

A single nucleotide polymorphism in hsa-miR-146a is responsible for the development of bronchial hyperresponsiveness in response to intubation during general anesthesia

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Abstract. Bronchial hyperresponsiveness (BHR) is the most common clinical manifestation identified in asthmatic patients, and intubation is the major factor that stimulates the airway of patients receiving general anesthetic. In the present study, nitric oxide synthase 1 (*NOS1*) was identified as a target gene of micro (mi)R-146a using *in silico* analysis and luciferase assay. Furthermore, the regulatory role of miR-146a was demonstrated by the observation that the *NOS1* expression level in pulmonary artery smooth muscle cells (PASMCs) transfected with miR-146a mimics was significantly downregulated and the *NOS1* expression level in PASMCs transfected with miR-146a inhibitors was significantly upregulated. Additionally, it was identified that a polymorphism in pri-miR-146 interfered with mature processing and reduced the quantity of mature miRNA. To assess the association between the polymorphism and the development of BHR, 563 patients with basic pulmonary diseases, such as asthma, emphysema or bronchitis were enrolled in the present study. Each participant received a general anesthetic and the development of BHR was evaluated. The miR-146a rs2910164 polymorphism CC genotype was identified to be significantly associated with an increased risk of BHR in response to intubation when compared with the GG or GC genotype (odds ratio, 0.38; confidence interval, 0.18-0.78). These findings indicate that the miR-146a rs2910164 polymorphism is associated with a decrease risk of BHR, and the CC genotype increased the level of *NOS1* expression, which was physiologically inhibited by wild-type miR-146a.

Introduction

Bronchial hyperresponsiveness (BHR), defined as an exaggerated sensitivity to a variety of physical, chemical or pharmacologic stimuli, is characterized by episodes of intermittent airflow obstruction caused by bronchospasm, which is also an important clinical feature of asthma, and may be apparent in certain other pulmonary diseases, such as chronic bronchitis and emphysema (1). In general, BHR occurs in response to a lower level of stimulus and is increased in magnitude when compared with healthy individuals. BHR is the most common clinical manifestation that is identified in asthmatic patients, and intubation is the major factor that stimulates the airway of the patients who receive general anesthesia (2). BHR is not uncommon and its prevalence in the general population has been estimated to be 13% in the general Chinese population (2). Additionally, BHR is reported in asymptomatic individuals and those individuals are considered to be susceptible to the development of certain medical disorders that are characterized by bronchospasms, such as in asthma (3). BHR in response to intubation represents a major risk, and in certain instances is life-threatening, to those patients who receive general anesthesia (2). Therefore, it is important to establish a biomarker to effectively predict the occurrence of bronchospasm during general anesthesia, which may facilitate the necessary actions to prevent BHR.

The neuronal nitric oxide synthase 1 gene, *NOS1* is responsible for the development of certain types of bronchospasm-associated airway obstruction, and was demonstrated to upregulate the expression level of *NOS2*, another regulatory factor that is involved in regulating the balance between bronchoconstriction and bronchodilation (4). The production of nitric oxide (NO) catalyzed by *NOS1*, is physiologically associated with bronchospasm as a neurotransmitter for non-adrenergic non-cholinergic (NANC) nerves (5). NANC mechanisms are believed to be essential in bronchoconstriction and bronchodilation control, and a defect in NANC bronchodilation underlies the molecular mechanism of BHR (6).

Micro (mi)RNAs are small non-coding RNA molecules, ~22 nucleotides long, and they function as inhibitors to suppress the expression of ≤30% human protein-coding genes at the post-transcriptional level by either translational repression or messenger (m)RNA degradation (7). Increasing evidence

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demonstrates that miRNAs are involved in controlling the balance between bronchoconstriction and bronchodilation (7,8).

MiR-146 is a family of miRNA precursors found in mammals, including humans. MiR-146 predominantly participates in the regulation of inflammation and other processes that are associated with the innate immune system (9). A previous study revealed that the minor C allele of the miR-146a rs2910164 polymorphism leads to a reduction in the expression of its mature form by causing mispairing within the miR-146a hairpin, resulting in a wide range of functional alterations (10). The majority of miR-146a rs2910164 studies were regarding the risk of various types of cancer (11-13), and only one described an association with a protection mechanism against asthma in an Asian population (14).

As *NOS1* functions as a regulator of bronchospasms and *NOS1* is a potential target of miR-146a, the miRNA/mRNA association was verified. In addition, the association between the miR-146a rs2910164 polymorphism and BHR in response to intubation in patients exhibiting basic pulmonary disease (such as asthma, emphysema and bronchitis) who received general anesthesia was verified.

Materials and methods

Participants. As shown in Table I, a total of 563 patients exhibiting basic pulmonary diseases, such as asthma, emphysema and chronic bronchitis, who received a general anesthetic (including intubation) prior to surgical intervention at the First Hospital of Dalian Medical University (Dalian, China) between September 2013 and September 2014 were enrolled in the present study. Tissue samples were snap-frozen immediately with liquid nitrogen, stored carefully, and subsequently were used to extract DNA and RNA for molecular analysis. Those patients who exhibited sinus bradycardia, atrioventricular block (degree II or III), and/or sick sinus syndrome were excluded from the present study. The study was approved by the Research Ethics Committee in China Medical University and informed consent was obtained from each patient.

Assessment of BHR. To assess BHR in response to intubation, auscultation was performed on either side of the chest at the second intercostal space in the parasternal line, the fifth intercostal space in the midclavicular line, the fourth intercostal space in the midaxillary line, prior to intubation and anesthesia and 5 min after intubation. BHR was diagnosed by the presence of wheezing, which was evaluated by an anesthesiologist blinded to the protocol. Wheezing was defined as high-pitched expiratory rhonchi that were audible in at least three of six auscultation sites.

Genotyping analysis. Genomic DNA was isolated from blood samples (5 ml) obtained between September 2013 and September 2014 using a Qiagen Genomic DNA extraction kit (Qiagen, Inc., Valencia, CA, USA). The rs2910164 G/C polymorphism was genotyped in duplicate using the TaqMan® Allelic Discrimination assay (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an ABI Prism® 7900 system (Applied Biosystems). In addition, the genotyping results were confirmed by direct sequencing in 10 random samples including five homozygote and five heterozygote individuals,

using the following primers: Forward, 5'-ATTTTACAGGGC TGGGACAG-3' and reverse, 5'-TAGCAGCAGCAGCAA GAGAG-3'.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using miRNeasy (Qiagen, Inc.). Trizol (Gibco; Thermo Fisher Scientific, Inc.) was used to isolate the total cellular RNA from tissue samples and PTENCE cells, according to the manufacturer's protocol, and subsequently RNeasy Qiagen columns (Qiagen Inc.) were used to purify the RNA. The RNA concentration was examined and the integrity was evaluated by agarose gel electrophoresis (gels run at 100 V for 1 h; agarose gel purchased from Invitrogen; Thermo Fisher Scientific, Inc., agarose gel electrophoresis equipment purchased from Sigma-Aldrich, St. Louis, MO, USA). A TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize the copy (c)DNA (CANCAC1) from RNA with a mixture of 2 µg total RNA, 1 µl miRNA-specific primers (25 µM) and doubly distilled (dd)H₂O (RNase-free) to a final volume of 10 µl. Subsequently, the mixture was denatured for 10 min at 70°C prior to the mixture being placed on ice, followed by the addition of the reaction buffer, including 11 µl ddH₂O (RNase-free), 4 µl deoxynucleotide phosphates (dNTPs) mix, 4 µl 5xRT buffer and 1 µl ReverTra Ace (100 units/µl; Toyobo Co., Ltd., Osaka, Japan). The mixture was then maintained for 60 min at 42°C, followed by an incubation for 10 min at 90°C. RT-qPCR was performed using an Applied Biosystems 7500 Sequence Detection system (ABI7500 SDS; Applied Biosystems, Thermo Fisher Scientific, Inc.). A TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize the cDNA and RT was performed using 1 µg total RNA. Quantitative amplification was performed using a Roche LightCycler480-II real-time thermal cycler (Roche Diagnostics) to determine the relative expression level of miR-146a and *NOS1* using RT² SYBR green/fluorescein qPCR Mastermix (Qiagen, Inc.). The experiment was repeated at least three times. SnoNA U6 served as an internal marker, and the 2^{-ΔΔC_q} method (15) was used to evaluate the results.

Cell culture, and mimic and inhibitor miRNA transfection. Human pulmonary artery smooth muscle cells (PASMCs) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and added to Dulbecco's modified Eagle's medium high glucose medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. The cells were passaged every 2 days. The miR-146a-5p miRIDIAN duplex mimics, hairpin inhibitors and negative control duplex mimics were purchased from Thermo Fisher Scientific, Inc., and the oligonucleotides (Takara Biotechnology Co., Ltd., Dalian, China) were transfected into human PASMCs by reverse transfection using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Western blotting. Total protein was separated by 10% SDS-PAGE (Roche Diagnostics, Basel, Switzerland) and the separated protein was transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked using 5% non-fat milk in phosphate-buffered saline (Invitrogen; Thermo Fisher Scientific, Inc.). Anti-NOS1 anti-

Table I. Demographic data and clinical characteristics of study participants.

Characteristic	BHR ⁺ (n=138)	BHR ⁻ (n=425)	P-value
Age (years)	56.3±9.7	58.4±10.1	NS
Gender (M/F)	72/66	276/149	NS
Height (cm)	163.3±7.5	164.1±10.8	NS
Weight (kg)	63.5±7.4	64.3±9.4	NS
Smoking status			
Current smoker	34	42	
Ex-smoker	52	89	
Non-smoker	52	294	<0.05
Basic pulmonary disease			
Emphysema	36	146	
Asthma	63	187	
Chronic bronchitis	39	92	<0.05
FVC (% of predicted value)	62.3±7.5	66.2±7.3	<0.01
FEV ₁ (% of predicted value)	43.6±5.3	53.5±5.9	<0.01
DLCO (% of predicted value)	66.4±5.5	69.4±6.2	<0.01

Data are shown as the means ± standard deviation. BHR, bronchial hyper-responsiveness; M, male; F, female; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 sec; DLCO, diffusing capacity of the lungs for carbon monoxide; NS, not significant.

Table II. Association between genotype frequency and the presence of BHR.

Variable	BHR ⁺ (n=138; %)	BHR ⁻ (n=425; %)	P-value
Genotype			
GG (reference)	60 (44)	167 (39)	
GC	69 (50)	192 (45)	0.999
CC	9 (6)	66 (16)	0.012
Combined			
GG + GC (reference)	129 (94)	359 (84)	
CC	9 (6)	66 (16)	0.009

BHR, bronchial hyper-responsiveness.

body and anti-β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the membrane was incubated with the primary antibody for 2 h at room temperature (dilution, 1:1,000). A secondary antibody (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:5,000, and incubated for 2 h at room temperature. The fluorescent signal was detected using an ECL detection kit (Applygen Technologies, Inc., Beijing, China). The integrated intensities of the protein bands were expressed relative to the protein levels of the control.

Luciferase reporter assay. A *NOS1* wild-type 3'-untranslated region (UTR) was amplified by PCR as described above and cloned into a pcDNA3 (Promega Corporation, Madison, WI, USA) that contained a firefly luciferase reporter gene, at a position downstream of the luciferase reporter. The vectors were designated as wild-type 3'UTR of *NOS1*. Site-directed mutagenesis was used to introduce a mutation to replace the wild-type seed sequence in the 3'UTR using the Site-Directed Mutagenesis kit

(Beijing SBS Genetech Co., Ltd., Beijing, China) and designated as mutant 3'UTR of *NOS1*. Human PSMCs were seeded in a 48-well plate, and transfected with miR-146a mimics and the wild-type or mutant 3'UTR of *NOS1*. pRL-TK (5 ng), a plasmid expressing *Renilla* luciferase (Promega Corporation), was also co-transfected and served as an internal control. After 48 h, the cells were collected and the luciferase reporter assay was performed in a TD-20/20 luminometer (Promega Corporation).

Statistical analysis. Deviations from Hardy-Weinberg equilibrium (HWE) were tested for using the χ^2 test for observed and expected values with the degrees of freedom adjusted by the number of estimated independent frequencies. Characteristics of the case and the control sample were compared using χ^2 test (binary variables), and Student's t-test or one-way analysis of variance (binary variables). To adjust for additional covariates, the data were reanalyzed by multiple logistic regression with BHR as the outcome variable. P<0.05 was considered to

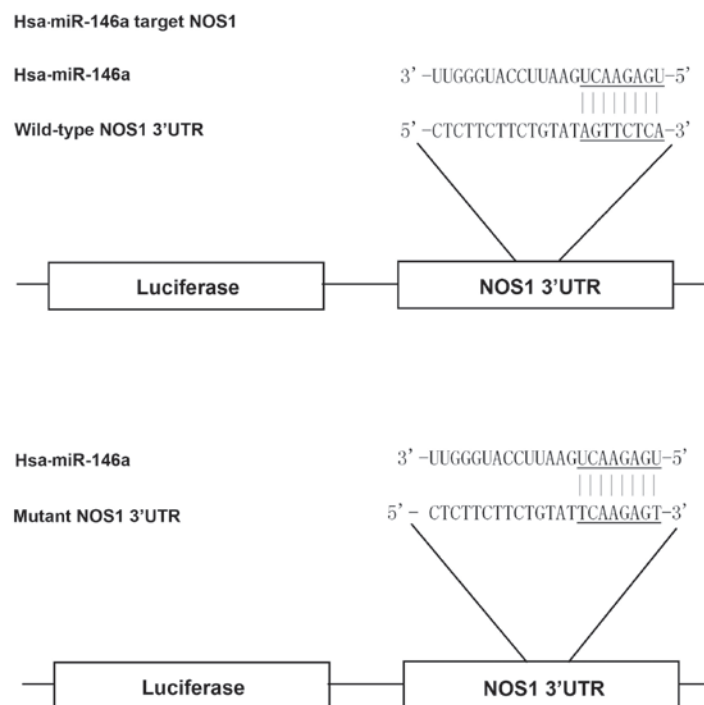


Figure 1. Sequence depiction of mature miR-146a, wild-type and mutant *NOS1*. *NOS1*, nitric oxide synthase 1; UTR, untranslated region.

indicate a statistically significant difference. For an identified association, the odds ratio (OR) and its 95% confidence interval (CI) was computed as an approximation of the relative risk. The statistical analyses were conducted using SPSS 21.0 software package (IBM SPSS, Armonk, NY, USA).

Results

Participant characteristics. In the present study, a total of 563 patients with basic pulmonary diseases, such as asthma, emphysema and chronic bronchitis who received a general anesthetic (including intubation) prior to surgical intervention at the First Hospital of China Medical University were enrolled, and the polymorphisms in the current study were in HWE ($P > 0.05$). A total of 138 were positive for BHR and 425 were negative. No difference was identified regarding age, gender, height, weight and presence of emphysema, asthma and chronic bronchitis between the BHR-positive and -negative groups (Table I). Additionally, these variables were included in the multivariate logistic regression analysis to evaluate the potential effects on the association between the rs2910164 G/C polymorphism and the risk of BHR in response to intubation in patients who received a general anesthetic.

Association between rs2910164 G/C polymorphism and presence of BHR in response to intubation. The genotype frequency of the rs2910164 G/C polymorphism among the BHR-positive and -negative groups, and the association with the presence of BHR are presented in Table II. The frequencies of the GG, GC and CC genotypes were 44, 50 and 6%, respectively, in the BHR-positive group, and 39, 45 and 16%, respectively, in the BHR-negative group. When the GG and GC genotype was used as the reference, it was noted that the

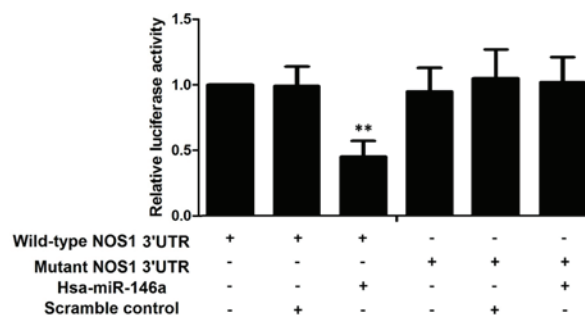


Figure 2. Luciferase assay demonstrated that miR-146a mimics over-expressing pulmonary artery smooth muscle cells and transfected with wild-type *NOS1* exhibited a significantly lower luciferase activity compared with those cells transfected with mutant *NOS1* (** $P < 0.01$). *NOS1*, nitric oxide synthase 1; UTR, untranslated region.

CC genotype was associated with a significantly increased risk of BHR (OR, 0.38; 95% CI, 0.18-0.78).

Effect of the rs2910164 G/C polymorphism on EGFR expression in lung cancer cells. Based on the computational analysis, *NOS1* was identified as a potential target gene of miR-146a (Fig. 1). To evaluate whether miR-146a targets the *NOS1* 3'UTR in PSMCs, reporter vectors carrying wild-type or mutant *NOS1* 3'UTR were constructed, as demonstrated in Fig. 1. The reporter vectors were subsequently used for transient transfection in PSMCs together with miR-146a mimics or scramble controls. As shown in Fig. 2, only the luciferase activity from the cells co-transfected with wild-type *NOS1* 3'UTR and miR-146a mimics was identified to be significantly lower than the control, whereas all other groups were compa-

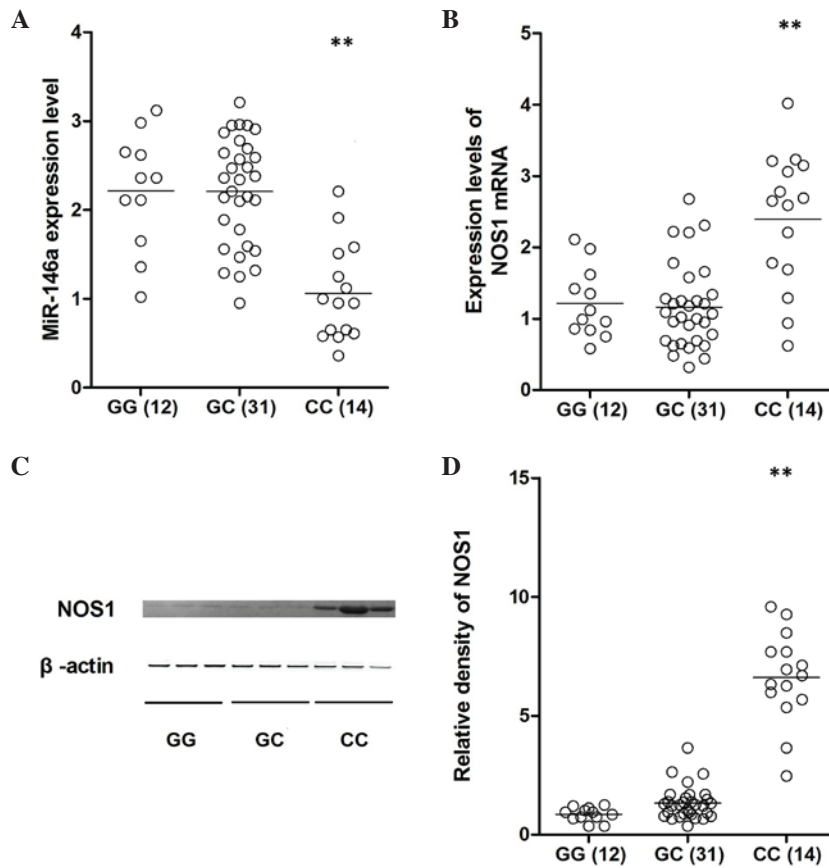


Figure 3. (A) miR-146a expression levels were comparable between the GG and GC genotype groups, and the two were significantly higher than that of CC group. (B) mRNA expression levels of *NOS1* were significantly lower in the GG and GC group when compared with the CC group. (C) Protein expression levels of NOS1 were significantly lower in the GG or GC group than in CC group as determined by western blot analysis. (D) Relative density of western blot results (** $P < 0.01$). NOS1, nitric oxide synthase 1.

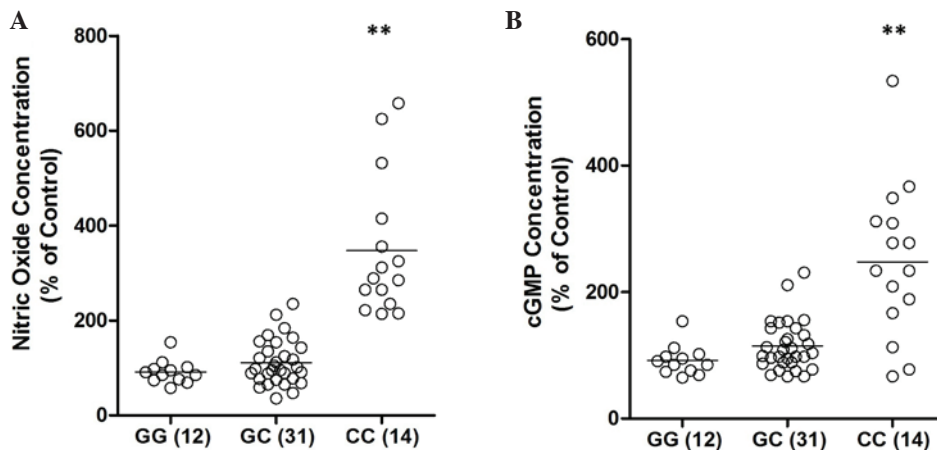


Figure 4. (A) Nitric oxide production was significantly lower in the GG and GC group when compared with the CC group. (B) cGMP levels were significantly lower in the GG or GC group than in the CC group (** $P < 0.01$). cGMP, cyclic guanosine monophosphate.

table. The results confirmed that *NOS1* was a valid target of miR-146a in PSMCs.

Determination of miR-146a and *NOS1* expression patterns in lung tissue samples with different genotypes. Lung tissue samples of three different genotypes (GG, $n=12$; GC, $n=31$; CC, $n=14$) were used to further investigate the impact of the polymorphism on *NOS1* expression levels. Using qPCR analysis, the

expression of miR-146a was identified to be comparable between the GG and GC groups, which were substantially higher than that of the CC group (Fig. 3A). In addition, the mRNA and protein expression levels of *NOS1* were determined in all genotype groups using qPCR and western blotting, which indicated that the level of *NOS1* expression was similar between the GG and GC groups, and the two were significantly lower than that of the CC group (Fig. 3B-D). To further confirm the effect of

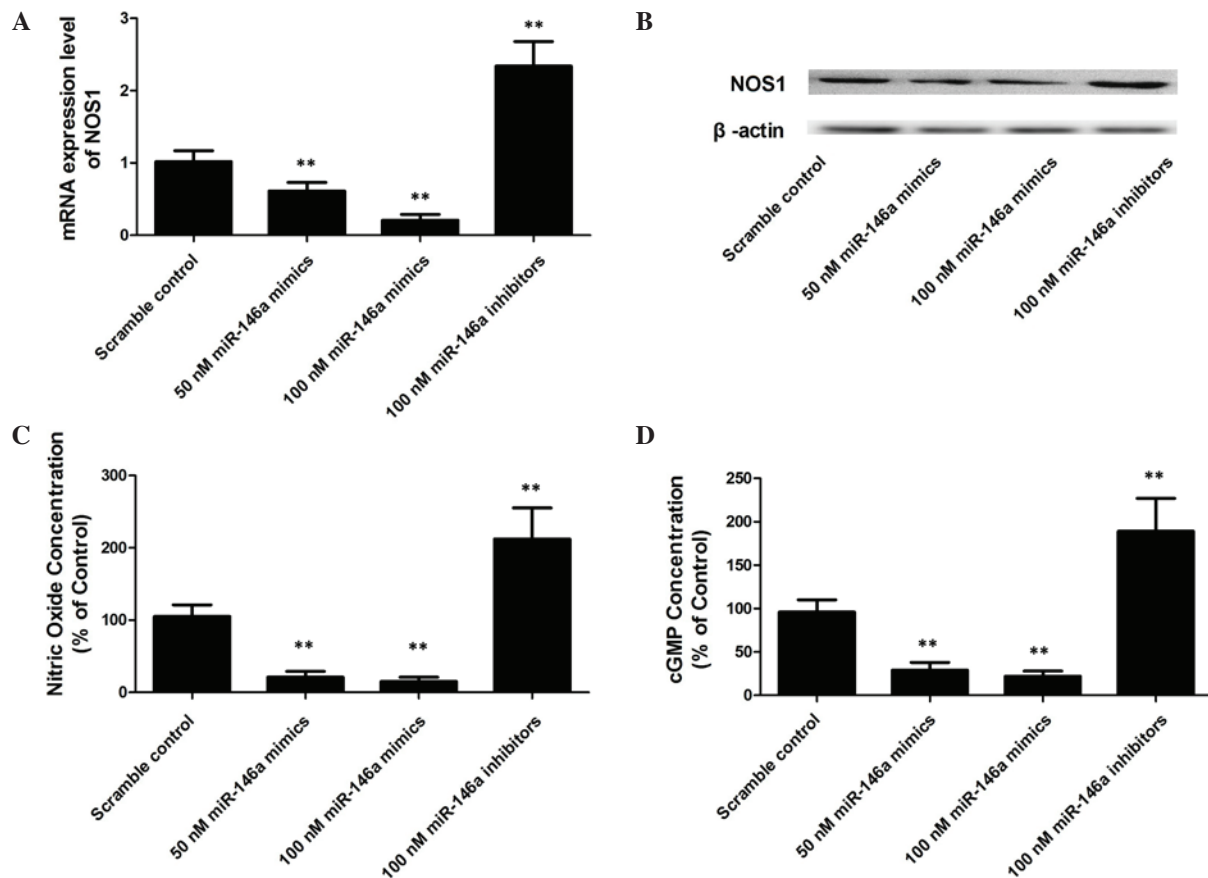


Figure 5. (A) mRNA expression of *NOS1* was significantly reduced in the PSMCs transfected with miR-146a mimics, but significantly higher in PSMCs transfected with miR-146a inhibitors. (B) Protein expression of NOS1 was significantly reduced in the PSMCs transfected with miR-146a mimics, but significantly higher in PSMCs transfected with miR-146a inhibitors. (C) NO production was significantly reduced in the PSMCs transfected with miR-146a mimics, but significantly higher in PSMCs transfected with miR-146a inhibitors. (D) The cGMP level was significantly reduced in the PSMCs transfected with miR-146a mimics, but significantly higher in PSMCs transfected with miR-146a inhibitors (** $P < 0.01$). NOS1, nitric oxide synthase 1; cGMP, cyclic guanosine monophosphate; PSMCs, pulmonary artery smooth muscle cells.

rs2910164 G/C polymorphism on the signaling pathway, the expression levels of NO and cyclic guanosine monophosphate (cGMP) were examined. As shown in Fig. 4, the concentration of NO and cGMP were consistently higher in the CC group than in the GG or GC groups.

Alternation of miR-146a significantly altered the expression of NOS1 and the production of its effectors, NO and cGMP. The regulatory role of the control of *NOS1* expression was further evaluated; the PSMCs were transfected with miR-146a mimics and inhibitors, and upregulation of miR-146a was identified to significantly decrease the expression level of *NOS1* (Fig. 5A and B) and reduced the production of NO (Fig. 5C) and cGMP (Fig. 5D) in the PSMCs in a dose dependent manner. While downregulation of miR-146a significantly increased the expression level of *NOS1* and the production of NO and cGMP in PSMCs (Fig. 5).

Discussion

miRNAs are crucial regulators that control the expression of thousands of human genes. *In vivo* and *in vitro* studies have shown that variants interfering with miRNA production processing or target recognition may be potentially functional polymorphisms that lose their control over the expression of various downstream

target genes, which may be the underlying molecular mechanism of the development of human pathologies (8,16-20). It has been previously reported that the miR-146a rs2910164 polymorphism affected the efficiency of premature miR-146a processing, leading to a reduction of mature miRNAs (11). Furthermore, statistical analyses indicated an association between the minor allele of the polymorphism (the C allele) and either the risk for or protection from various diseases (including, cancer, arthritis and asthma) (11-14,19). Jazdzewski *et al* (10) demonstrated that this allele reduces the expression level of mature miR-146a by ~2-fold and inhibits its target genes, including Toll-like receptors, factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1 (17,21,22). In the present study, online *in silico* analysis was conducted to identify the potential target of miR-146a, which is functionally associated with BHR in response to physical stimuli, and found that *NOS1* is a candidate. To assess whether miR-146a targets the *NOS1* 3'UTR in PSMCs, reporter vectors carrying wild-type or mutant *NOS1* 3'UTR were constructed. As shown in Fig. 2, only the luciferase activity, from the cells co-transfected with wild-type *NOS1* 3'UTR and miR-146a mimics, was significantly lower than the control; all the other groups were comparable. The results confirmed that *NOS1* was a valid target of miR-146a in PSMCs. Therefore, the present study hypothesized that C allele carriers exhibit increased expression levels of *NOS1*, which contributes

to the molecular mechanism underlying the differential acute response to intubation. In the current study, the carrier of the CC genotype of the miR-146a gene was identified to be associated with protection from BHR in response to intubation (OR, 0.38; 95% CI, 0.18-0.78).

The neuronal NOS gene, *NOS1* has been reported to be associated with airway obstruction and bronchial inflammation, and it promotes NOS2 expression (4). NO is a potent vasodilator, and neurally-derived NO, catalyzed by *NOS1*, is functionally associated with bronchospasm, by modulating the balance between bronchoconstriction and bronchodilation, as a neurotransmitter for the NANC nervous system (5). NANC mechanisms are important in maintaining the homeostasis of the respiratory system, and a defect in NANC bronchodilation has been indicated to be involved in the pathogenesis of certain bronchospasm-based diseases, such as asthma (6). Additionally, *NOS1* is detectable in a wide spectrum of human tissues, particularly in bronchial smooth muscle cells (23). In mice, *NOS1* was detected in epithelium of the trachea, the removal of which was found to decrease the expression of *NOS1*, indicating that bronchial epithelial cells are the major source of *NOS1* production (23). An *in vivo* study showed that the inhibitory NANC relaxation of ovalbumin (OVA)-exposed animals was not significantly affected by an NOS inhibitor, indicating that the neural NO-induced relaxation was markedly impaired by repeated antigen exposure (24). Markedly reduced NO production was observed in *NOS1* knock-out mice that were challenged with OVA, when compared with that of the wild-type controls (25). In another study, Martinez *et al* (26) identified that stimulus exposure caused airway hyper-responsiveness via compromised expression of bronchoprotective *NOS1*. These data demonstrated a regulatory role of *NOS1* in the mechanism underlying the development of bronchospasms via promoting the production of NO. In the current study, lung tissue samples were collected from different genotypes, and the expression of miR-146a was identified to be comparable between the GG and GC groups, which were substantially greater than the CC group. Additionally, the mRNA and protein expression levels of *NOS1* were determined in all of the genotype groups using qPCR and western blotting, finding that *NOS1* expression levels were similar between the GG and GC groups, and the two were significantly lower than that of the CC group (Fig. 3B-D). To further demonstrate the effect of the rs2910164 G/C polymorphism on the signaling pathway, the expression levels of NO and cGMP were evaluated. As shown in Fig. 4, the concentration of NO and cGMP were consistently higher in the CC group when compared with the GG or GC group.

In the present study, multiple logistic regressions analysis are included to reduce the false positive results from controlling potential confounding factors. Spurious association as a result of population stratification did not occur, as the ethnicity of all subjects was Chinese Han, which is assumed to be a homogenous population. Functional analysis was performed to support the association study. However, the sample size was limited, as only 563 patients were included in the statistical analysis, and such a limited sample size may have compromised the statistical power of the association study. In addition, as all of the participants were patients who had received general anesthetic prior to surgical intervention the study may contain selection bias.

The association between the rs2910164 polymorphism and BHR is notable, and further additional case-control studies in other ethnic groups are warranted to confirm the finding of the current study. Furthermore, investigations of other study cohorts are required to gain insight into the molecular mechanisms underlying this association and the role of miR-146a single nucleotide polymorphisms in bronchospasm-based diseases.

In conclusion, the rs2910164 C allele is associated with BHR in response to intubation in the patients who receive general anesthetic. Thus, the rs2910164 C allele may serve as a novel biomarker and as a potential therapeutic target to predict and treat BHR.

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