determined in those samples. Using real-time PCR, we found that the expression level of miR-146a was comparable between the individuals carrying the rs2910164 GG and GC genotypes. Both of these genotypes had a significantly elevated expression of miR-146a compared with the CC

| Genotype     | PH (N=281) | NC (N=325) | P value |
|--------------|------------|------------|---------|
| Age (years)  | 60.3±7.4   | 59.8±8.1   | NS      |
| Gender (M/F) | 192/89     | 213/112    | NS      |
| Height (cm)  | 162.2±7.2  | 163.3±7.8  | NS      |
| Weight (kg)  | 64.2±6.8   | 66.2±6.4   | NS      |
| Smoking (pack*years) | 46.4±6.3 | 23.4±3.8 | < 0.01 |
| FVC (% of predicted value) | 82.5±6.7 | 90.3±6.3 | < 0.01 |
| FEV₁ (% of predicted value) | 73.2±5.4 | 83.4±6.6 | < 0.01 |
| DLCO (% of predicted value) | 70.1±5.4 | 82.6±5.2 | < 0.01 |
| Genotype     |            |            |         |
| GG (reference)| 132 (46.9%)| 133 (40.9%)|         |
| GC           | 126 (44.8%)| 140 (43.1%)| 0.563   |
| CC           | 23 (8.3%)  | 52 (16.0%) | 0.0038  |
| Combined     |            |            |         |
| GG+GC (reference) | 258 (91.7%)| 273 (84.0%)|         |
| CC           | 23 (8.3%)  | 52 (16.0%) | 0.0042  |
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The rs2910164 G allele showed a dominant influence on the expression of miR-146a in our lung tissue samples, as depicted in Figure 1A.

Based on the results of an in-silicon analysis, COX-2 was identified as a virtual target gene of miR-146a (Fig. 2A), which was confirmed by the results of the luciferase reporter assay. The luciferase activity in the PASMCs cotransfected with the miR-146a mimics and vector-containing wild-type 3'UTR of COX-2 was substantially lower than the control. The luciferase

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**Fig. 1.** A. Expression level of miR-146a (lung tissues) in each genotype group; B. mRNA expression level of COX2 (lung tissues) in each genotype group.

**Fig. 2.** A. Schematic comparison of the "seed sequence" in 3' UTR of COX2 and hsa-miR-146a; B. Hsa-miR-146a could suppress the luciferase activity of WT1 COX2 3'UTR but not mutant COX2 3'UTR.

**Fig. 3.** A. Protein expression level of COX2 (lung tissues) in each genotype group; B. Relative density of western blot in A by densitometry analysis. C. PGI₂ production level (lung tissues) in each genotype group.

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activity in the cells transfected with the miR-146a mimics and vector-containing mutant 3’UTR of COX2 showed no difference compared with the control, as depicted in Figure 2B. We examined the expression levels of COX-2 and PGI\(_2\) in the lung tissue samples and found that, consistent with the pattern of miR-146a, significantly higher levels of COX2 mRNA/protein expression and PGI\(_2\) were noted in individuals with the CC genotype compared with GG or GC, as depicted in Figures 1B, 3A and 3B. Additionally, we found that the protein expression level of COX-2 was significantly higher in the CC than GG and GC genotype groups using an immunohistochemistry analysis (Fig. 4).

To further confirm the miRNA-mRNA regulatory relationship, we transfected the PASMCs with either the miR-146a mimics, its inhibitors or anti-COX-2 siRNA. As depicted in Figure 5, we examined the expression level of COX-2 using real-time PCR and a western blot. We showed that the upregulation of miR-146a caused by transfection of the mimics substantially suppressed both mRNA/protein expression of COX-2 and production of PGI\(_2\) in the human PASMCs. The inhibitory effect was similar to the anti-COX-2 siRNA of the same concentration. Consistently, the downregulation of miR-146a by the transfection of its inhibitors significantly promoted mRNA/protein expression levels of COX-2 and production of PGI\(_2\) in the PASMCs (Figs. 5A, B and C).
Additionally, we evaluated the viability and apoptosis status of the cells with various treatments. We found that a decrease in COX-2 expression by the transfection of miR-146a mimics or anti-COX-2 siRNA significantly promoted the viability of PASMCs by inhibiting apoptosis. An increase in COX-2 by the transfection of miR-146a inhibitor substantially suppressed the proliferation of the cells by inducing apoptosis (Fig. 6).

**Discussion**

Numerous miRNAs and genes have been reported to be associated with the development of PAH [15-18]. In this study, we performed a case-control association study to identify the relationship between the rs2910164 polymorphism and risk of PAH. We showed that the CC genotype of the rs2910164 polymorphism was significantly associated with a decreased risk of PAH after adjusting for several known risk factors. Additionally, we validated COX-2 as a target of miR-146a in PASMCs and determined the differential expression of COX-2 and miR-146a in each rs2910164 genotype group. Furthermore, we showed that rs2910164 in miR-146a was significantly correlated with the levels of both PGI₂ and COX-2. The expression levels of miR-146a are closely correlated with the viability and apoptosis status of PASMCs.

Accumulating evidence suggests that miRNAs function as key regulators in a wide range of biological processes such as proliferation, differentiation, tissue fibrosis and repair [19-21]. Variants in miRNAs have been reported to be correlated with a number of human diseases [22-24]. As shown by Jazdzewski et al, a C allele rs2910164 polymorphism significantly compromises the processing and maturing of miRNA and results in an approximately 2-fold decrease in production of mature miR-146a than a G allele in a thyroid cell line [25]. A C allele of an rs2910164 polymorphism presents approximately 60 bp away from the first nucleotide on the passenger strand of pre-miR-146a and causes mispairing within the mature hairpin. In this study, the expression level of miR-146a was determined in the lung...
tissues harvested from the 39 participants genotyped as GG (n=12), GC (n=16), or CC (n=11). We found that the expression level of miR-146a was comparable between the individuals carrying the rs2910164 GG and GC genotypes. Both of these genotypes had significantly elevated expressions of miR-146a compared with the CC genotype. Moreover, we performed a computational analysis and identified COX-2 as a potential target of miR-146a, which was further confirmed by the luciferase reporter assay.

Prostanoids are part of a family of oxygenated fatty acids that originate from arachidonic acid. Arachidonic acid is mainly derived from the phospholipids of a cell membrane and is primarily catalyzed by phospholipases. Arachidonic acid is converted to PGH$_2$ by cyclooxygenase (COX) and then used to synthesize individual prostanoids such as PGD$_2$, PGF$_2$, PGE$_2$, TXA$_2$, and PGI$_2$. In total, there are eight prostanoid receptors. They can be classified into three categories based on their function, which is mostly determined by the different members of the G protein-coupled receptor family they activate and the reactions they trigger [26]. PGI$_2$ functions as a potent vasodilator by increasing intracellular cAMP [27]. In this study, we validated COX-2 as a target of miR-146a in PASMCs and showed that the expression level of COX-2 and production of PGI$_2$ were negatively correlated with the expression level of miR-146a. It has been shown that G protein-coupled receptors activated by cAMP stimulate mitogenesis partly via extracellular signal-regulated kinases (ERKs), which are members of the mitogen-activated protein (MAP) kinase family [28]. MAP kinases, in turn, phosphorylate and regulate the activity of nuclear proteins and key enzymes, which eventually regulate the expression of the genes that are critical in controlling cell proliferation [28]. Additionally, G protein-coupled receptors can also activate other members of the MAP kinase family to regulate apoptosis. As a result, activation of G protein-coupled receptors may induce programmed cell death [28]. In this study, we found that the decrease in COX-2 expression by transfection of miR-146a mimics or anti-COX-2 siRNA significantly promoted the viability of PASMCs by inhibiting apoptosis, while increase of COX2 by transfection of miR-146a inhibitor substantially suppressed the proliferation of the cells by inducing apoptosis (Fig. 6).

Despite showing that miR-146a acts as a principal regulator of COX-2 expression in PASMCs and that rs2910164 polymorphisms are significantly associated with the risk of PAH, the complicated nature of the disease also suggests that the disease pathogenesis might be heterogeneously associated with multiple variants at different gene loci, similarly to other polygenic medical disorders. The effect of an rs2910164 polymorphism on the susceptibility of human disease might be associated with genetic backgrounds and residential environment [29]. The conflicting result of the association between rs2910164 and PAH could be attributed to the distinct genotype frequency in different ethnic populations (e.g., the rs2910164 G allele in Caucasians: 76.58 vs Asians: 47.88%) [30]. Several other polymorphisms within the pathway of COX-2 production, including rs20417, rs20432, rs5275 and rs4648310, have been proven to be functional and are associated with a risk of various human diseases [31-38]. They might also be responsible for the development of PAH. Therefore, further studies involving a more comprehensive panel of polymorphisms in different ethnic populations are warranted to determine the underlying molecular mechanism of PAH. Additional functional analyses are necessary to support the results of the current study in an animal model.

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Disclosure Statement

The authors claim no conflict of interest
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