

## Original Article

# MicroRNA-188 inhibits proliferation migration and invasion of prostate carcinoma by targeting at MARCKS

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**Abstract:** Recent evidences suggested that miRNAs process key roles in the biological behaviors and the development of prostate carcinoma (PC). However, its molecular mechanism in PC is still remained largely unclear. In this study, the expression level of miR-188 and the protein expression of MARCKS were detected by RT-PCR and western blot, respectively. Moreover, we determined the oncogene or anti-oncogene role of miR-188 in the PC through gain and loss function assay of miR-188 using transfection of miR-188 mimics and inhibitor. Meanwhile, luciferase reporter vectors with miR-188 mimics were constructed and transfected into PC cells. Our data indicated that the expression of miR-188 was dramatically reduced in human PC tissues and cell lines and the increased miR-188 levels obviously inhibited the proliferation, migration and invasion of PC cells and negatively regulative with MARCKS. Together, our findings proved that miR-188 plays an inhibitive role in PC of proliferation, migration and invasion at least in part via suppressing MARCKS expression, which could be regarded as a promising therapeutic target in PC.

**Keywords:** miR-188, MARCKS, prostate carcinoma, proliferation, invasion

## Introduction

Prostate carcinoma (PC), one of most common malignant tumor, is with high morbidity and mortality in worldwide [1]. Despite recent advances include adjuvant chemotherapy, radiotherapy and extensive tumor resection, the prognosis of those patients with their 5 years survival rates still are very unfavorable [2, 3]. Thus, it is an urgent need to investigate the molecular mechanism of cancer tumorigenesis and the more appropriate therapeutic targets in PC.

MicroRNAs (miRNAs), usually about 19-24 nucleotide RNAs, were found to act an important role in a series of biological processes in life such as cell proliferation, migration, invasion, and tumorigenesis development [4, 5]. And in the recent years, miRNAs have been determined to play the important regulators in series of human cancer [6]. And miR-188 has been reported to be significantly down-regulated and acts a role as a suppressor in many cancer types, including gastric cancer [7], glioma

[8], non-small-cell lung cancer [9], and hepatocellular carcinoma [10]. More and more evidences suggest miR-188 acts a key role in cancer development. However, the functional mechanism of miR-188 in PC is not well understood.

Myristoylated alanine-rich protein kinase C substrate (MARCKS) is rich in expression as the protein kinase C (PKC) substrate that binds actin and calmodulin (CaM) to regulate actin dynamics [11]. In the recent studies, MARCKS has been reported to participate in a variety of cellular processes by regulating the actin cytoskeletal structure, such as cell adhesion, migration, metastasis, membrane transport and movement [12]. In human hepatocellular carcinoma tissues, the down-regulated expression of MARCKS has been determined, and down-regulated MARCKS could enhance the migration in human hepatic stellate cells [13]. Meanwhile, MARCKS was also been found to play a critical role in TPA-mediated migration in neuroblastoma cells [14]. The increased phosphorylation of MARCKS contributes to unre-

sponsiveness to paclitaxel therapy in breast cancer [15].

The current study aimed to explore the role of miR-188 in PC and elucidate its potential molecular mechanism. Collectively, in this study, miR-188 was found to be markedly down-regulated in PC tissues and cell lines, and exert tumor-suppressing functions in PC. Furthermore, MARCKS was identified as a direct functional target of miR-188 in PC.

### Materials and methods

#### *Clinical samples and cell culture*

Thirty-five PC specimens and adjacent non-tumor tissues were collected under the protocols approved by Ethics and Scientific Committees of The First Affiliated Hospital of Nanjing Medical University (Nanjing, China), and we got the informed consents from all patients during May 2017 to July 2018 in The First Affiliated Hospital of Nanjing Medical University.

The human PC cell lines (DU145, PC3, PCa, LNCaP) and the human normal prostatic epithelial cell line (RWPE-1) were obtained from the Shanghai Institute of Cell Bank (Shanghai, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin (10,000 U/ml) in 5% CO<sub>2</sub> incubator at 37°C and 95% humidity.

#### *Quantitative real-time PCR (qRT-PCR)*

Total tissue and cell RNA were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was then synthesized using the GoScript™ Reverse Transcription kit (Madison, WI, USA). Quantitative real-time PCR (q-PCR) was carried out according to the protocol of GoTaq® qPCR Master mix kit (Madison, WI, USA). The U6 small nuclear RNA and GAPDH mRNA were used to normalize the expression for miR-188 and MARCKS mRNA, respectively. The sequences of primers used were: MARCKS forward primer: 5'-GTG GTG CCA GGT ACT GGT TT-3', reverse primer: 5'-CGA CCT GTT GGA ATG GTG GT-3', GAPDH forward primer: 5'-GAA AGC CTG CCG GTG ACT AA-3', reverse primer: 5'-GCA TCA CCC GGA GGA GAA AT-3', U6 small nuclear RNA for-

ward primer: 5'-CTC GCT TCG GCA GCA CA-3', reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'; miR-188 forward primer: 5'-CAC ATC CCT TGC ATG GTG GAG-3', reverse primer: 5'-GCA AAC CCT GCA TGT GGG AG-3'.

#### *miRNA transfection, MARCKS silencing and overexpression*

The miR-188 mimics (miR-188), miR-188 inhibitor, miR negative control (miR-NC) and inhibitor negative control (Inhibitor-NC) (GenePharma, Shanghai, China) were transfected using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) following the manufactures' instructions. After transfection, qRT-PCR was used to detect the efficiency. siRNA for MARCKS was designed and transfected to cells. In addition, PC cells with pcDNA3.1-MARCKS were transfected to overexpress MARCKS. Afterward, the proliferation and invasion in PC cells co-transfected both wild-type (Wt), MARCKS over-expressed miR-NC or miR-188 were detected by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide assay (MTT; Beyotime, Shanghai, China) and transwell assay, respectively.

#### *Cell viability assay by MTT*

Cells (10<sup>4</sup>/96-well plate) were plated and cultured. And then the cells were transfected at a final concentration of 50 nM/μL of miR-188 mimics (miR-188) or miR-NC. Cell viability was detected daily for 4 days using the MTT assay (C0009, Beyotime Institute of Biotechnology, Shanghai, China).

#### *Colony formation assay*

In this study, we used the colony formation assay to detect the capacity of cell proliferation. Briefly, 48 hours after cell transfection, 100 μL PC3 cells (5×10<sup>4</sup> cells/mL) was seeded into a six-well plate for 14 days. After fixed with 10% formaldehyde for 20 min, the forming colonies were then stained with 0.1% crystal violet (Sigma-Aldrich Co.).

#### *Transwell invasion assay*

48 hours after cell transfection, PC cells (5×10<sup>4</sup>) were seeded onto the upper chamber. After incubation for 48 h, the non-invasive cells were removed from the upper side. And the

lower surfaces of the filters were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet (Sigma-Aldrich Co.) and the number of migratory cells was counted.

## Western blot analysis

After rinsed with PBS, the PC cells were lysed in extraction buffer for 15 min, and then 12,000 r min<sup>-1</sup> is centrifuged at low temperature for 15 min, supernatant is sucked and loaded into a new centrifuge tube. An Enhanced BCA Protein Concentration Assay Kit (P0009, Beyotime, Shanghai, China) was used to detect the protein concentration. Approximately 30 µg/line protein was separated by 10% SDS-PAGE and then electrotransferred to PVDF membranes. Blots were blocked with 5% BSA for 1.5 h at 37°C followed by incubated overnight with the primary antibodies at 4°C. After that, bands were incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature and then visualized with Tanon 5200 Chemiluminescence imaging system. The amounts of the target proteins were analyzed using Image J analysis software and normalized to according control. Results were obtained from three independent experiments.

## Luciferase reporter assay

Firstly, wide-type and mutation of MARCKS containing putative binding site of miR-188 were synthesized and integrated into a psiCHECK-2 dual-luciferase vector to form the psiCHECK-2-MARCKS-3'-UTR-wild type (MARCKS-wt) or psiCHECK-2-MARCKS-3'-UTR-mutant (MARCKS-mut) reporter vector. PC3 and DU145 (1×10<sup>5</sup> cells/24-well plate) were transfected with the luciferase reporter gene plasmids together with miR-188, miR-188 inhibitors, inhibitor-NC or miR-NC. The firefly luciferase activity was measured and normalized to Renilla signals at 48 h post-transfection.

## Statistical analysis

All experimental results were expressed by mean ± standard deviation, and one-way ANOVA followed by Tukey-Kramer multiple comparisons was carried out with Graphpad Prism statistical software. *P* values less than 0.05 were considered as statistically significant difference between groups.

## Results

*miR-188 was significantly down-regulated and MARCKS was significantly up-regulated in PC tissues and cell lines*

Firstly, qRT-PCR analysis was performed to determine the miR-188 expression in PC cell lines and tissues. miR-188 showed a significantly down-regulated in PC tissues (**Figure 1A**, *P*<0.05) and cell lines (**Figure 1B**, *P*<0.05, respectively) compared with that in normal human prostate tissue and the normal human prostatic epithelial cell line (RWPE-1). Meanwhile, the results from western blot showed that the protein expression of MARCKS was significantly up-regulated in human PC tissues (**Figure 1C**) and cell lines (**Figure 1D**).

## *Inhibiting proliferation of PC cell lines by miR-188*

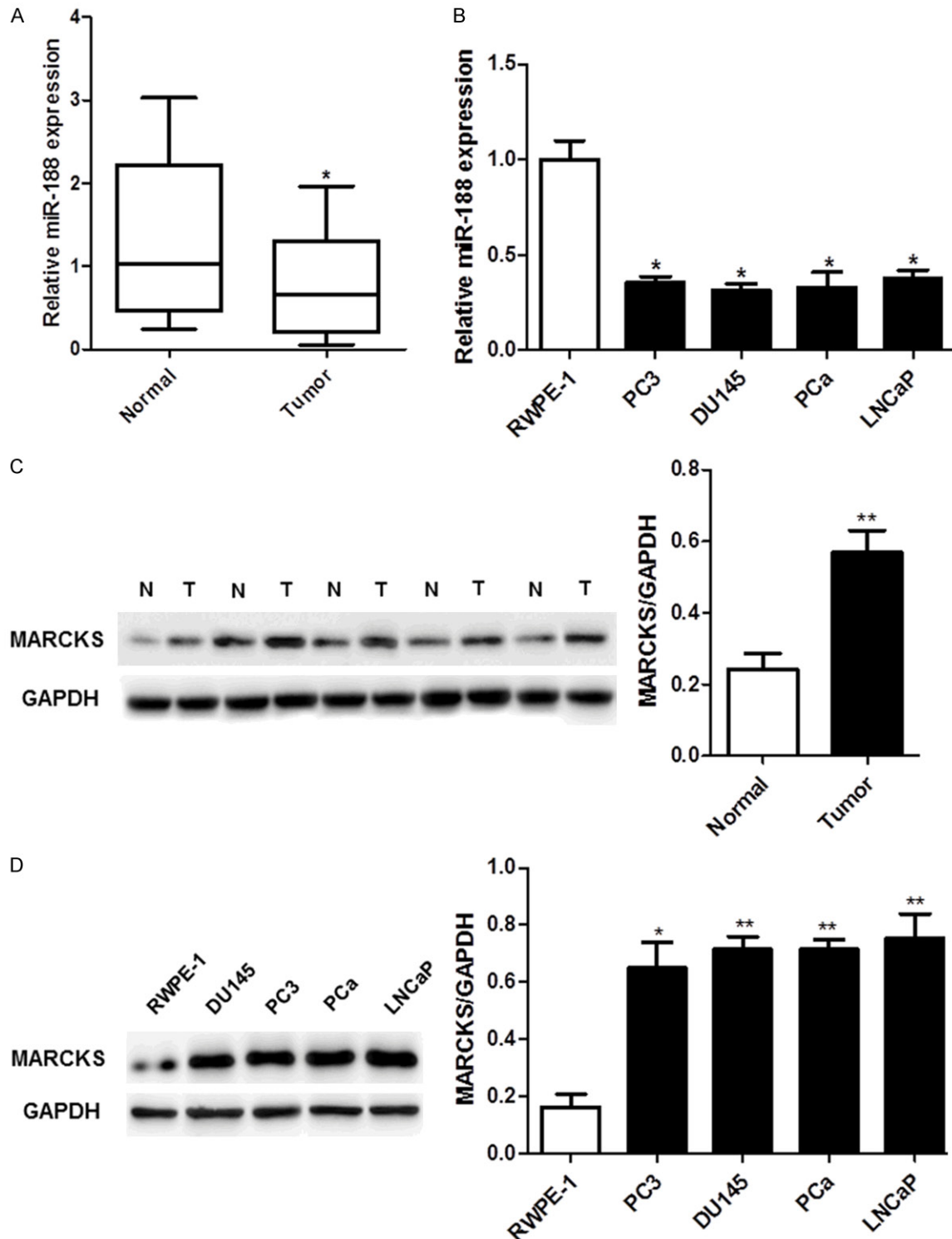
To explore the potential role of miR-188 in PC, the transfection efficiency was determined according to the level of miR-188 using qRT-PCR. As shown in **Figure 2A**, it was found a significantly increased expression of miR-188 after miR-188 mimics transfection. Further investigations from the MTT assay showed miR-188 mimics could inhibit the viability of PC3 and DU145 cells (**Figure 2B**). Additionally, the result from colony formation assay was further verified that miR-188 mimics notably inhibited the colonies and miR-188 inhibitor dramatically promoted formation of colonies (**Figure 2C**).

## *Inhibiting invasion of PC cells by miR-188*

Transwell assay was used to determine the invasive capacities in PC cells. As shown in **Figure 3**, a dramatic decrease of migration in miR-188 mimics group compared to the miR-NC (*P*<0.05). Moreover, it was observed that cell transfected with miR-188 inhibitor exhibited a dramatic increase in migration compared with the inhibitor-NC (*P*<0.05).

## *MARCKS is a potential target of miR-188 during its inhibition on PC cells*

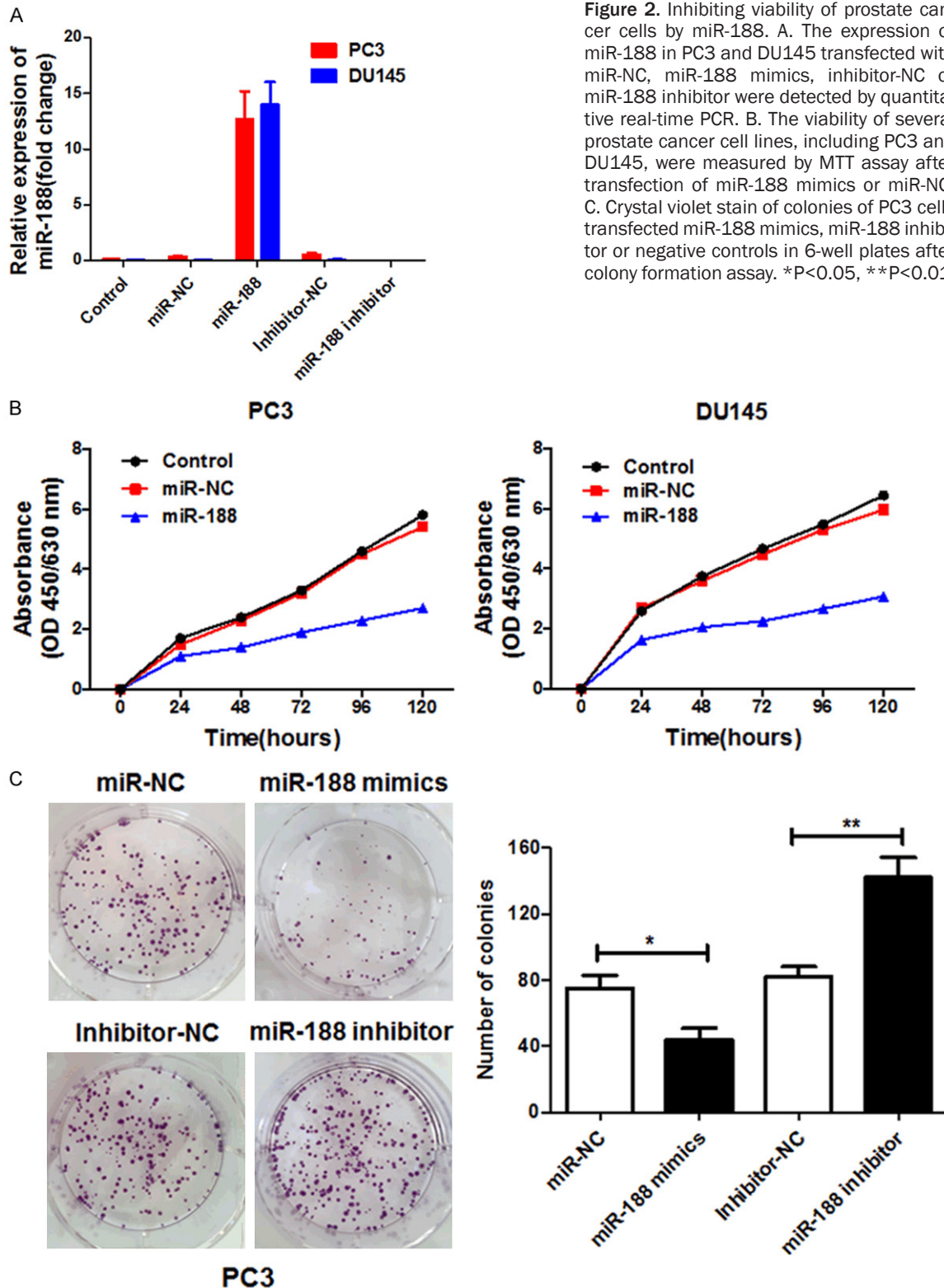
The mRNA expression of MARCKS in PC3 and DU145 cells transfected with miR-188 mimics resulted in significant decrease compared with miR-NC group, whereas miR-188 inhibitor notably increased the MARCKS mRNA (**Figure 4A**).



**Figure 1.** miR-188 was down-regulated in human prostate cancer tissues and prostate cell lines. A. Expressions of miR-188 in human prostate cancer tissues and adjacent non-tumor tissues were detected by quantitative real-time PCR. B. Expressions of miR-188 in several prostate cell lines and normal human prostatic epithelial cell line (RWPE-1) were detected by quantitative real-time PCR. C. Protein of MARCKS in human prostate cancer tissues and adjacent non-tumor tissues were detected by western blot. D. Protein of MARCKS in several prostate cell lines and normal human prostatic epithelial cell line (RWPE-1) were detected by western blot. \* $P < 0.05$ , \*\* $P < 0.01$ .

In addition, in order to further detect whether miR-188 could effect on MARCKS expression.

The results from luciferase reporter indicated that up-regulated expression of miR-188 could

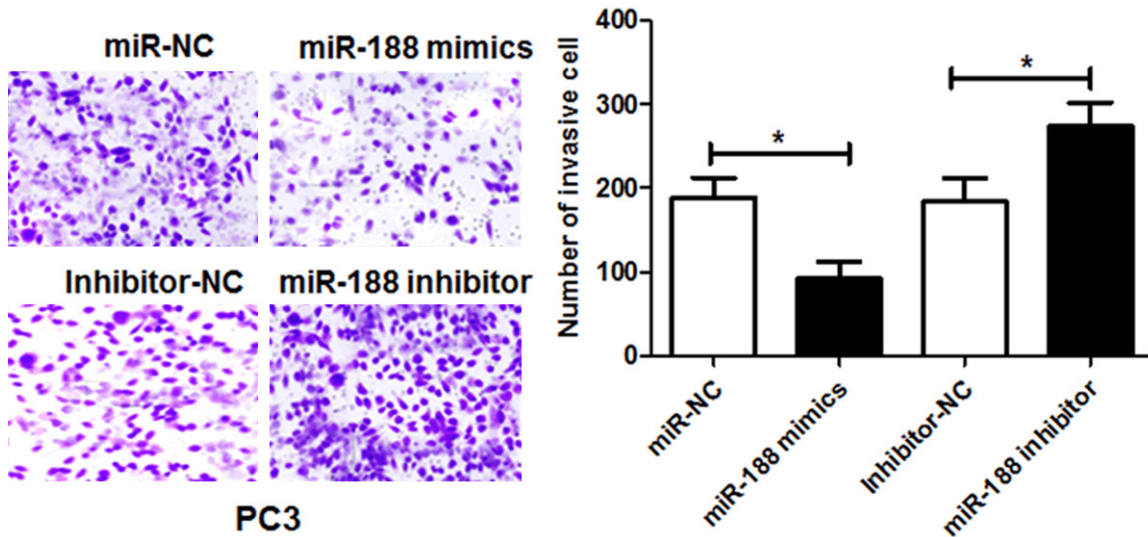


**Figure 2.** Inhibiting viability of prostate cancer cells by miR-188. A. The expression of miR-188 in PC3 and DU145 transfected with miR-NC, miR-188 mimics, inhibitor-NC or miR-188 inhibitor were detected by quantitative real-time PCR. B. The viability of several prostate cancer cell lines, including PC3 and DU145, were measured by MTT assay after transfection of miR-188 mimics or miR-NC. C. Crystal violet stain of colonies of PC3 cells transfected miR-188 mimics, miR-188 inhibitor or negative controls in 6-well plates after colony formation assay. \* $P < 0.05$ , \*\* $P < 0.01$ .

significantly inhibit the activity of reporter gene, whereas miR-188 inhibitor dramatically increased it (Figure 4B).

Furthermore, we determined the protein expression of MARCKS in PC3 and DU145 cells using western blot. Based on the results, we





**Figure 3.** Invasion of prostate cancer cells were inhibited by miR-188. After 48 h of post-transfection of miR-188 mimics or miR-NC into PC3 cells, PC3 cells in transwell membrane were stained by crystal violet after transwell invasive assay. \* $P < 0.05$ , \*\* $P < 0.01$ .

found a significant decrease in MARCKS expression in miR-188 mimics-transfected group (Figure 4C). Conversely, as shown in Figure 4D, miR-188 inhibitor-transfected group displayed higher expression of MARCKS compared with the negative control group. Next, we explored the suppressive efficacy of miR-188 on MARCKS by using MARCKS knockout PC cells. As shown in Figure 5A, in the group of siRNA transfection, the expression of MARCKS was almost completely silent.

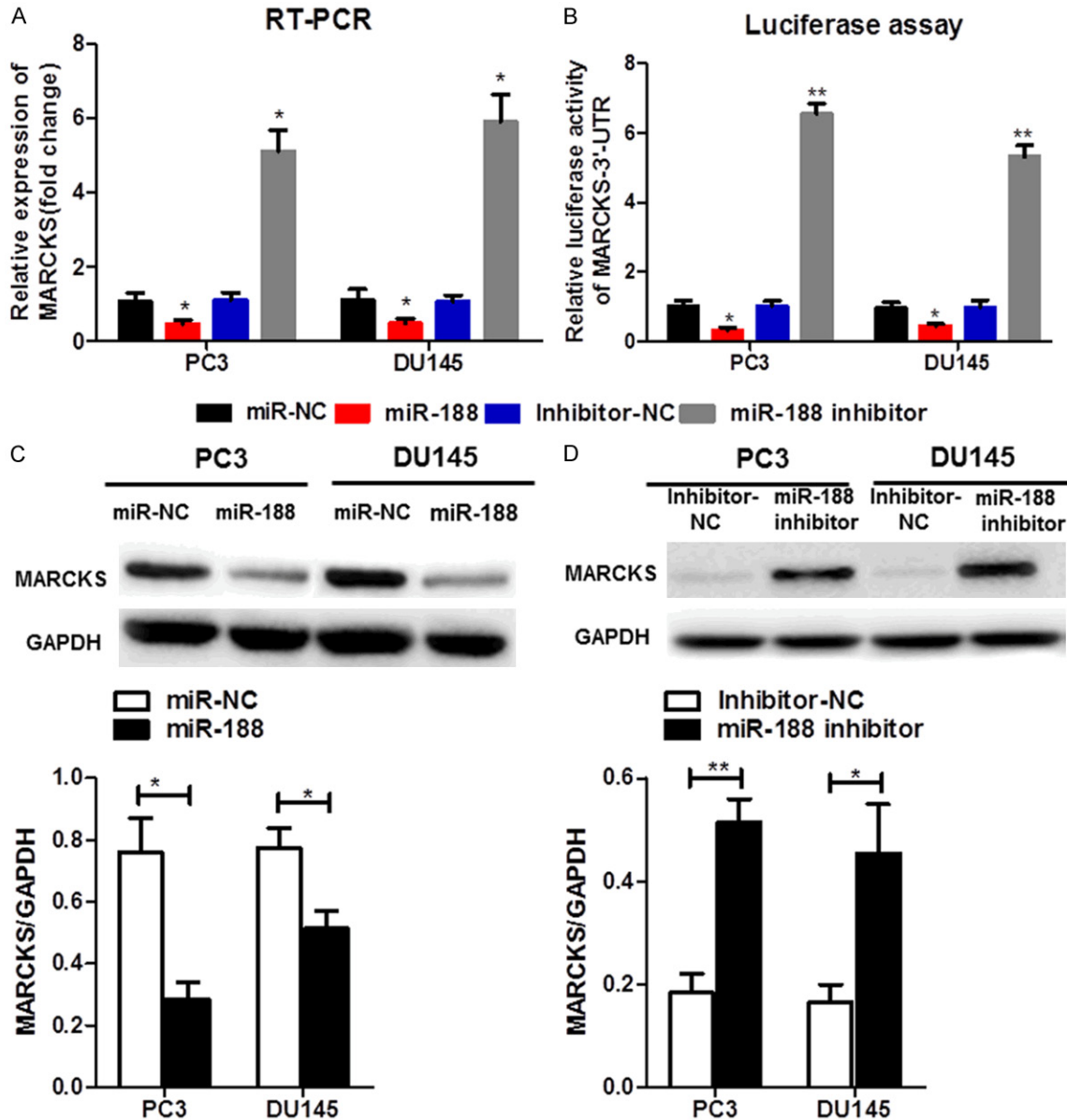
To further confirm the potential target of miR-188 on MARCKS-3'-UTR, we constructed MARCKS-Wt and MARCKS-Mut reporter vectors. As expected, luciferase reporter assay revealed MARCKS as a direct target of miR-188 in PC (Figure 5C). Afterward, MTT assay and transwell assay showed that MARCKS overexpression significantly rescued inhibitory effect of miR-188 on viability (Figure 5D) and invasion (Figure 5E) in PC3 cell.

## Discussion

Emerging studies have indicated that PC shows a very high invasive capacity [16]. Recently, a large number of reports have confirmed that miRNAs play an important role in tumorigenesis and development, also act as an oncogene or tumor suppressor gene and are involved in the regulation of tumor growth, differentiation,

angiogenesis, apoptosis, and invasion and metastasis [17], which its role in the development of PC complements and enriches the mechanism of PC [18]. Down-regulated miR-188 has been found in many cancers, such as gastric cancer, glioma and colorectal carcinogenesis [19]. Based on the above studies, we speculated that miR-188 could be used as a biomarker for tumor diagnosis and prognosis. In some individual patients, the level of miR-188 should be examined to help determine the extent of pathological tumor development [20]. In this study, we first examined the expression levels of miR-188 and MARCKS in human PC tissues and PC cells. Compared with normal human prostate tissues and normal human prostatic epithelial cell line (RWPE-1), the levels of miR-188 in PC tissues and PC cells were significantly decreased, while the expression of MARCKS was notably increased, suggesting that miR-188 and MARCKS both play important roles in development process of PC. Therefore, in order to further examine the role of miR-188 in PC, we used MTT assay and found that the miR-188 transfection group significantly inhibited PC cell viability at different time points. Similarly, cell cloning and invasion experiments demonstrated that miR-188 significantly inhibited the cloning and invasion of PC cells.

As we all know, miRNAs usually exert their regulatory effects by targeting downstream genes

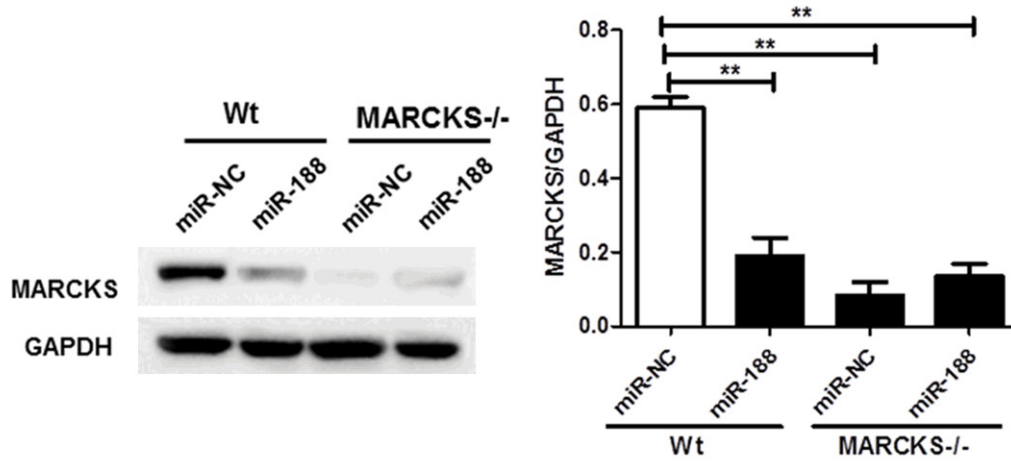


**Figure 4.** MARCKS is a potential target of miR-188 during its inhibition on prostate cancer cells. A. mRNA of MARCKS in cells transfected with miR-188 mimics, miR-188 inhibitor or their negative controls were detected by quantitative real-time PCR. B. luciferase activities of MARCKS were detected in prostate cancer cells transfected with psicheck-2-MARCKS-3'-UTR and miR-188 mimics, miR-188 inhibitor or negative controls. C. Proteins of MARCKS in PC3 and DU145 cells transfected with miR-188 mimics or mir-NC were detected by western blot. D. Proteins of MARCKS in cells transfected with miR-188 inhibitor or inhibitor-NC were also detected. \*P<0.05, \*\*P<0.01.

[21]. Therefore, the identification of target genes of miRNAs plays an important role in the diagnosis and treatment of tumors [22]. Based on the results of bioinformatics analysis and luciferase reporter genes, we determined that MARCKS is a target for miR-188 in PC. Thus, it is reasonable to speculate that miR-188 could inhibit the PC cell proliferation, migration and invasion by targeting MARCKS. qRT-PCR and

western blot analysis determined that the expression of MARCKS was decreased in PC cells transfected with miR-188 mimics, and the expression of MARCKS was increased in cells transfected with miR-188 inhibitor. To further scientifically and rigorously confirm this, we constructed a wild-type plasmid and a mutant plasmid (MARCKS-Wt or MARCKS-Mut) of the luciferase reporter vector, and the results

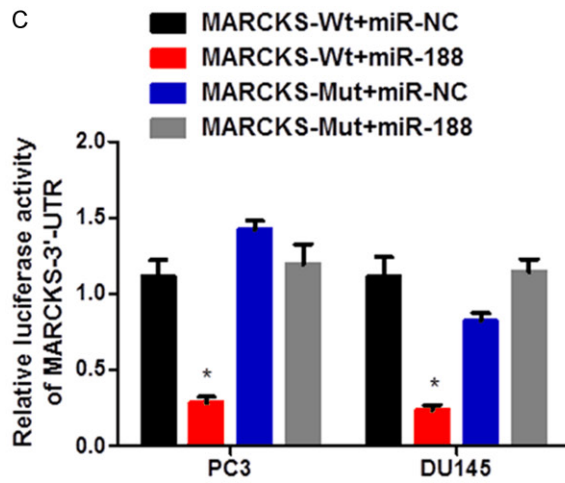
A



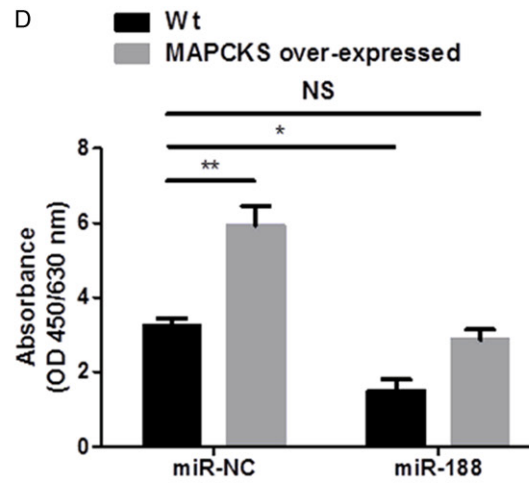
B

Predicted consequential pairing of target region (top) and miRNA (bottom)	
Position 396-403 of MARCKS 3' UTR	5' ...GAUGGUGGGUCAAAGGGAUA...
hsa-miR-188-5p	3' GGGAGGUGGUACGUUCCUAC

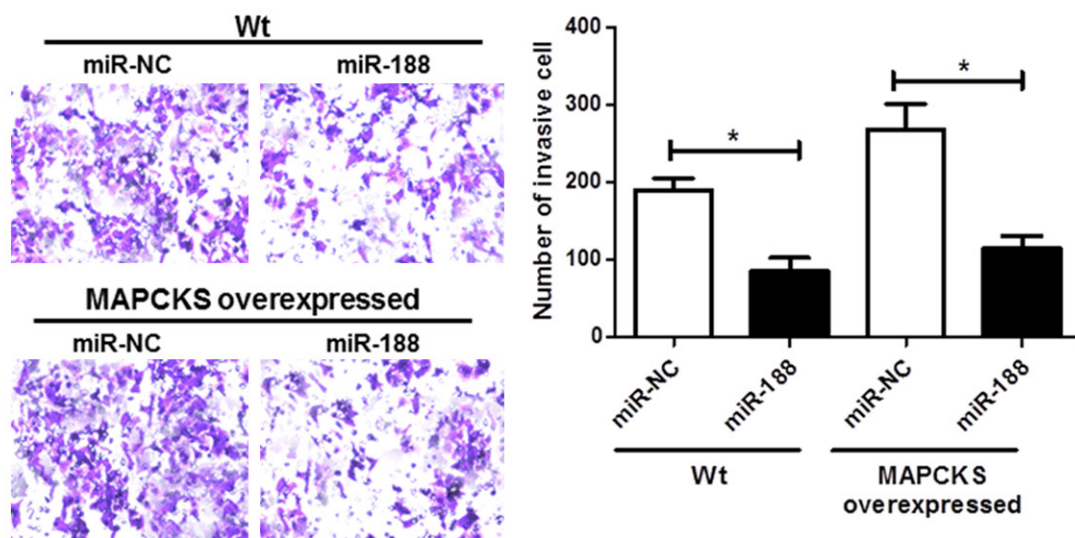
C



D



E





**Figure 5.** miR-188 binding to MARCKS-3'-UTR to inhibit prostate cancer cells. A. Total proteins were extracted from Wt and MARCKS-/- and then expressions of MARCKS were measured by western blot. B. The miR-188-binding site in the MARCKS-3'-UTR. C. psicheck-2-MARCKS-3'-UTR-Wt (MARCKS-Wt) and psicheck-2-MARCKS-3'-UTR-Mut (MARCKS-Mut) reporter vectors were constructed. Luciferase activities in MARCKS-Wt or MARCKS-Mut PC3 cells transfected with miR-188 or miR-NC. D, E. Viability and invasion of both Wt and MARCKS overexpressed prostate cancer cells transfected with miR-NC or miR-188 were investigated by MTT assay and transwell assay, respectively. \*P<0.05, \*\*P<0.01.

showed that luciferase activity in MARCKS-Wt transfected miR-188 mimics was significantly lower than the MARCKS-Wt luciferase activity in the transfected miR-NC group. In addition, it is found no significant difference in the luciferase-Mut activity between the transfected miR-188 mimics group and the luciferase-Mut activity in the transfected miR-NC group, suggesting that miR-188 can inhibit PC by targeting MARCKS.

In summary, the study availed a better understanding of the function of miR-188 and MARCKS in PC. We confirmed the down-regulated level of miR-188 in PC and revealed the role of miR-188 in PC cell proliferation and invasion. These findings indicated a tumor suppressor role of miR-188 in PC development and may serve as a potential therapeutic target in PC.

## Acknowledgements

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Written informed consent was obtained from all enrolled subjects.

## Disclosure of conflict of interest

None.

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