

## Original Article

# Role and clinical significance of miRNA-381 in prostate cancer

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**Abstract:** Objective: This study was to investigate the role and mechanism of miRNA-381 in prostate cancer. Methods: Totally 51 prostate cancer patients diagnosed and operated in our hospital from February 2013 to August 2015 were enrolled. And, 39 healthy volunteers were set as controls. Cancer tissues, the corresponding peritumoral tissues and blood specimens were collected. Quantitative RT-PCR was applied to detect the changes of COX-2 mRNA and miRNA-381 expression. ELISA was applied to detect COX-2 protein expression in blood. Dual luciferase reporter assay was applied to verify whether miRNA-381 could direct bind to COX-2 mRNA. MTT assay was applied to detect the cell proliferation of agomiR-381 and siRNA transfected LNCaP human prostate cells. Western Blot was applied to detect the changes of COX-2 protein expression in agomiR-381 transfected LNCaP human prostate cells. Results: The expressions of COX-2 mRNA and protein were significantly up-regulated in cancer tissues and blood in prostate cancer patients, while the relative expression of miRNA-381 was significantly down-regulated. Up-regulated expression of miRNA-381 resulted in significantly down-regulated expression of COX-2 mRNA and protein and decreased cell proliferation rate of LNCaP cells. Dual luciferase reporter showed that miRNA-381 regulated the expression of COX-2 by binding to the 3'-UTR. The cell proliferation rate of LNCaP cells were also decreased after siRNA silencing COX-2 expression. Conclusion: The expression of COX-2 was significantly up-regulated in cancer tissues and blood in prostate cancer patients and this increase may be related to the down-regulated expression of miRNA-381. Our data suggests that miRNA-381 may regulate cell proliferation and expression of related proteins in prostate cancer cells through COX-2.

**Keywords:** mir-381, COX-2, prostate cancer

### Introduction

Prostate cancer (PCa) is a common cancer of the male urinary system and the incidence rate ranks as the highest in USA [1]. The mortality ranks as the second among male cancers in USA [2]. About 5% of prostate cancer cases were detected by pathological sections of prostatic hyperplasia [3].

Digital rectal examination combined with prostate specific antigen (PSA) testing is widely recognized as the best screening method for the early detection of prostate cancer [4-7]. Biomarkers play an important role in the diagnosis process, and many biological markers associated with prostate cancer have been confirmed by previous investigators [8, 9]. Previous studies found that the up-regulated expression of

cyclooxygenase-2 (COX-2) plays an important regulatory role in the occurrence and development of prostate cancer, and the pathogenesis of most prostate cancer are accompanied by a progressive increase in COX-2 [10, 11]. It is found that up-regulated expression of miRNAs can inhibit the expression of COX-2 [12] and ultimately inhibit the formation, proliferation and metastasis of tumor cells. However, the regulatory effect of miRNA-381 on COX-2 in prostate cancer has been rarely reported.

In this study, qRT-PCR, Western Blot, MTT, cell transfection, gene bioinformatics prediction, and ELISA were applied to detect the expression of COX-2 mRNA and protein in tumor tissues and blood in prostate cancer patients. The relationship between COX-2 and miRNA-381 expression was analyzed, in order to explore

the mechanism of miRNA-381 in the development of prostate cancer.

### Materials and methods

#### Subjects

Totally 51 prostate cancer patients diagnosed and operated in our hospital from February 2013 to August 2015 were enrolled. And, 39 healthy volunteers were set as controls. Cancer tissues and the corresponding peritumoral tissues were collected from prostate cancer patients. Blood specimens were collected from prostate cancer patients and healthy controls. The age of prostate cancer patients was from 49 to 86 years, with a median age of 65.6 years. The age of control group was from 45 to 81 years, with a median age of 62.5 years. Among the 51 cases prostate cancer patients, 37 cases were hospitalized due to progressive difficulty in urinating, 4 cases were due to urinary frequency and urgency, 4 cases were due to painless gross hematuria, 3 cases were due to progressive urination difficulties associated with painless gross hematuria, 2 cases were due to urinary frequency, urgency, dysuria and painless gross hematuria, and 1 case was due to urinary frequency associated with painless gross hematuria. All of the 51 prostate cancer patients were the first time onset prostate cancer patients, without any treatment history by hormone, traditional Chinese medicine, radiotherapy and chemotherapy, etc, and they were all pathologically diagnosed. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of the Affiliated Hospital of Sichuan Medical University.

#### Reagents and instruments

The miRcute miRNA Isolation Kit, miRcute miRNA First-strand cDNA Synthesis Kit, miRcute miRNA qPCR Detection Kit, Super Real PreMix (SYBR Green) and TIAN Script II First-strand cDNA Synthesis Kit were all purchased from TIANGEN Biotech (Beijing) Co, Ltd (Beijing, China). miRNeasy Serum/Plasma Kit was purchased from Guangzhou Jianlun Biological Technology Co. Ltd. (Guangzhou, China). BCA Protein Assay Kit was purchased from Real-Times (Beijing) Biotechnology Co. Ltd (Beijing, China). ELISA Kit for COX-2 was purchased from Kaibo Biochemical Reagent Co. Ltd. (Shanghai, China). Cell proliferation and cytotoxicity as-

say kit was purchased from JRDUN Biotechnology (Shanghai, China). Rabbit anti-human COX-2 primary antibody (ab15191), rabbit anti-human  $\beta$ -actin primary antibody (ab129348) and goat anti-rabbit secondary antibody were all purchased from Abcam Inc. (MA, USA). Trizol was purchased from Yeasen Co, Ltd (Shanghai, China). The iQ5 real-time PCR detection systems were purchased from Bio-Rad Corporation (Hercules, CA, USA). Dual-Luciferase<sup>®</sup> Reporter Assay System was purchased from Promega Corporation (Madison, WI, USA). Image lab3.0 software was purchased from Bio-Rad Corporation (Hercules, CA, USA).

#### Quantitative RT-PCR

Total RNA was extracted by Trizol according to the manufacturer's protocol. RNA was reversely transcribed into cDNA, which was used as a template for PCR to detect the expression of COX-2 and miRNA-381. The expression of  $\beta$ -Actin and U6 were set as internal controls to COX-2 and miRNA-381, respectively. The primer sequences were as follows: COX-2: Sense: 5'-CAGCCATACAGCAAATCCTTG-3'; Anti-sense: 5'-CAAATGTGATCTGGATGTCAAC-3',  $\beta$ -actin: Sense: 5'-CACCAGGGCGTGATGGT-3'; Anti-sense: 5'-CTCAAACATGATCTGGGTCAT-3', miRNA-381: Sense: 5'-ACACTCCAGCTGGG-TATACAAGGGCAAGCT-3'; Anti-sense: 5'-TGGTGTCGTGGAGTCG-3', U6: Sense: 5'-CTCGCTCGGCAGCACA-3'; Anti-sense: 5'-AACGCTTCACGA-ATTGCGT-3'. Quantitative PCR for COX-2 and  $\beta$ -actin were performed with the following procedure: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. And quantitative PCR for miRNA-381 and U6 were performed with the following procedure: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The relative expression was calculated by  $2^{-\Delta\Delta CT}$  method.

#### ELISA assay

Serum was separated from blood sample by centrifugation at 3000 rpm for 10 min. Then, 50  $\mu$ L samples (1:4 dilution) or standard reference solutions were added to the corresponding wells. HRP-conjugated detection antibody (100  $\mu$ L) was added to each well, sealed and incubated in constant temperature incubator for 1 h. After washing for 5 times, 50  $\mu$ L substrate A and B was added to each well. After incubation at 37°C for 15 min, 50  $\mu$ L termination solutions were added, and OD value of

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each well at 450 nm wavelength were determined within 15 minutes.

### *Bioinformatics prediction*

The miRanda, TargetScan, PicTar, MiRanda, and BibiServ were applied for predicting COX-2 upstream regulatory miRNAs, and the possible regulatory sites were also predicted.

### *Dual luciferase reporter assay*

Wild type and mutated type of the predicted miRNA-381 binding site in the 3'-UTR of COX-2 gene were chemically synthesized *in vitro*, with adding *Spe*-1 and *Hind* III cleavage sites at both ends. The two DNA fragments were cloned into pMIR-REPORT luciferase reporter plasmid, and the mutated 3'-UTR seed region containing plasmid was set as a control. The 0.8 µg plasmids with 3'-UTR or mutated 3'-UTR were transfected into 293T cells by liposome method, and then transfected with agomiR-381 (100 nM). After cultured for 24 h, the fluorescence value was measured by GloMax 20/20 luminometer. Renilla fluorescent activity was set as internal control, and the procedures were strictly performed according to the instructions for dual luciferase report system kit.

### *Human prostate cancer cell line LNCaP transfection*

The  $3 \times 10^5$  LNCaP cells in logarithmic growth phase were seeded in 24-well plates, cultured in antibiotic-free 10% FBS F12/DMEM medium at one day before transfection. Cell transfection was performed when the cell density reached about 70%. Plasmids, siRNA, or agomiR was added into 50 µL OptiMemi medium and incubated for 5 min. At the same time, 1 µL lipo2000 was also added to 50 µL OptiMemi medium and incubated for 5 min. Then the two mixtures were combined together and were added to cells. After incubation for 20 min, culture medium was added and co-cultured for 6 h. Then, the culture medium was replaced with fresh 10% FBS F12/DMEM medium and continued to culture. Cells were collected at 48 h after transfection, and the expression levels of targeted COX-2 mRNA and protein were detected.

### *Western blot*

The total protein was extracted and the protein concentration was determined by BCA protein assay kit. The 20 µg samples were subjected to 10% SDS-PAGE for Western Blot analysis.

The primary antibodies, including anti-COX-2 antibody (1:1000) and anti-β-actin antibody (1:5000) were added and incubated overnight at 4°C. Then, secondary goat anti-rabbit antibodies (1:3000) was added and incubated at room temperature for 1 h. Membrane was placed in ECL solution for color development. And, image was obtained by gel imaging system and analyzed by image lab3.0 software. The relative content of COX-2 protein was calculated as the ratio of COX-2 gray value to β-actin gray value.

### *MTT assay*

Cells were seeded in a 96-well plate at the density of  $2 \times 10^3$ /well, and each sample was provided with 3 parallel wells. The 20 µL of 5 g/L MTT reaction solution was added to each well at 24 h, 48 h, and 72 h. Then, 150 µL DMSO was added to each well at the last day, the absorbance value of each well was read at a wavelength of 490 nm after 4 h incubation. The cell proliferation curves were drawn.

### *Statistical analysis*

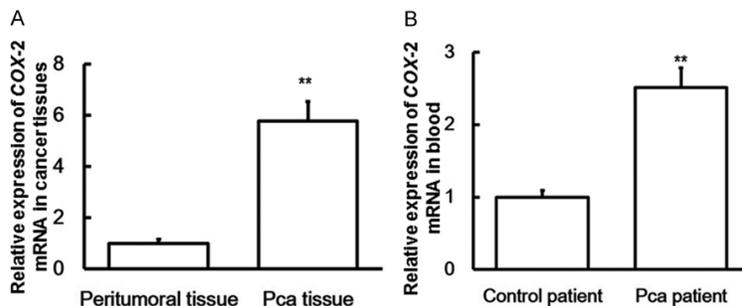
All statistical analyses were performed by using the Statistical Package for Social Sciences software (SPSS, Windows version release 18.0; SPSS Inc.; Chicago, IL, USA). Data were presented as mean ± standard deviation. All data were analyzed with normality test. One-way ANOVA was applied for multiple sets of measurement data analysis. LSD and SNK method were applied when there was homogeneity of variance, and Tamhane's T2 or Dunnett's T3 method was applied when there was not homogeneity of variance. A *P* value < 0.05 was considered statistically significant.

## Results

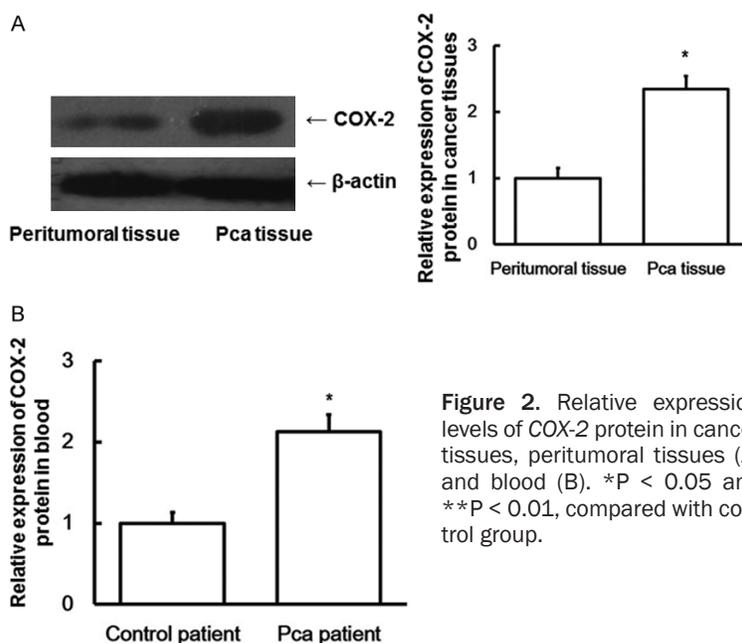
### *Changes of COX-2 mRNA expression in cancer tissues and blood samples*

The qRT-PCR was applied to detect the expression of COX-2 mRNA in cancer tissues, peritumoral tissues, and blood samples. As shown in **Figure 1A**, compared with that in the peritumoral tissues, COX-2 mRNA expressions in cancer tissues were significantly increased in prostate cancer patients (*P* < 0.05). As shown in **Figure 1B**, compared with that in control patients, COX-2 mRNA expressions in blood sample of prostate cancer patients were significantly increased (*P* < 0.05). This result indicat-

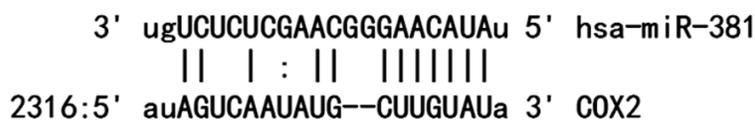
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**Figure 1.** Relative expression levels of COX-2 mRNA in cancer tissues, peritumoral tissues (A) and blood (B). \*P < 0.05 and \*\*P < 0.01, compared with control group.



**Figure 2.** Relative expression levels of COX-2 protein in cancer tissues, peritumoral tissues (A) and blood (B). \*P < 0.05 and \*\*P < 0.01, compared with control group.



**Figure 3.** The predicted specific regulatory binding sequences of miRNA-381 to COX-2.

ed that COX-2 may play a certain role in the regulation of prostate cancer.

### *Changes of COX-2 protein expression in cancer tissues and blood samples*

Western Blot and ELISA were applied to detect the expression of COX-2 protein in cancer tissues, peritumoral tissues and blood samples. As shown in **Figure 2A**, compared with

that in peritumoral tissues, COX-2 protein expressions in cancer tissues were remarkably increased in prostate cancer patients (P < 0.05). Similarly, COX-2 protein expressions in blood samples of prostate cancer patients were significantly higher than those of control patients (P < 0.05) (**Figure 2B**). This result indicated that the expression of COX-2 protein was also up-regulated in cancer tissues and blood samples of prostate cancer patients, which was consistent with the trend of mRNA.

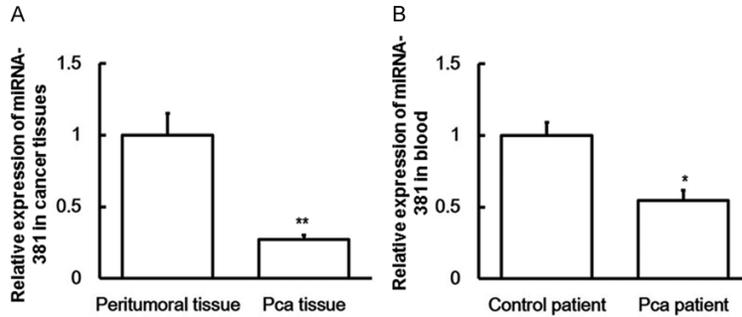
### *COX-2 is one of the target genes of miRNA-381*

The COX-2 upstream regulatory miRNAs were predicted with target gene prediction softwares. Results showed that there may be targeted regulatory relationship between miRNA-381 and COX-2, and the specific regulatory binding sequences as shown in **Figure 3**.

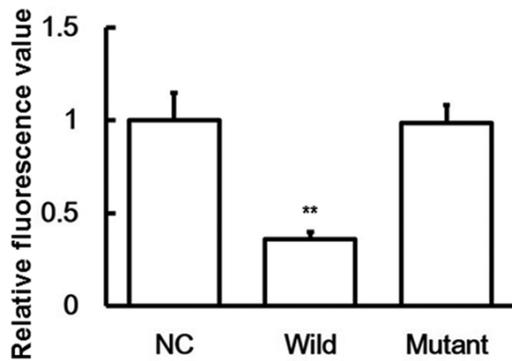
### *Changes of miRNA-381 expression in cancer tissues and blood samples*

The qRT-PCR was applied to detect the expression of miRNA-381 in cancer tissues, peritumoral tissues and blood samples. As shown in **Figure 4A**, compared with that in the peritumoral tissues, miRNA-381 expressions in cancer tissues were significantly decreased in prostate cancer patients (P < 0.05). And, miRNA-381 expressions in blood samples of prostate cancer patients were significantly lower than those of control patients (P < 0.05) (**Figure 4B**). These results indicated that miRNA-381 may play a certain role in the regulation of prostate cancer, and this regulatory function may be acted through negatively regulating the transcription level of targeted COX-2 gene.

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**Figure 4.** Relative expression levels of miRNA-381 in cancer tissues (A) and blood (B). \* $P < 0.05$  and \*\* $P < 0.01$ , compared with control group.



**Figure 5.** Comparison of fluorescence values in dual luciferase reporter assay. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with cells transfected with wild type COX2.

### Dual luciferase reporter assay

Dual luciferase reporter assay was applied to verify the relationship between miRNA-381 and COX-2. As shown in **Figure 5**, the fluorescence value was significantly decreased after co-transfecting wild type COX-2 and miRNA-381 ( $P < 0.05$ ), while there was no significant difference in the mutant COX-2 transfected group ( $P > 0.05$ ). This result indicated that miRNA-381 may regulate the expression of COX-2 through binding to the 3'-UTR of COX-2.

### Effect of agomiR-381 transfection on LNCaP cells

As shown in **Figure 6**, after transfected with agomiR-381, the expressions of miRNA-381 and COX-2 mRNA were significantly increased (**Figure 6A**) and decreased (**Figure 6B**) in LNCaP cells, respectively. And, the cell proliferation was also slowed down significantly (**Figure 6C**). This result indicated that up-regulated expression of miRNA-381 may inhibit cell prolif-

eration of LNCaP cells through regulating COX-2 expression.

### Effect of siRNA transfection on LNCaP cells

As shown in **Figure 7**, after transfected with siRNA, the expression of COX-2 mRNA (**Figure 7A**) and protein (**Figure 7B**) were significantly decreased in LNCaP cells. And, the cell proliferation was also slowed down significantly (**Figure 7C**). This result indicated

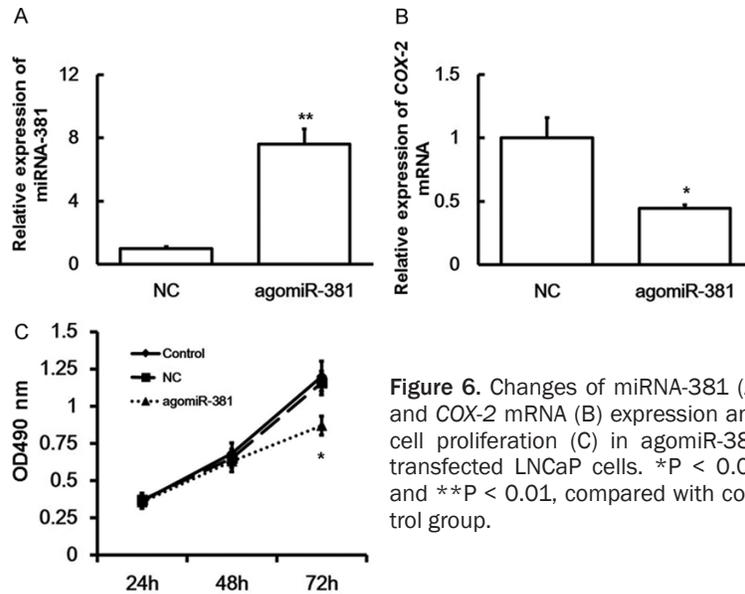
that down-regulated expression of COX-2 may inhibit cell proliferation of LNCaP cells.

## Discussion

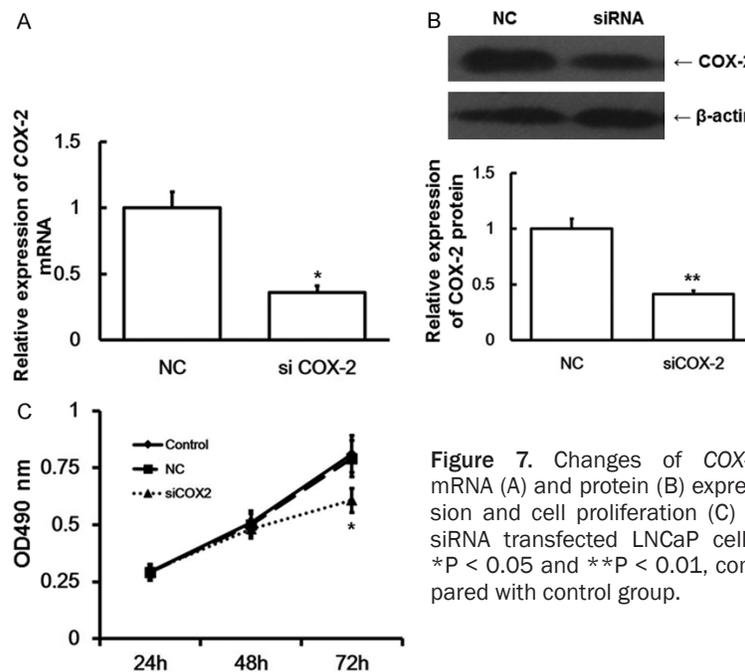
In this study, we detected the expression levels of COX-2 mRNA and protein and miRNA-381 in cancer tissues and blood, preliminary explored the biological functions of miRNA-381 and COX-2, and studied the molecular mechanisms of miRNA-381 on prostate cancer.

Chronic inflammation is generally considered as one of the physiological causes of tumors [12]. The COX, especially the COX-2, plays an important role in the development of chronic inflammation. COX-2 was induced by physical, chemical, biological and other external stimuli, which works as the necessary key rate-limiting enzyme to catalyze the synthesis of prostaglandins (PGs) to involve in inflammation reaction [13]. Previous studies show that expression change of COX-2 is closely related to the development of many kinds of tumors. The COX-2 inhibitors are developed for antipyretic analgesic treatments, and also often for adjuvant anti-tumor therapy [14-16]. In this study, a significant increase of COX-2 mRNA and protein expression was observed in prostate cancer tumor tissue, which is consistent with previous studies, indicating that the change of COX-2 expression is related to the development of prostate cancer, and the abnormal expression of COX-2 may be a key to the pathogenesis of prostate cancer [17, 18]. Another obvious feature of tumor is invasion and metastasis [19, 20]. In theory, it is possible to determine the COX-2 mRNA and protein in the blood depends on blood or tissue fluid, which may indirectly reflect the malignant degree and metastasis of prostate cancer cells.

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**Figure 6.** Changes of miRNA-381 (A) and COX-2 mRNA (B) expression and cell proliferation (C) in agomiR-381 transfected LNCaP cells. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with control group.



**Figure 7.** Changes of COX-2 mRNA (A) and protein (B) expression and cell proliferation (C) in siRNA transfected LNCaP cells. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with control group.

After prediction, we found that miRNA-381 was closely related to COX-2. It is likely that miRNA-381 is an upstream regulation miRNA of COX-2. Endogenous, small, non-encoding miRNAs may splice and inhibit the translation of COX-2 mRNA [21]. miRNAs regulate the activities of protein encoding genes, which play an important role in the development of diseases [22, 23]. The expression of miRNA-381 was significantly downregulated in colon cancer and the down-regulated expression of miRNA-381 led

to up-regulated expression of liver receptor homologue-1 (LRH-1), and resulted in proliferation and invasion of colon cancer cells [24]. miRNA-381 could inhibit pituitary tumor growth [25]. miRNA-381 and multi-drug resistance gene 1 (MDR1) genes were closely related, and played an important role in multi-drug resistance [26]. miRNA-381 could inhibit the activity of renal cancer cell Cdc2, by working together with miRNA-424 to target on *WEE1* gene [27]. And, miRNA-381 was also closely related to the pathogenesis of lung adenocarcinoma [28]. These findings suggest that miRNA-381 may have a very close relationship with the pathogenesis of tumors. Our results showed that the expressions of miRNA-381 were significantly decreased in cancer tissues and blood in prostate cancer patients. Considered that the expressions of COX-2 were abnormally increased in cancer tissues and blood, we hypothesize that down-regulated expression of miRNA-381 may be one of reasons for COX-2 up-regulation, which may further affect the biological characteristics of prostate cancer cells. To further study the molecular mechanism, we analyzed proliferation of LNCaP cells after agomiR-381 transfection. Results showed that up-regulated expression of miRNA-381

resulted in the decreased expression of COX-2 and cell proliferation rates of LNCaP cells. Dual luciferase reporter assay was applied to verify the directly binding and the specific binding sites of miRNA-381 to COX-2 mRNA. The results showed that miRNA-381 can directly bind to the targeted seed region in 3'-UTR of COX-2 mRNA and regulate the expression of COX-2.

In summary, our findings suggest that miRNA-381 may play a role in the pathogenesis of

prostate cancer through regulating the expression of COX-2.

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### Disclosure of conflict of interest

None.

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