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A Variant in the Precursor of MicroRNA-146a is Responsible for Development of Erectile Dysfunction in Patients with Chronic Prostatitis via Targeting NOS1

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The morbidity of erectile dysfunction (ED) has been found to be substantially increased in patients with chronic prostatitis (CP). Accumulating evidence shows that single-nucleotide polymorphism (SNP) located in pre-miRNA or mature microRNA may affect the processing of microRNA (miRNA) and alter the expression of the miRNA, as well as its target gene. In this study we investigated the association between rs2910164 G/C polymorphism and risk of ED in patients with CP, as well as the underlying molecular mechanism.

Material/Methods: Computational analysis was used to search for the target of miR-146a, and the luciferase reporter assay system was used to validate NOS1 to be the target gene of miR-146a. We also treated PC-3 cells with miR-146a mimics/inhibitors to verify the negative regulatory relationship between miR-146a and NOS1, and real-time PCR and Western blot analysis were used to estimate the expression of the NOS1 mRNA and miR-146a.

Results: The binding site of miR-146a was found to be located within the 3'-UTR of the NOS1 by searching an online miRNA database (*www.mirdb.org*), and luciferase reporter assay was done to confirm that NOS1 is a direct target gene of miR-146a. We also found that mRNA and protein expression level of NOS1 in PC-3 cells treated with miR-146a mimics and NOS1 siRNA was substantially down-regulated compared with scramble control, while cells treated with miR-146a inhibitors showed increased expression of NOS1. In addition, 705 people were recruited for our research – 342 CP patients with ED and 363 CP patients without ED – and we found that the presence of minor allele of rs2910164 polymorphism is significantly associated with reduced risk of ED in patients with CP.

Conclusions: The findings indicate a decreased risk of ED in patients with CP who are carriers of miR-146a rs2910164 C allele, and this association might be due to its ability to compromise the expression of miR-146a, and thereby increase the expression of its target gene, NOS1.

MeSH Keywords: Erectile Dysfunction • MicroRNAs • Prostatitis

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Background

As a very common condition, CP affects a wide age range of men. The estimated prevalence of CP ranges from 2% to 10%, while the overall lifetime prevalence reaches 9–16%. CP has detrimental influence on life quality comparable to that of diabetes mellitus, Crohn's disease, angina, and myocardial infarction [1]. CP symptoms include sexual dysfunction, a variable degree of voiding, inflammation of the prostate, and pelvic pain (localized to the urethra, perineum, and prostate) [2].

There is a consensus that CP is related to significant sexual dysfunction [3]. In investigated patients with CP, erectile dysfunction (ED), which is defined as failure to obtain or/and maintain a penile erection needed for satisfactory sexual performance, is claimed to be the most common sexual dysfunction [3]. The reported CP-associated ED prevalence varied from 15.0% to 48.3%, depending on the populations sampled and the evaluation tools used [4].

As short non-coding RNAs, microRNAs, consisting of approximately 22 nucleotides, are encoded by the genome and remained highly conserved in higher eukaryotes' evolution. MicroRNAs take part in the regulation of gene expression at the post-transcriptional level by instructing the complex RISC to target mRNAs, leading to mRNA decay and translational inhibition [5]. MicroRNAs can influence the expression levels of a variety of genes [6]5, but the absence of perfect complementary between microRNAs and their target mRNAs impedes the accurate prediction of the targeted mRNAs, consequently, the *in silico* prediction tools need to be further optimized [7]. Increasing evidence indicates that microRNAs participate in numerous pathological and physiological processes, as well as responses to xenobiotics such as drug-induced cardiotoxicity [8].

Significantly, it has been demonstrated that many aging-related genes that can be manipulated by numerous miRNAs were involved in the A-ED pathogenesis. Consequently, miRNAs might take part in A-ED onset by influencing the function of various proteins at the upstream level [9]. Our previous research has confirmed the profile of miRNAs in the corpus cavernosum (CC) of rats with A-ED. Consequently, up-regulation of miR-200a was found in the CC of rats with A-ED, and its main target pathway was confirmed as eNOS/NO/PKG, which is considered an important pathway in the physiology of normal erection, as shown by bioinformatic analysis [10].

In 2007, bioinformatic analyses predicted a correlation between the occurrence of SNPs in miR-SNPs (miRNA target sites) or miRNAs and pathogenesis and Wu et al. later experimentally validated the hypothesis in different human cancer types [11,12]. Furthermore, SNPs have been shown to be less frequent in miRNAs or their target sites when compared with other parts of the genome. The negative selection of sequence variations in miRNAs indicates their importance in critical cellular processes such as the regulation of gene expression [11]. Different scenarios accounting for the influence of miR-SNPs are conceivable. On the one hand, the expression of a variety of different genes could be affected by SNPs in a miRNA-coding sequence, for instance, due to a damaged maturation or processing of the miRNA. On the other hand, the existing binding sites or newly created binding sites could be modulated by the SNPs in target sites, taking effect on 1 or only a few specific target molecules. At the same time, in the human genome, miR-SNPs are considered as a unique group of functional polymorphisms. Researchers have come to acknowledge their patho-mechanistic role and examine their biological relevance [13].

NOS1 has been reported to be functionally involved in the pathogenesis of the development of ED in chronic prostatitis, and it has been also shown that NOS1 is a target of miR-146a in PC-3 cells [14,15]. One polymorphism in the miR-146a has been shown to be able to compromise the processing of the miRNA and decrease its expression level [16]. In this study, we verified the miR-146a/NOS1 relationship in PC-3 cells, and tested the association between miR-146a rs2910164 polymorphism and the risk of ED in chronic prostatitis.

Material and Methods

Subjects

We recruited 705 CP patients in the Department of Urology, Third Xiang Ya Hospital Affiliated to Central South University (Changsha, China) for our research, including 342 CP patients with ED and 363 CP patients without ED, from September 2013 to November 2014. A peripheral blood sample was collected from each participant. A total of 64 prostate tissue samples were collected from patients who were diagnosed with benign prostate proliferation and who received surgical intervention at our hospital. Prior to study initiation, we collected information on participants, such as specific questions regarding the psychosocial problems, medical history that included problem assessment and symptoms, a digital rectal examination (DRE) of the pelvic floor muscles and prostate, and a focused physical examination that containing post-massage urine analysis and culture or expressed prostatic secretions and pre-massage urine. Written informed consent was obtained from all patients or their first-degree relatives before the surgery. The Human Research Ethics Committee of Central South University (Changsha, China) approved this research. The research process was in conformity with the latest vision of the Declaration of Helsinki.

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Genotyping by TaqMan genotyping kit

Standard PCR protocols were used to amplify the DNA specimens, and the ABI 3730xl sequencing platform was used to sequence the PCR products, as described previously. ChromasLite software and DNAMAN (Applied Biosystems, Foster City, CA) were used to analyze the results of the sequencing.

RNA isolation and real-time PCR

TRIzol reagent (Invitrogen, Life Technologies, CA) was used to extract the total RNA from the tissue samples and PC-3 cell lines in accordance with the manufacturer's protocol. M-MLV Reverse Transcriptase (Promega, WI) was used to perform the synthesis of cDNA with the RNA-extracted and random primers (Takara, Shiga, Japan) according to the standard protocol. The SYBR Green Real Master Mix kit (Tiangen, Beijing, China) was used to perform the qRT-PCR assays following the standard protocol of the manufacturer. The Bulge-Loop miRNA qRT-PCR Primer Set (RiboBio, Guangzhou, China) was used to perform the reverse transcription and qRT-PCR for miR-146a following the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression of the NOS1 mRNA and miR-146a. All reactions were run 3 times.

Cell culture and transfection

RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone, UT) was used to incubate PC-3 cells under a humidified atmosphere of 5%CO_2/95% air at 37°C. When the cells reached 80% confluence, the lipofectamine 2000 (Invitrogen, Life Technologies, CA) was used to transfect the PC-3 cells with miR-146a mimics and inhibitors and NOS1 siRNA in accordance with the manufacturer's instructions. Each test was repeated 3 times.

NO production assay

Indirect competitive ELISA was used to test the specificity and presence of antibodies, as described before. ENR-BSA dissolved in 0.5 M carbonate buffer (100 ng per well, 100 µL) was used to coat the microplates at 4°C for 12 h, and 1% casein in PBS (w/v, 250 µL/well) was used to wash and block the plates, and incubated for 60 min at 37°C. The blocking solution was carefully removed, and the competitor in PBS buffer or PBS buffer was added to each well, and incubated for 60 min at room temperature. PBS was used to wash the plates 3 times, and 100 µL of anti-mouse IgG-HRP conjugate at a dilution of 1 1000 in 1% skim milk (Boster, China) was used to incubate the plates for 60 min. We used 100 µL of TMB solution and washing buffer to wash the plates for 5 min. To halt the color development, we used 100 µL of 2N H_2SO_4, and a microtiter plate reader (Thermo Scientific, MA) was used to detect the plates based on the absorbance at 450 nm, according to the manufacturer's protocol.

Luciferase assay

The 3'UTR of NOS1 genes were amplified by PCR, and the PCR products were inserted into the XhoI and XbaI sites of the pmirGlo plasmid (Promega, WI) according to standard protocol. The KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) was used to construct the pmirGlo plasmids with mutated NOS1 3'UTR, in accordance with the manufacturer's protocol. The mutagenesis was inserted into the same site of a control vector (Ambion, Cambridgeshire, UK) at the same time. Lipofectamine 2000 (Invitrogen, Life Technologies, CA) was used to transfect the PC-3 cells with miR-146a mimics (50 nM) and plasmid (5 ng/mL) in accordance with the manufacturer's recommendation. The Dual-Glo luciferase assay kit (Promega, WI) was used to detect the luciferase activity based on the manufacturer's instructions. All tests were performed 3 times.

Western blot analysis

After all treatments, the PC-3 cells were harvested. SDS buffer (Beyotime, Shanghai, China) was used to resuspend the cells to obtain the total protein extracts from PC-3 cells and tissue samples in accordance with the manufacturer's instructions. We used 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to separate the protein, and then transferred it to an Immobilon-P transfer polyvinylidene fluoride membrane (Millipore, Bedford, MA) for 2 h (120 V). We used 5% bovine serum albumin (BSA) prepared with TBS-T buffer to treat the membrane for 120 min at room temperature. Primary antibody anti-NOS1 at a dilution of 1:2000 (Boster, China) and anti-β-actin at a dilution of 1:5000 (Boster, China) containing 1% BSA was used to incubate the membrane for 12 h at 4°C. A secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) at a dilution of 1: 10 000 (Boster, China) containing 1% BSA was used to incubate the membrane for 6 h at 4°C. HRP substrate (BeyoECL plus A/B, Beyotime, Shanghai, China) was used to visualize the membrane according to the recommendations of the supplier. Each test was repeated in triplicate.

Statistical analysis

All data are shown as the mean ±SD (standard deviation). P values less than 0.05 were considered statistically significant. SPSS 16.0 software (SPSS Inc, Chicago, IL) was used to perform the statistical analyses. Logistic regression analysis was used to perform the genotyping analysis.

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Table 1. Demographic and genotypic characteristics of Chronic prostatitis (CP) patients with or without erectile dysfunction (ED).

	CP patients with ED (n=342)	CP patients without ED (n=363)	Significance
Age range (mean ±SD)	18–74 (47.53±12.3)	21–79 (48.37±6.5)	0.254
Cigarettle smoking: n,(%)	76 (22.22%)	85 (23.42%)	0.71
Fasting glucose (mg dl^{-1})	91.54±18.6	92.32±13.4	0.521
Blood pressuresys (mmHg)	134.14±15.3	132.95±12.3	0.254
Blood pressuredias (mmHg)	78.15±7.2	77.34±6.8	0.125
Body mass index (kg m^{-2})	26.38±2.4	25.95±4.1	0.092

Table 2. Association between genotypes (% in parentheses) and allele frequencies of rs2910164 polymorphisms and risk of major erectile dysfunction (ED) in the patients with Chronic prostatitis (CP).

	CP patients with ED (n=342)	CP patients without ED (n=363)	Significance
rs2910164 genotype			
GG	140 (41%)	196 (54%)	Genotypes: OR: 0.61 95% CI (0.52–0.79) P<0.001
GC	150 (44%)	145 (40%)	
CC	52 (15%)	22 (6%)	
GC/CC	202 (59%)	267 (46%)	
Frequency			
G	430 (62%)	537 (74%)	Alleles: OR: 0.60 95% CI (0.56–0.76) P<0.001
C	254 (39%)	189 (26%)	

Results

Characteristics of the participants

We recruited 705 people for our research: 342 CP patients with ED and 363 CP patients without ED. The demographic and clinicopathological of participants, such as age range, cigarette smoking, fasting glucose, blood pressure, and body mass index (BMI) were collected, and summarized in Table 1. The *t* test was used to perform the statistical analysis between these 2 groups, and no difference was observed with respect to age range (P=0.254), cigarette smoking (P=0.71), fasting glucose (P=0.521), blood pressure (P=0.254 and P=0.125), or BMI (P=0.092). We divided all the participants into 4 groups by rs2910164 genotype: GG, GC, CC, and GC/CC, and logistic regression test was used to analyze the SNP type, and found that rs2910164 polymorphism was significantly associated with the risk of ED (95% CI 0.52–0.79, OR 0.61, P<0.001). We also divided all the participants into 2 groups by frequency: G and C. Logistic regression analysis was used to analyze the frequency, showing that the frequency was significantly associated with the risk of ED (95% CI 0.56–0.76, OR 0.60, P<0.001) (Table 2).

NOS1 is a target of miR-146a

MiR-146a is reportedly related to a variety of diseases by acting on different signaling pathways. In this study, we aimed at understanding the relationship between the miR-146a level of CP patients with ED and its mechanism. By searching the literature and performing computational analysis to search for the possible target gene of miR-146a, we identified NOS1 as a virtual target of miR-146a, with potential binding site in the 3'UTR of NOS1, as shown in Figure 1.

Using luciferase assay, we further confirmed that NOS1 was the target gene of miR-146a. As shown in Figure 2, the luciferase activity of cells transfected with wild-type NOS1 was significantly up-regulated compared with scramble control, and the luciferase activity of cells transfected with mutant NOS1 was comparable with the scramble control, suggesting that NOS1 was the target gene of miRNA-146a.

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Figure 1. Schematic comparison between miR-146a and the "seed sequence" in the 3'UTR of NOS1.

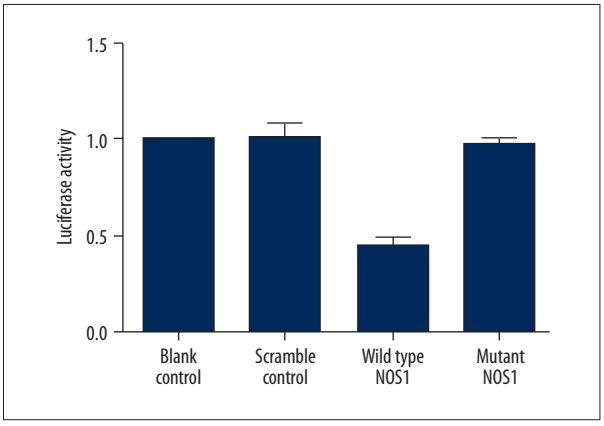

Figure 2. Luciferase activity reporter assay was conducted to verify NOS1 as the direct target gene of miR-146a as well as to validate the regulatory relationship between miR-146a and NOS1. The luciferase activity of cells transfected with wild-type NOS1 was significantly lower compared with scramble control, and the luciferase activity of cells transfected with mutant NOS1 was comparable with the scramble control.

The negative regulatory relationship between NOS1 and hsa-miR-146a

The regulatory relationship between NOS1 and miRNA-146a was tested using real-time PCR, as shown in Figure 3. We confirmed the negative regulatory relationship between miR-146a and NOS1, and the negative correlation coefficient was –0.5093 (r=–0.5093).

Determination of expression of miR-146a and NOS1 mRNA and protein with different genotypes

Real-time PCR and Western blot analysis were used to estimate the impacts of rs2910164 polymorphism on the expression of miRNA-146a mRNA, NOS1 mRNA and protein with the prostate tissues divided into 3 different genotypes (GG n=39, GC n=20, CC n=6). As shown in Figure 4, the expression of miRNA-146a mRNA of cells carrying GC and CC genotype was similar, and both were evidently lower than in the GG group, indicating that rs2910164 polymorphism is significantly associated with the expression of miR-146a mRNA. We also found the

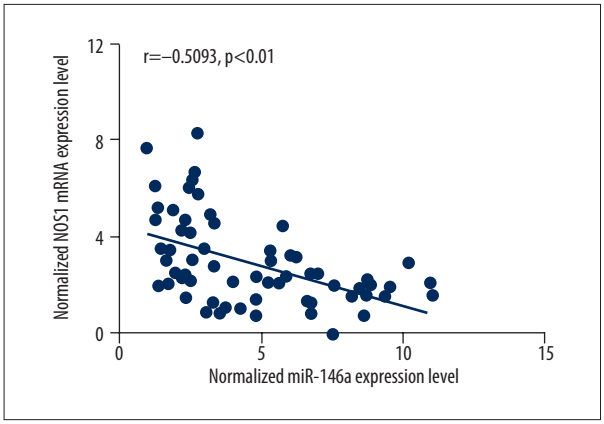

Figure 3. The expression level of NOS1 mRNA and the expression of miR-146a were measure using real-time PCR to validate the miRNA-mRNA regulatory relationship. We confirmed the negative regulatory relationship between miR-146a and NOS1 mRNA, and the negative correlation coefficient was –0.5093 (r=–0.5093).

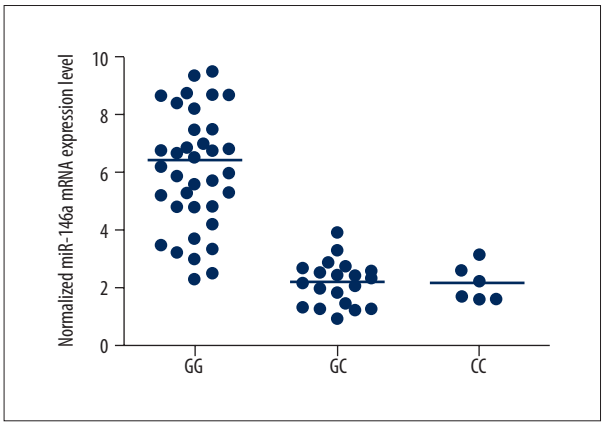

Figure 4. Real-time PCR was used to measure the expression of miR-146a mRNA with different genotypes, the expression of miRNA-146a mRNA of cells carried GC and CC genotype was similar, and both were evidently lower than in the GG group.

expression of NOS1 mRNA (Figure 5A) and protein (Figure 5B) of cells with GC and CC genotype was comparable, and both were remarkably up-regulated compared with GG carriers, indicating that rs2910164 polymorphism is significantly associated with the expression of NOS1 mRNA and protein.

Determination of NO production and NOS1 mRNA and protein level in different groups

To further confirm the regulatory relationship between miR-146a and NOS1 in NO production, we transfected cells with miR-146a mimics, NOS1 siRNA, and miR-146a inhibitors. We found that the NO production (Figure 6A, 6D) of cells treated

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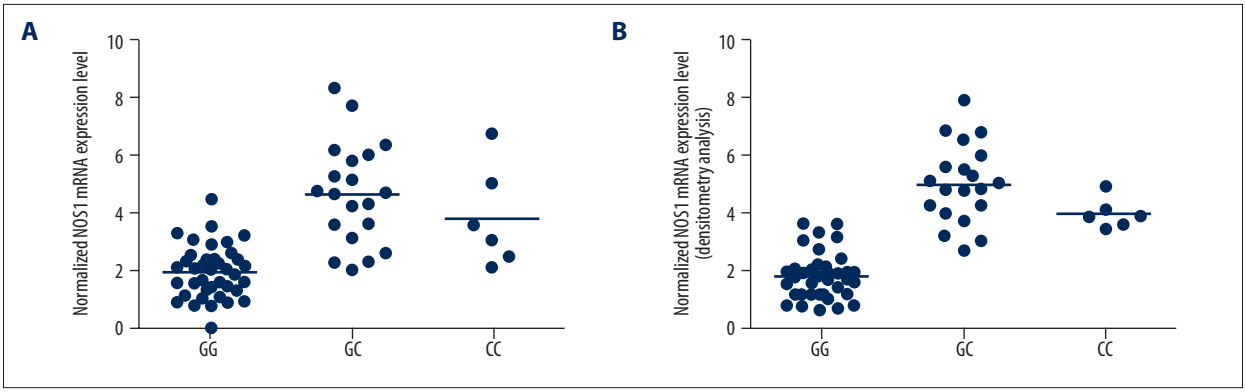

Figure 5. Real-time PCR and Western blot were used to estimate the impacts of rs2910164 polymorphism on the expression of NOS1 mRNA and protein. We found the expressions of NOS1 mRNA (**A**) and protein (**B**) of cells with GC and CC genotype were comparable, and both were remarkably up-regulated compared with GG carriers.

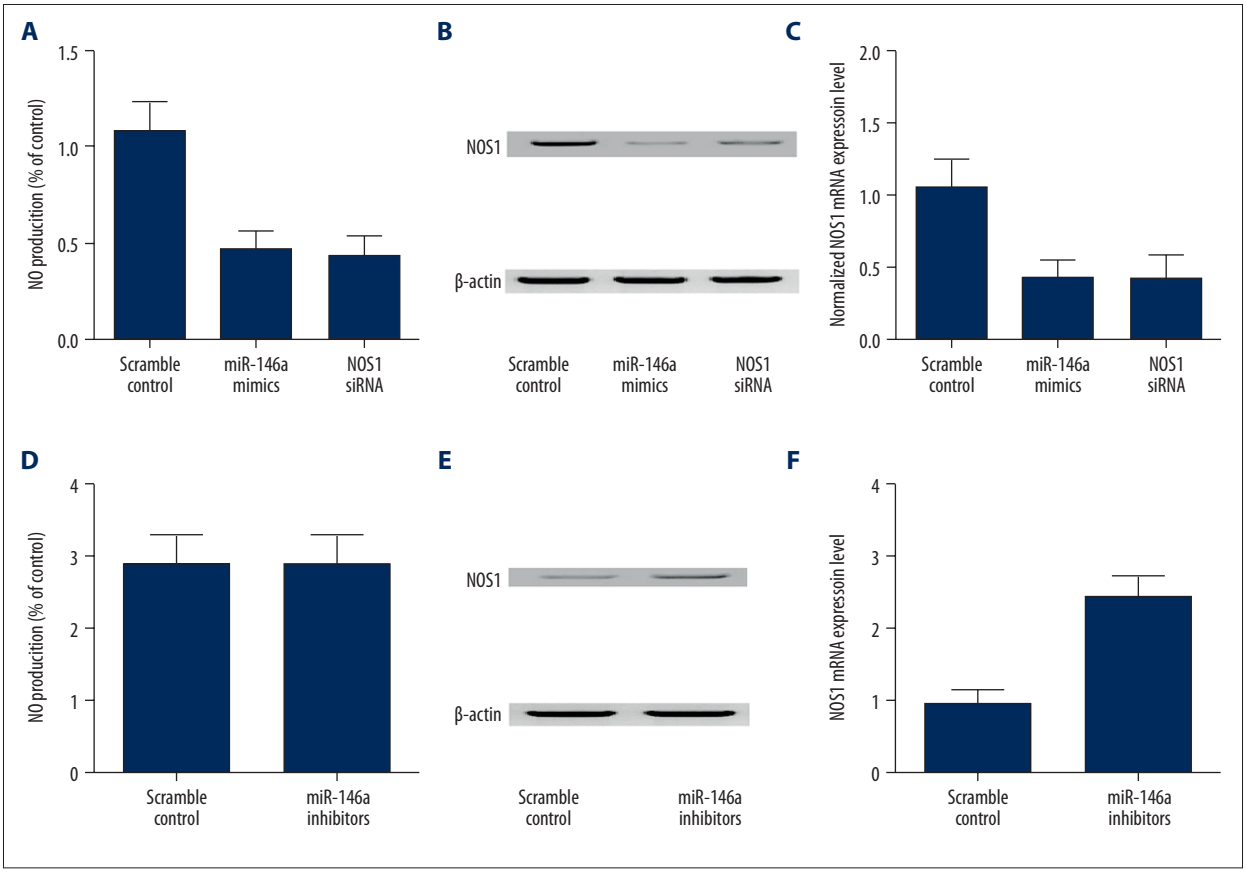

Figure 6. (**A–F**) Real-time PCR and Western blot were used to estimate effects of miR-146a on the expression of the NOS1 mRNA and protein and NO production. We revealed a clearly up-regulated NOS1 mRNA and protein expression level and NO production in the miR-146a inhibitors treatment group and lowered NOS1 mRNA and protein expression level and NO production in the miR-146a mimics and NOS1 siRNA treatment group, both compared with the scramble controls.

with miRNA-146a mimics and NOS1 siRNA was much lower compared with scramble controls, and the NO production was up-regulated when cells were treated with miRNA-146a inhibitors, suggesting that the expression of miRNA-146a can down-regulate NO production. The qRT-PCR and Western blot analysis results revealed significantly enhanced NOS1 mRNA (Figure 6C, 6F) and protein (Figure 6B, 6E) expression levels in the miR-146a inhibitors treatment group, lower NOS1 mRNA (Fig. 6C and 6F) and protein (Figure 6B, 6E) expression level in the miR-146a mimics treatment group, and NOS1 siRNA both

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compared with the scramble controls, confirming that miRNA-146a inhibits the expression of NOS1.

Discussion

Previous research suggested that miR-146a rs2910164 polymorphism is associated with an increased risk of ischemic stroke by decreasing mature miR-146aproduction [17]. Located in the stem region opposite the mature miR-146a sequence, the rs2910164 polymorphism has a change from G to C in the passenger strand, leading to lower transcriptional activity of pre-miR-146a, abnormal binding of target mRNAs, and decreased amount of mature miR-146a. Consequently, the inhibition of target genes taking part in the cytokine and Toll-like receptor signaling pathway (IRAK1, TRAF6) was reduced and activity of nuclear factor (NF)-κB, which is involved in the process of atherosclerosis, was impaired [18,19]. Several recent studies have explored the association between cancer risk and miR-146a rs2910164 G/C polymorphism. In a meta-analysis, Lian reported that susceptibility to breast cancer among Europeans might be increased by the CC homozygote of rs2910164 [20]. However, another meta-analysis reported no clear correlation between breast cancer risk and has-miR-146a rs2910164 polymorphism [21]. Similarly, no significant association between breast cancer risk and rs2910164 polymorphism was found in a very large meta-analysis in 2013 [22]. In a systematic review, Chen concluded that evidence quality was too low to identify the association between lung cancer and rs2910164 SNP [23]. Several meta-analyses pooled the association between cancer risk in general and rs2910164 polymorphism [24], and some of them reported a correlation between gastrointestinal cancer susceptibility and the rs2910164 polymorphism [25]. In the present study, we collected peripheral blood and determined the genotype of 705 participants. We next performed logistic regression analysis, and found that rs2910164 was significantly associated with risk for CP patients with ED (OR 0.61, 95% CI 0.52-0.79, and P value was less than 0.001 among GG, GC, and CC groups). In addition, we conducted *in silico* analysis to validate that NOS1 was the target of miR-146a, and we further constructed a vector containing full-length NOS1 3'UTR, and carried out luciferase assay by transfecting the PC-3 cells with wild-type NOS1 3'UTR and mutant NOS1 3'UTR. We found the luciferase activity of the cells was much lower following co-transfection with wild-type NOS1 3'UTR than that of controls, and the activity of the cells co-transfected with mutant NOS1 3'UTR was comparable to that of controls.

As an important regulator of cardiac function, nitric oxide (NO) is involved in direct posttranslational modification of protein thiols (S-nitrosylation) and the activation of cyclic guanosine monophosphate-dependent signaling pathways [26]. NO generated by the activity of neuronal nitric oxide synthase (NOS1) participates in S-nitrosylation of key sarcoplasmic reticulum (SR) Ca2+ handling proteins [27]. Particularly, nitrosylation of both cardiac and skeletal muscle ryanodine receptors (RyR2 and RyR1, respectively) enhances their activation and release properties [28].

Recent studies have demonstrated that the gaseous neurotransmitter nitric oxide (NO) plays a significant role in the pathogenesis of depression. At least 3 subtypes of NO synthase (NOS): NOS1 (also known as nNOS), NOS2 (also known as iNOS), and NOS3 (also known as eNOS) [29]. NOS1 is found in both GABAergic interneurons and glutamatergic pyramidal cells [30]. It has been found that there is linkage between depression and the genomic locus of NOS1 on chromosome 12q24 [31]. A single-nucleotide polymorphism present in NOS1 has been found to be related to general psychological distress by a recent genome-wide association study of mood disorders [32]. The correlation between NOS1 and mood disorders is further supported by evidence of reduced NOS1expression in the hypothalamus and the locus coeruleus in people with depression [33–36]. In this study, we confirmed the negative regulatory relationship between miR-146a and NOS1; the negative correlation coefficient was –0.5093 (r=–0.5093) using real-time PCR. Furthermore, we found that exogenous expression of miR-146a was significantly down-regulated in the cells genotyped as GG or GC compared with the CC group, and the expressions of NOS1 mRNA and protein were significantly up-regulated in the cells genotyped as GG or GC compared with the CC group. We performed real-time PCR and Western blot analysis to determine the expression level of NOS1 mRNA and protein in different groups, and found that NOS1 mRNA and protein levels were clearly down-regulated subsequent to transfection with miR-146a mimics and NOS1 siRNA, and were clearly up-regulated following transfection with miR-146A inhibitors. NO production levels were clearly down-regulated subsequent to transfection with miR-146a mimics and NOS1 siRNA, and were clearly up-regulated after transfection with miR-146A inhibitors.

ED is a common urological disorder, especially in men with diabetes and aged men. During physiological process of erection, cyclic guanosine monophosphate (cGMP) accumulation in cavernous smooth muscle cells (CSMCs) is triggered by nitric oxide (NO) release, followed by increased blood flow into the corpus cavernosum, and smooth muscle cells relax to create an erection. ED may result from dysregulation of cGMP accumulation and NO synthesis. The cGMP concentration was elevated by shRNA or siRNA-mediated knockdown of *PIN*, a key repressor for nNOS, leading to improved erectile dysfunction in aged rats [37]. In addition, aberrantly increased expression level of CX43 (connexin43) is usually related to ED. Nerve degeneration along with a decrease in nNOS has been reported to be observed in penile tissue from ED patients, indicating

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that reduced NO derived from endothelium and neurons plays a role in this condition. NOS1 polymorphisms have also been found to be related to responsiveness to sildenafil in ED [16,38]. The reduced nNOS level in penile tissue and the ultrastructural changes in penile cavernous tissue might be the key mechanisms of ED induced by prolactinoma in rats [39–41].

Conclusions

The findings indicated a decreased risk of ED in the patients with CP who are carriers of miR-146a rs2910164 C allele. This association might be due to its ability to compromise the expression of miR-146a, and thereby increase the expression of its target gene, NOS1.

Conflict of interest

None.

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