miR-589-5p is downregulated in prostate cancer and regulates tumor cell viability and metastasis by targeting CCL-5

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Abstract. Prostate cancer is one of the most common human malignancies, which represents a serious threat to health, and microRNAs (miRNAs/miRs) have been reported to be closely associated with the progression and development of prostate cancer. The present study aimed to investigate the expression patterns, functions and underlying mechanisms of miR-589-5p in prostate cancer. The results demonstrated that the expression levels of miR-589-5p were downregulated in prostate cancer tissues and cell lines. Overexpression of miR-589-5p inhibited cell viability, migration and invasion in prostate cancer cells. Subsequently, chemokine (C-C motif) ligand 5 (CCL-5) was identified as a direct target gene of miR-589-5p, which was highly expressed at the mRNA and protein levels in prostate cancer tissues and cells. Furthermore, CCL-5 mRNA was negatively correlated with miR-589-5p expression in prostate cancer tissues. Silencing CCL-5 promoted the apoptosis, and inhibited the migration and invasion of prostate cancer cells. Taken together, these results indicated that miR-589-5p may act as a tumor suppressor in prostate cancer by targeting CCL-5, thus suggesting that miR-589-5p may be a novel and reliable molecular marker for the diagnosis and prognosis of prostate cancer.

Introduction

Prostate cancer is one of the most common human malignancies and the second leading cause of cancer-associated mortality in men in western countries (1,2). According to an updated estimate, prostate cancer has the highest incidence amongst male tumors in 2018, accounting for 19% of male malignant tumors, while this disease has been reported as the second leading cause of cancer-associated mortality (3). In recent years, the incidence of prostate cancer has greatly increased in China (4,5). Furthermore, since the average life span is increasing and due to increased westernization of lifestyles, the incidence of prostate cancer is also increasing annually in China; notably, prostate cancer has been listed as one of the fastest growing tumors in China in the 21st century (6). Prostate cancer predominantly occurs in older men, and the median age of patients is ~72 years old; in addition, prostate cancer is the third most common major malignant tumor of the urogenital system, following bladder and renal tumors worldwide (7). The morphological characteristics and structure of prostate cancer are usually complex, and there is no obvious clinical symptom in the early stages; therefore, it is very difficult to make a clear diagnosis of prostate cancer (8). At present, the determination of prostate-specific antigen (PSA) in clinical serum samples is the most important diagnostic tool for prostate cancer. However, it has previously been reported that PSA is not the gold standard for diagnosis of prostate cancer. Although the sensitivity is high (~78.7%), the specificity is only 59.2%, the false negative rate is 38-48% and the false positive rate is 25%; therefore, prostate cancer can easily escape diagnosis or be misdiagnosed (9). Notably, rectal examination, imaging examination and other examination methods are also used in the clinical diagnosis of prostate cancer; however, these examinations do not achieve the desired results for the diagnosis of early prostate cancer, and may cause unnecessary injury and pain to patients (10,11). At present, there are various methods for the treatment of prostate cancer, including surgical treatment, hormone therapy, radiotherapy, etc. Although these methods are widely used in clinical treatment, there are several limitations in the treatment and prognosis of prostate cancer (12-14). Therefore, research has recently paid more attention to the prevention and treatment of prostate cancer. Exploration of the molecular mechanisms underlying the development of prostate cancer, and identification of novel methods for the treatment of prostate cancer, have recently been focuses of research.

Numerous studies have reported that microRNAs (miRNAs/miRs) serve an irreplaceable role in the progression and development of prostate cancer, and have significant impacts on the biological behaviors of prostate cancer, including proliferation, differentiation, invasion and hormone dependence (15,16). It has important application value and potential to provide reliable molecular markers for the diagnosis and prognosis of prostate cancer, which

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has unlimited potential as a target for treatment. miRNAs, which contain 17-25 nucleotides, suppress gene expression by binding to the 3'-untranslated regions (3'UTRs) of target genes, and have roles in various biological processes, including development, differentiation, cell proliferation and apoptosis (17-19). Distinct miRNA expression profiles have been identified in human prostate cancer tissues and cell lines, and it has been reported that miRNAs may act as either tumor suppressors or oncogenes in prostate cancer, depending on the functions of their target genes (20,21). Therefore, it is important to investigate the abnormal expression of miRNAs and their functions in prostate cancer, in order to provide effective and novel therapeutic targets for anti-prostate cancer therapy.

miR-589-5p has previously been reported to be frequently abnormally expressed in numerous types of human cancer (22,23). However, to the best of our knowledge, the expression and biological effects of miR-589-5p in prostate cancer have yet to be elucidated. Therefore, the present study aimed to investigate the expression levels of miR-589-5p in prostate cancer tissues and cell lines. In addition, the functions and underlying molecular mechanisms in prostate cancer were explored.

Materials and methods

Prostate cancer tissues. The present study was approved by the Ethical Committee of Nanjing Medical University (Huai'an, China). All patients provided written informed consent. A total of 30 prostate cancer tissues and corresponding adjacent normal tissues were obtained from patients who underwent surgical resection between June 2016 and April 2017 at the Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University (Huai'an, China); their average age was 45±7 years. old; none of the patients received pre-operative chemotherapy or radiotherapy. All fresh tissues were stored at -80˚C for further study.

Cell culture and transfection. The DU-145 and PC3 human prostate cancer cell lines, and RWPE-1 normal prostate epithelial cell line were purchased from the Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in an incubator containing 5% CO_2.

DU-145 and PC3 cells were seeded into 6-well plates at a density of $4x10^5$ cells/well. Once cells reached 60-70% confluence, transfection with synthesized miR-589-5p mimics negative control (NC), miR-589-5p mimics, miR-589-5p inhibitors NC, miR-589-5p inhibitors, chemokine (C-C motif) ligand 5 (CCL-5) small interfering RNAs (si-CCL-5) and negative control siRNA (siRNA NC) were synthesized by Invitrogen; Thermo Fisher Scientific, Inc and was performed with Lipofectamine® 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM for 24 h, according to the manufacturer's protocols. The sequences were as follows: miR-589-5p mimics forward, 5'-TTTTGGAGT TTTTTTTGGTTTTT-3' and reverse, 5'-CAAAAACAAAAC CAA-AATCA-3'; miR-589-5p inhibitors forward, 5'-TGTTGG AGAGCCAAGTGGTATTT-3' and reverse, 5'-CCTGAACAG AACCGGACTCA-3'; miR-589-5p mimics NC or miR-589-5p inhibitors NC forward, 5'-GGCTGCATTGGCTGGCGA AACCCGUC-3' and reverse, 5'-ATGCGUGCCCTGCTGTTG C-TCCATGTCG-3'; si-CCL5 forward 5'-GCGTCGAGTTTG TCACGAGA-3', reverse 5'-TGACACTCCTTTACTGTGCT-3'. Transfected cells were cultured for 24, 48 or 72 h under the same conditions as aforementioned.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from prostate cancer tissues and cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min, according to the manufacturer's protocol. For detection of miR-589-5p expression, TaqMan MicroRNA Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to synthesize cDNA according to the manufacturer's protocol. The expression levels of miR-589-5p were examined using TaqMan MicroRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. To quantify CCL5 mRNA expression, the PrimeScript RT reagent kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize cDNA according to the manufacturer's protocol. Subsequently, CCL-5 mRNA was amplified using SYBR Premix Ex Taq (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. U6 and GAPDH were used as internal controls for miR-589-5p and CCL-5, respectively. The PCR primers used in the present study were as follows: miR-589-5p, forward 5'-CGAGGTCAG CGTGATTTCATGG-3', reverse 5'-TGTGTCCAAGTCCCA GCCAGAG-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'; CCL-5, forward 5'-CAGTCGTCTTTGTCACCCGA-3', reverse 5'-TGTAACTGC TGCTGTGTGGT-3'; and GAPDH, forward 5'-ACAACTTTG GTATCGTGGAAGG-3' and reverse 5'-GCCATCACGCCA CAGTTTC-3'. The thermocycling conditions comprised one cycle at 95˚C for 30 sec, followed by 42 cycles of amplification (95˚C for 3 sec and 60˚C for 30 sec). Fold-changes in miR-589-5p and CCL-5 mRNA expression were calculated using the $2^{-\Delta\Delta Cq}$ method (24), and RT-qPCR reactions were performed in triplicate.

Cell viability assay. Cell viability was evaluated using the Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Briefly, DU-145 and PC3 cells at a density of $3x10^3$ cells/well were cultured in a 96-well plate and transfected with miR-589-5p mimics NC, miR-589-5p mimics, miR-589-5p inhibitors NC or miR-589-5p inhibitors. After 24, 48 or 72 h at 37˚C and 5% CO_2, 10 μl CCK-8 reagent was added to each well and the cells were incubated for another 4 h. The optical density was observed at a wavelength of 450 nm using a microplate reader (LNB, Shanghai, China).

Cell apoptosis. DU-145 and PC3 cells at a density of $1x10^5$ cells/well were cultured in a 6-well plate for 48 h post-transfection. Subsequently, the cells were detached,

washed with PBS and centrifuged at 12,000 x g for 5 min. The cells were then incubated with 500 μl buffer, 5 μl fluorescein isothiocyanate-Annexin V and 5 μl propidium iodide (PI) for 1 h at 37˚C, according to the manufacturer's protocols (Shanghai Yeasen Biotechnology, Co., Ltd., Shanghai, China). Cell apoptosis were analyzed using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Wound-healing assay. For the wound-healing assay, DU-145 and PC3 cells were seeded into a 6-well plate at a density of $1x10^5$ cells/well post-transfection for 24 h. Once cell confluence reached 90%, the cell monolayers were wounded in the surface of the plates using a 200 μl pipette tip and washed with serum-free medium to remove floating cells. The cells were then cultured in a serum-free medium for cell recovery. Images of the cells were captured at 0 and 48 h under an inverted microscope.

Transwell invasion assay. The Transwell invasion assay was performed to evaluate the invasive capacity of prostate cancer cells. Following transfection for 24 h, DU-145 and PC3 cells at a density of $5x10^4$ cells/well in 100 μl serum-free medium were seeded into the upper chambers of a Transwell system (8 μm; Corning Corporation, Corning, NY, USA), which were coated with Matrigel (BD Pharmingen; BD Biosciences). Culture medium containing 20% FBS was added to the lower chambers. After 48 h at 37˚C, the cells remaining on the upper compartments were removed, whereas the invaded cells were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 10% crystal violet at room temperature for 15 min. Cell numbers were obtained from five fields per membrane under a light microscope (Olympus Corporation, Tokyo, Japan). The following equations were used to analyze findings: Invasion rate (%) = invasive cells/total cells.

Target prediction for miR-589-5p and luciferase reporter assay. The candidate target genes of miR-589-5p were analyzed using the miRNA target prediction programs PicTar (pictar.mdc-berlin.de/) and TargetScan (www.targetscan.org/). The 3'UTR sequences of CCL-5 containing the putative binding site for miR-589-5p were amplified from human genomic DNA, and cloned into the pGL3 vector (Promega Corporation, Madison, WI, USA), in order to obtain the recombinant vectors pGL3-CCL-5-wild-type (wt) and pGL3-CCL-5-mutant (mut). Subsequently, DU-145 and PC3 cells ($1x10^5$) were plated in 48-well plates for 24 h, and psiCheck-2 with the wild-type (WT) or mutant (MUT) 3'-untranslated region (UTR) of CCL-5 was cotransfected with miR-589-5p mimics or miR-NC at a concentration of 50 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, cells were collected and subjected to a luciferase assay using the Dual-Luciferase Reporter Assay kit according to the manufacturer's protocol (Promega Corporation).

Western blotting. Total protein was extracted from tissues and transfected cells using radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Subsequently, the supernatants were collected following centrifugation at 12,000 x g for 20 min at 4˚C, and the protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Vazyme, Piscataway, NJ, USA). Loading buffer was added to the supernatant of samples and the proteins were denatured at 100˚C for 5 min. Equal amounts of protein (30 μg) were then separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for at room temperature for 1 h, washed four times with Tris-buffered saline plus 0.1% Tween (TBST; 15 min/wash), and incubated overnight at 4˚C with primary antibodies. The following primary antibodies were used: Anti-CCL-5 (cat. no. ab189841; 1:1,000), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. ab32503; 1:1,000), Bcl-2 (cat. no. ab196495; 1:1,000), caspase-3 (cat. no. ab90437; 1:1,000), caspase-9 (cat. no. ab25758; 1:1,000), matrix metalloproteinase (MMP)-2 (cat. no. ab37150; 1:1,000), MMP-9 (cat. no. ab73734; 1:1,000) and GAPDH (cat. no. ab9485; 1:1,000) (all from Abcam, Cambridge, UK). Subsequently, the membranes were washed with TBST three times (15 min/wash) and incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. ab6721; 1:2,000; Abcam) for 2 h at room temperature. Protein bands were examined using the Bio-Rad Imaging system (Hercules, CA, United States) with an enhanced chemiluminescence western blotting substrate kit (ProteinTech Group, Inc., Wuhan, China) and the results were measured using ImageJ software 1.48 (National Institutes of Health, Bethesda, MD, USA). GAPDH were used as a loading control.

Statistical analysis. All data are expressed as the means ± standard deviation, and all experiments were repeated at least three times. Statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Multiple group comparisons were conducted using one-way analysis of variance and Tukey's test. Student's t-test was used to compare only two groups. The association between CCL-5 and miR-589-5p expression in prostate cancer was evaluated using Spearman's correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-589-5p is downregulated in prostate cancer tissues and cell lines. In order to explore the role of miR-589-5p in prostate cancer, RT-qPCR was performed to evaluate its expression in prostate cancer tissues and cells. As presented in Fig. 1A, miR-589-5p was markedly downregulated in prostate cancer tissues compared with in corresponding adjacent normal tissues. In addition, the expression levels of miR-589-5p were examined in prostate cancer cells (DU-145 and PC3) and in RWPE normal prostate epithelial cells. The results demonstrated that, compared with in RWPE cells, the expression levels of miR-589-5p were significantly decreased in DU-145 and PC3 cells (Fig. 1B). These findings indicated that miR-589-5p may act as a tumor suppressor in prostate cancer progression and development.

miR-589-5p inhibits viability and induces apoptosis of prostate cancer cells. The present study demonstrated that miR-589-5p was downregulated in prostate cancer cells. To further explore the role of miR-589-5p in the progression and development of prostate cancer, miR-589-5p mimics NC, miR-589-5p mimics,

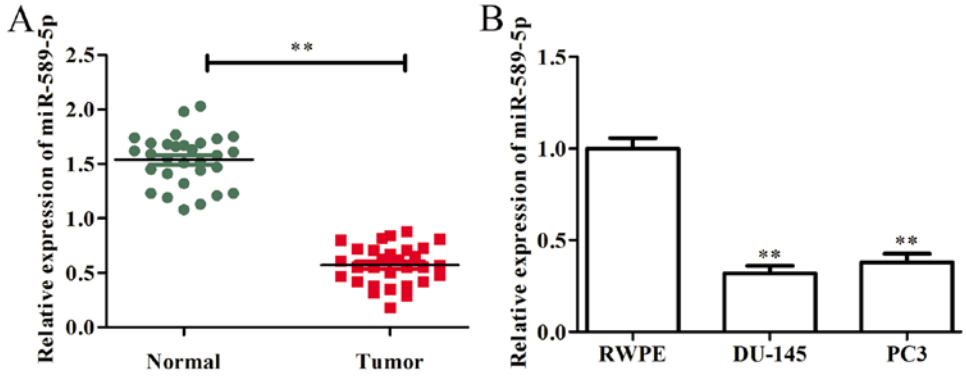

Figure 1. miR-589-5p is downregulated in prostate cancer tissues and cells. (A) miR-589-5p expression was detected in 30 prostate cancer tissues and matched adjacent normal tissues using RT-qPCR. (B) miR-589-5p expression was detected in prostate cancer and normal prostate epithelial cells using RT-qPCR. Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. **P<0.01 compared with the control group. miR-589-5p, microRNA-589-5p; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

miR-589-5p inhibitors NC and miR-589-5p inhibitors were transfected into DU-145 and PC3 cells. After transfection for 48 h, transfection efficiency was determined by RT-qPCR. As shown in Fig. 2A, the expression levels of miR-589-5p were significantly upregulated following transfection with miR-589-5p mimics, whereas they were downregulated post-transfection with miR-589-5p inhibitors, as compared with in the NC-transfected DU-145 and PC3 cells.

CCK-8 assay was performed to evaluate the effects of miR-589-5p on the viability of DU-145 and PC3 cells; the results revealed that the viability of DU-145 and PC3 cells transfected with miR-589-5p mimics was markedly suppressed compared with in cells transfected with the NC, whereas miR-589-5p inhibitors significantly promoted the viability of DU-145 and PC3 cells compared with the NC group (Fig. 2B).

The induction of apoptosis is a critical process through which miRNAs exert anticancer effects (25). Therefore, the association between miR-589-5p and DU-145 and PC3 cell apoptosis was revealed by flow cytometry. Post-transfection with miR-589-5p mimics NC, miR-589-5p mimics, miR-589-5p inhibitors NC and miR-589-5p inhibitors, PI and Annexin V double staining was conducted to evaluate apoptosis. As shown in Fig. 2C, miR-589-5p mimics promoted apoptosis, whereas miR-589-5p inhibitors inhibited apoptosis of DU-145 and PC3 cells compared with in the NC groups.

Tumor cell apoptosis is mediated by various molecules that inhibit (e.g. Bcl-2) or induce (e.g. Bax and caspases) cell death (26). Therefore, the present study detected the protein expression levels of Bcl-2, Bax, caspase-3 and caspase-9. The results demonstrated that pro-apoptotic proteins, including Bax, caspase-3 and caspase-9, were increased, whereas the expression levels of the anti-apoptotic protein Bcl-2 were significantly decreased in DU-145 and PC3 cells transfected with miR-589-5p mimics (Fig. 2D). Conversely, transfection with miR-589-5p inhibitors exerted the opposite effects on DU-145 and PC3 cells.

miR-589-5p suppresses the migration and invasion of prostate cancer cells. Wound-healing and Transwell invasion assays were performed to investigate the effects of miR-589-5p on migration and invasion of DU-145 and PC3 cells transfected with miR-589-5p mimics NC, miR-589-5p mimics, miR-589-5p inhibitors NC and miR-589-5p inhibitors. The results indicated that the migratory capacity of DU-145 and PC3 cells transfected with miR-589-5p mimics was markedly suppressed compared with cells transfected with miR-589-5p mimics NC, whereas transfection of DU-145 and PC3 cells with miR-589-5p inhibitors notably increased cell migration into the wound (Fig. 3A). Furthermore, a Transwell invasion assay revealed that miR-589-5p mimics markedly suppressed DU-145 and PC3 cell invasion. However, when DU-145 and PC3 cells were transfected with miR-589-5p inhibitors, invasion was significantly increased compared with in cells in the miR-589-5p inhibitors NC group (Fig. 3B).

During the progression and development of metastasis in prostate cancer, MMPs serve a significant role (27). In the present study, the protein expression levels of MMP-2 and MMP-9 were detected in DU-145 and PC3 cells transfected with miR-589-5p mimics NC, miR-589-5p mimics, miR-589-5p inhibitors NC and miR-589-5p inhibitors. The results indicated that miR-589-5p mimics markedly suppressed the protein expression levels of MMP-2 and MMP-9, whereas the expression levels of MMP-2 and MMP-9 were significantly enhanced following transfection with miR-589-5p inhibitors (Fig. 3C).

CCL-5 is a direct target of miR-589-5p in prostate cancer cells. This study aimed to explore the molecular mechanisms underlying the effects of miR-589-5p on prostate cancer cell viability, apoptosis, migration and invasion. Firstly, PicTar and TargetScan were used to predict the possible target gene of miR-589-5p; the results demonstrated that CCL-5 may be a potential target gene of miR-589-5p (Fig. 4A). Furthermore, a luciferase reporter assay was performed to confirm whether miR-589-5p binds to the 3'UTR of CCL-5; the results demonstrated that miR-589-5p directly binds to the 3'UTR of CCL-5 following co-transfection with miR-589-5p mimics and luciferase reporter vector; ectopic expression of miR-589-5p decreased the luciferase activity of CCL-5 3'UTR-wt, whereas it did not affect that of CCL-5 3'UTR-mut (Fig. 4B).

To further confirm whether CCL-5 was a direct target gene of miR-589-5p, RT-qPCR and western blotting were performed to evaluate the mRNA and protein expression levels of CCL-5

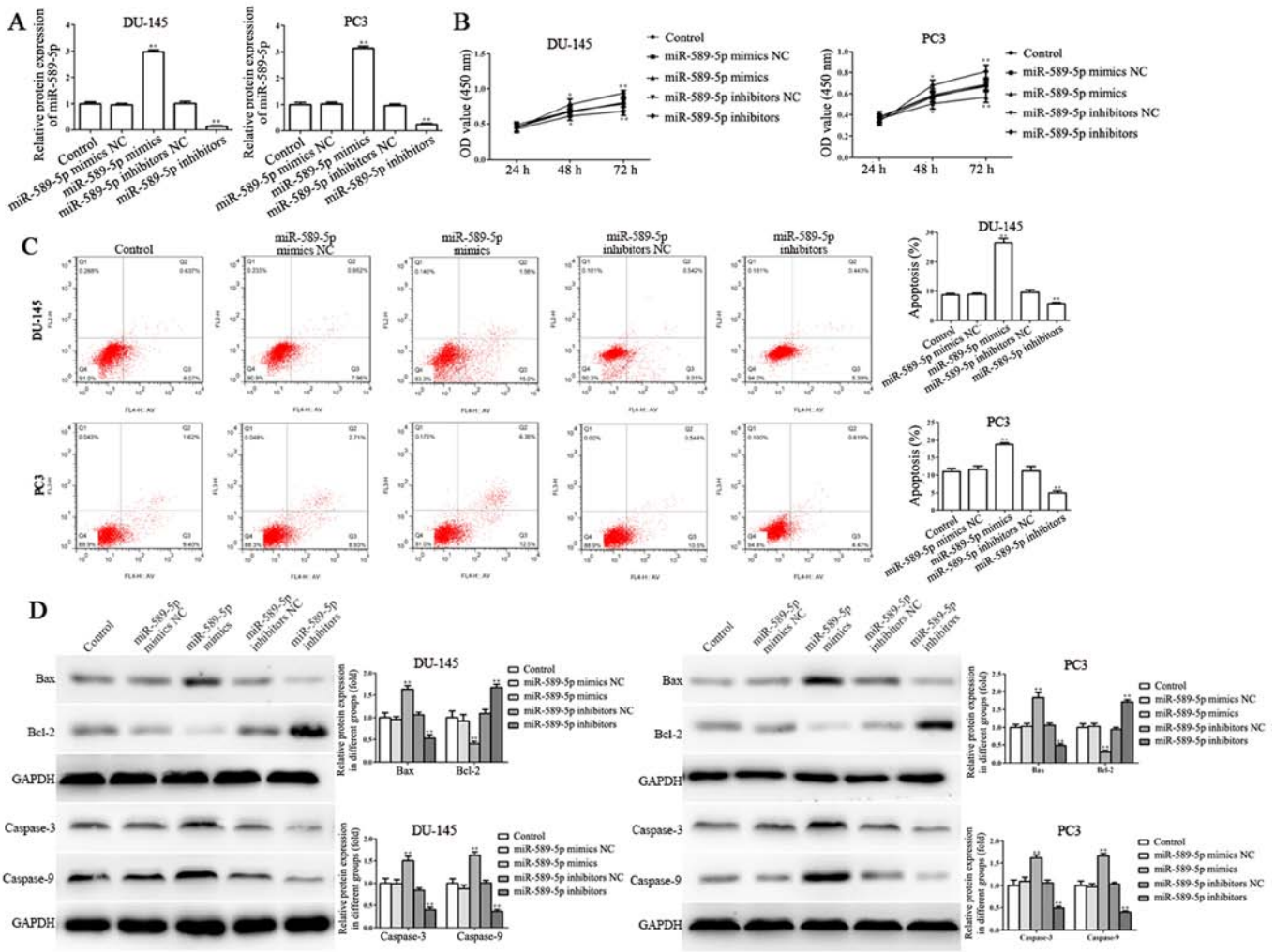

Figure 2. miR-589-5p inhibits viability and induces apoptosis of prostate cancer cells. (A) Transfection efficiency was assessed by reverse transcription-quantitative polymerase chain reaction post-transfection with miR-589-5p mimics or miR-589-5p inhibitors in DU-145 and PC3 cells. (B) Viability of DU-145 and PC3 cells was determined post-transfection with miR-589-5p mimics or miR-589-5p inhibitors for 24, 48 and 72 h using the Cell Counting kit-8 assay. (C) Apoptosis assays were performed by flow cytometry post-transfection with miR-589-5p mimics or miR-589-5p inhibitors for 48 h in DU-145 and PC3 cells. (D) Protein expression levels of Bax, Bcl-2, caspase-3 and caspase-9 were examined in DU-145 and PC3 cells transfected with miR-589-5p mimics or miR-589-5p inhibitors for 48 h; band intensity was semi-quantified using ImageJ software. Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. *P<0.05, **P<0.01 compared with the control group. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; miR-589-5p, microRNA-589-5p; NC, negative control; OD, optical density.

in DU-145 and PC3 cells post-transfection with miR-589-5p mimics NC, miR-589-5p mimics, miR-589-5p inhibitors NC and miR-589-5p inhibitors. The results revealed that overexpression of miR-589-5p significantly decreased the mRNA and protein expression levels of CCL-5, whereas knockdown of miR-589-5p increased the mRNA and protein expression levels of CCL-5 in DU-145 and PC3 cells (Fig. 4C and D). These findings strongly indicated that CCL-5 was the direct target gene of miR-589-5p.

CCL-5 is upregulated in prostate cancer tissues and cells. To further evaluate the role of CCL-5 in prostate cancer, the mRNA and protein expression levels of CCL-5 were determined in prostate cancer tissues and cells by RT-qPCR and western blotting. As shown in Fig. 5A-D, the mRNA and protein expression levels of CCL-5 were significantly upregulated in prostate cancer tissues and cells compared with in the corresponding adjacent normal tissues and normal prostate epithelial cells, respectively. In addition, the association between CCL-5 and miR-589-5p expression in prostate cancer was evaluated using Spearman's correlation analysis; the results demonstrated that CCL-5 mRNA expression was negatively correlated with miR-589-5p expression in prostate cancer tissues (Fig. 5E).

Knockdown of CCL-5 suppresses viability, migration and invasion, and induces apoptosis of prostate cancer cells. In order to investigate the role of CCL-5 in the regulation of viability, apoptosis, migration and invasion in DU-145 and PC3 cells, si-CCL-5 was transfected into DU-145 and PC3 cells using Lipofectamine® 2000 Reagent. Subsequently, RT-qPCR was performed to determine transfection efficiency; the results demonstrated that si-CCL-5 markedly inhibited the mRNA and protein expression levels of CCL-5 in DU-145 and PC3 cells (Fig. 6A and B). In addition, flow cytometry was performed to assess the effects of CCL-5 on

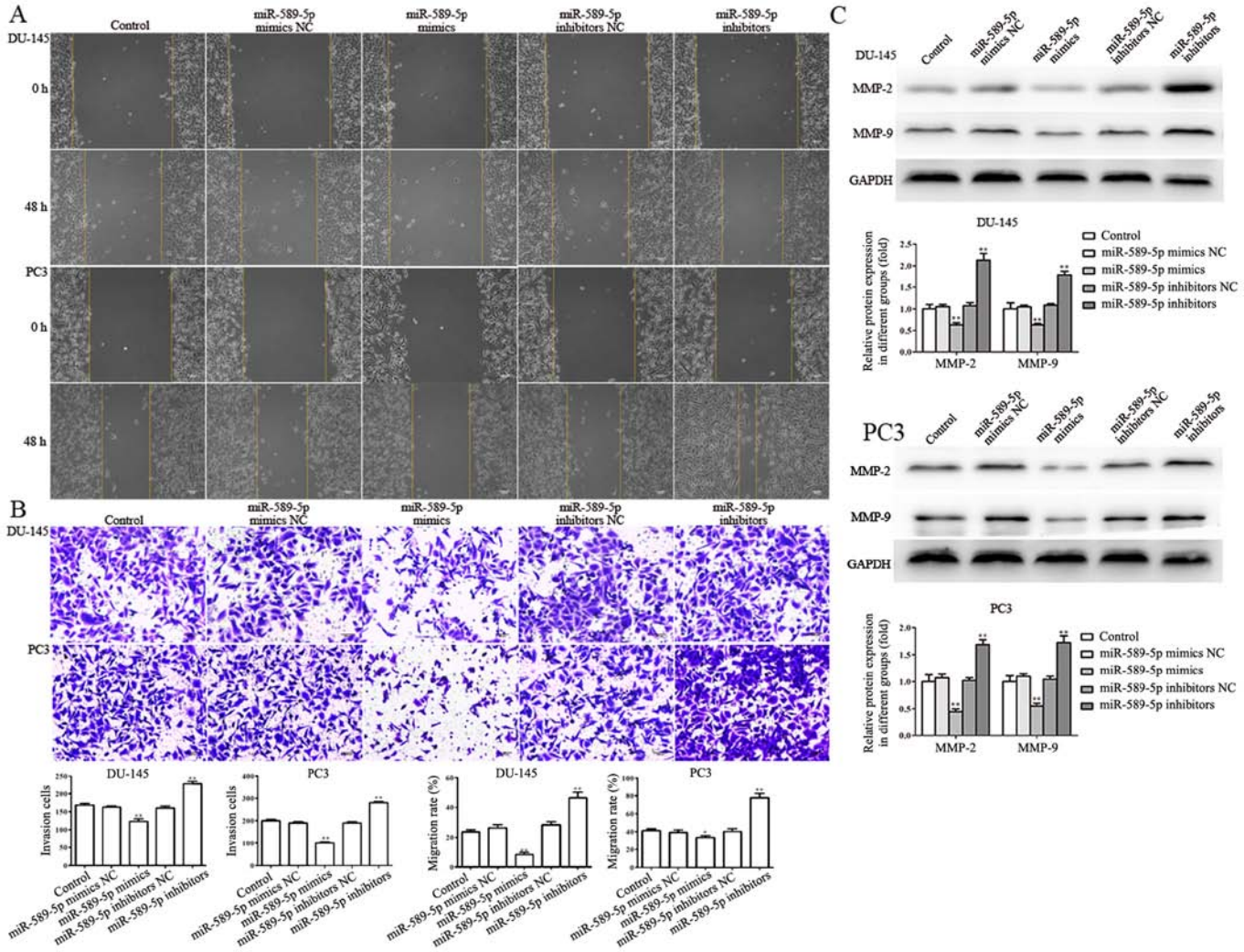

Figure 3. miR-589-5p suppresses the migration and invasion of prostate cancer cells. (A) Migratory abilities of DU-145 and PC3 cells transfected with miR-589-5p mimics or miR-589-5p inhibitors for 48 h were examined by wound-healing assay (magnification, x200). (B) Invasive abilities of DU-145 and PC3 cells transfected with miR-589-5p mimics or miR-589-5p inhibitors for 48 h were evaluated by Transwell invasion assay (magnification, x200). (C) Protein expression levels of MMP-2 and MMP-9 were examined in DU-145 and PC3 cells transfected with miR-589-5p mimics or miR-589-5p inhibitors for 48 h; band intensity was semi-quantified using ImageJ software. Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. *P<0.05, **P<0.01 compared with the control group. miR-589-5p, microRNA-589-5p; MMP, matrix metalloproteinase; NC, negative control.

the apoptosis of DU-145 and PC3 cells. The results verified that si-CCL-5 markedly promoted the apoptosis of DU-145 and PC3 cells; the proportions of apoptotic cells were 15.67±3.87 and 19.27±4.29 compared with in the control group (7.86±4.13 and 11.17±5.31), respectively (Fig. 6C). Furthermore, wound-healing, and Transwell invasion assays were performed to examine the effects of CCL-5 on the migration and invasion of DU-145 and PC3 cells. As shown in Fig. 6D and E, it was revealed that CCL-5, as a target gene for miR-589-5p, may serve an essential role in the progression and development of prostate cancer.

Discussion

Prostate cancer is the most common malignancy in men, and is the second leading cause of cancer-associated mortality in men. In addition, the incidence of prostate cancer increases with age (28). There are no symptoms during the early stage of prostate cancer, and symptoms occur only when the tumor obstructs the urethra or bladder neck (29). Therefore, early diagnosis and treatment are important factors in determining the cure rates and prognosis of tumor patients, and searching for reliable and effective molecular markers to guide the diagnosis and prognosis of prostate cancer is a popular topic in clinical research. miRNAs are non-coding short-chain RNAs, which have significant roles as oncogenes and tumor suppressor genes in the development of various types of cancer (30,31). It has previously been reported that miR-589-5p acts as a tumor suppressor in non-small cell lung cancer (22). In addition, the long non-coding RNA (lncRNA) putative fatty acid-binding protein 5-like protein 3 (FABP5P3) can bind to miR-589-5p, which serves a tumor-enhancing role in hepatocellular carcinoma; the lncRNA FABP5P3/miR-589-5p/zinc finger MYND-type containing 19 axis contributes to hepatocellular carcinoma cell proliferation, migration and invasion (23). In addition, miR-589-5p can regulate mitogen-activated protein kinase kinase kinase 8 expression in hepatocellular carcinoma (32). However, the role and mechanism of miR-589-5p

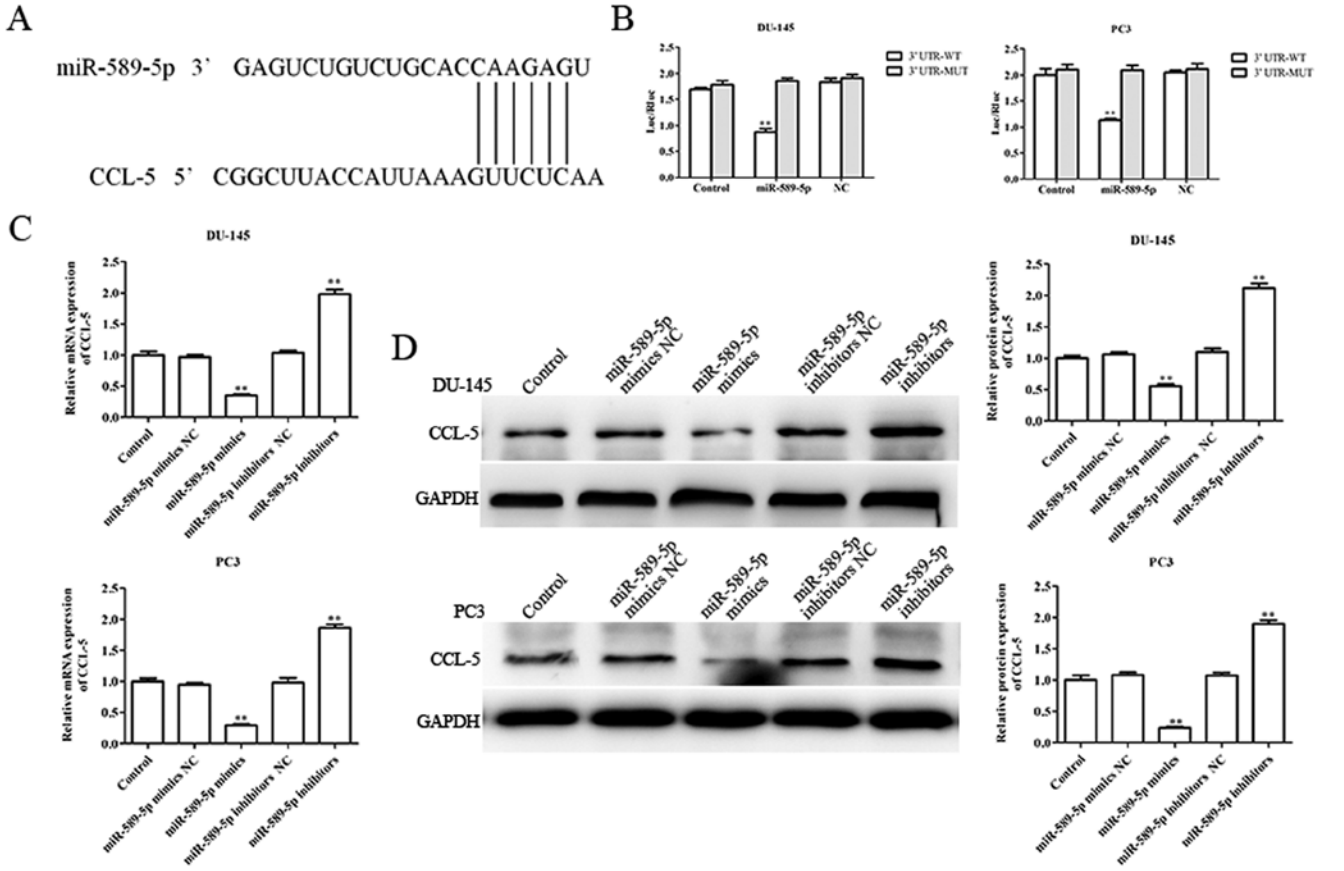

Figure 4. CCL-5 is a direct target gene of miR-589-5p in prostate cancer cells. (A) Prediction of the binding between miR-589-5p and CCL-5, as determined by TargetScan. (B) Luciferase reporter assays were performed to verify the binding of miR-589-5p to the 3'UTR of CCL-5. (C) Reverse transcription-quantitative polymerase chain reaction and (D) western blotting were performed to evaluate the mRNA and protein expression levels of CCL-5 following transfection with miR-589-5p mimics or miR-589-5p inhibitors. Band intensity was semi-quantified using ImageJ software. Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. **P<0.01 compared with the control group. 3'UTR, 3'-untranslated region; CCL-5, chemokine (C-C motif) ligand 5; miR-589-5p, microRNA-589-5p; MUT, mutant; NC, negative control; Rluc, Renilla luciferase; WT, wild-type.

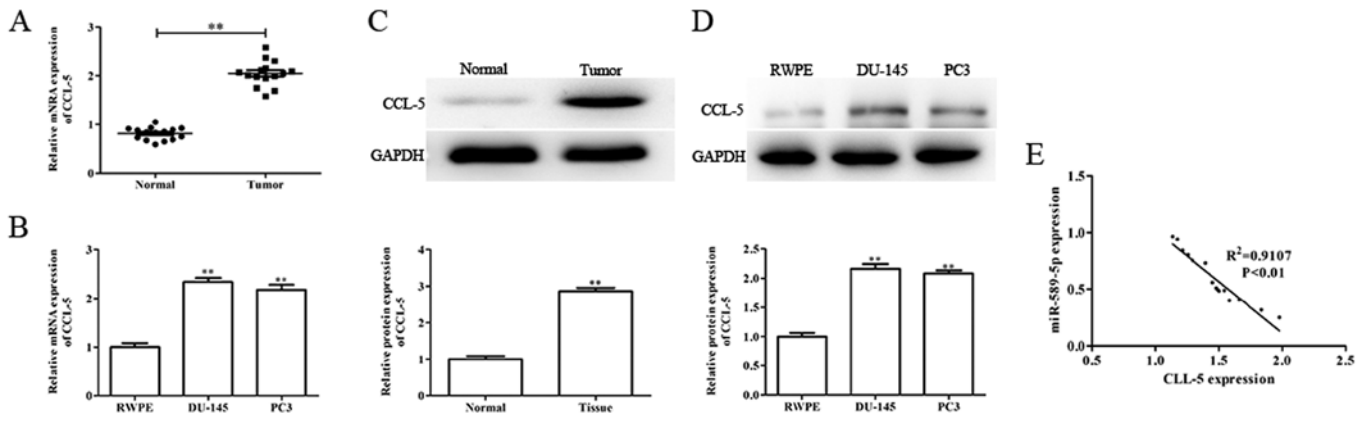

Figure 5. CCL-5 is upregulated in prostate cancer tissues and cells. (A) RT-qPCR and (B) western blotting were performed to detect the mRNA and protein expression levels of CCL-5 in 15 prostate cancer tissues and matched adjacent normal tissues. (C) RT-qPCR and (D) western blotting were performed to detect the mRNA and protein expression levels of CCL-5 in prostate cancer cell lines and normal prostate epithelial cells. Band intensity was semi-quantified using ImageJ software. (E) Analysis of the association between miR-589-5p and CCL-5 expression in prostate cancer tissues. Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. **P<0.01 compared with the control group. CCL-5, chemokine (C-C motif) ligand 5; miR-589-5p, microRNA-589-5p; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in prostate cancer remain elusive. The present study confirmed that the expression levels of miR-589-5p in prostate cancer tissues and cell lines were abnormally downregulated, thus suggesting that miR-589-5p may act as a tumor suppressor involved in the progression and development of prostate cancer.

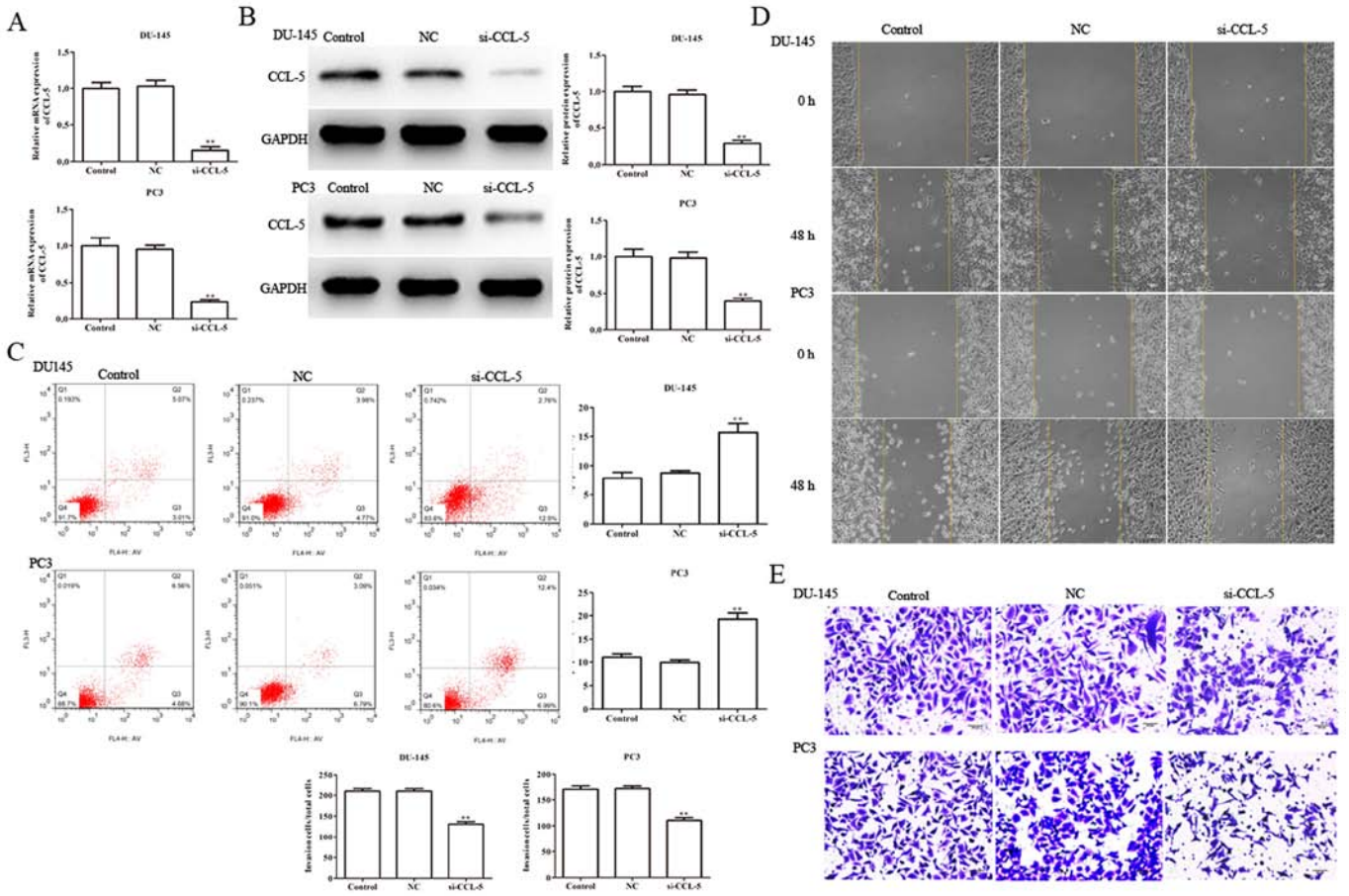

Figure 6. Silencing of CCL-5 suppresses viability, migration and invasion, and induces apoptosis of prostate cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blotting were performed to assess knockdown efficiency post-transfection with si-CCL-5 in DU-145 and PC3 cells. (C) Apoptosis assays were performed using flow cytometry post-transfection with si-CCL-5 for 48 h in DU-145 and PC3 cells. (D) Migratory abilities of DU-145 and PC3 cells transfected with si-CCL-5 for 48 h were examined by wound-healing assay (magnification, x200). (E) Invasive abilities of DU-145 and PC3 cells transfected with si-CCL-5 for 48 h were evaluated by Transwell invasion assay (magnification, x200). Data are presented as the means ± standard deviation of three independent experiments, each experiment was performed in triplicate. **P<0.01 compared with the control group. CCL-5, chemokine (C-C motif) ligand 5; miR-589-5p, microRNA-589-5p; NC, negative control; si, small interfering RNA.

Apoptosis is a programmed cell death controlled by intracellular signals (33). There are two classic apoptotic pathways: The death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway. Alterations in mitochondrial outer membrane permeabilization can result in the release of various pro-apoptotic factors from the intermembrane space to the cytoplasm, which further activates caspases and induces cell apoptosis (34). Furthermore, upregulation of the Bax:Bcl-2 ratio results in the release of cytochrome *c* from the mitochondria and activates mitochondria-dependent caspases to induce apoptotic cell death (35). In the present study, overexpression of miR-589-5p markedly inhibited the viability of DU-145 and PC3 prostate cancer cells, whereas knockdown of miR-589-5p promoted DU-145 and PC3 cell viability. In addition, miR-589-5p mimics increased the protein expression levels of Bax, caspase-3 and caspase-9, and decreased the protein expression levels of Bcl-2 in DU-145 and PC3 cells, whereas miR-589-5p inhibitors exhibited the opposite effects.

Tumor invasion and metastasis are biological characteristics of malignant tumors, and are the main causes of mortality in patients with cancer (36). The present results confirmed the inhibitory role of miR-589-5p in the invasion and metastasis of DU145 and PC3 cells by wound-healing and Transwell invasion assays. It has been reported that tumor invasion and metastasis are a series of complex, multi-step and multi-factor dynamic process between tumor cells and host cells or the extracellular matrix (ECM) (37,38). A series of tissue barriers are encountered in the process of tumor invasion and metastasis; these barriers are made up of basement membrane, mesenchyme and matrix in the ECM. Among the enzymes involved in the destruction of the ECM, MMPs are the most closely associated with tumor invasion and metastasis (39-41). The present study revealed that miR-589-5p mimics significantly suppressed the expression levels of MMP-2 and MMP-9, whereas the expression levels of MMP-2 and MMP-9 were promoted following transfection with miR-589-5p inhibitors.

According to the strict complementary nature of base pairs, miRNAs can target and bind mRNAs, and can inhibit the expression of target genes through degrading target mRNAs or inhibiting the translation progress. These functions of miRNAs participate in the progression and development of cancer (42). CCL-5 is a chemokine that belongs to the chemokine C-C family, which is mainly expressed in T lymphocytes, macrophages, platelets, synovial fibroblasts, renal tubular epithelial cells and certain types of tumor cells (43). Previous studies have

detected CCL-5 expression in tissues and serum samples from patients with pancreatic cancer, lung cancer, melanoma, etc., and it is correlated with disease progression (44,45). In addition, CCL-5, as a target gene, takes part in the development of numerous types of tumor (46,47). In this study, TargetScan and PicTar were used to analyze the target genes of miR-589-5p, and a luciferase reporter assay confirmed that CCL-5 may be a potential candidate of miR-589-5p. Furthermore, the role of CCL-5 in the progression and development of prostate cancer was evaluated. Upregulation of CCL-5 was observed in prostate cancer tissues and cell lines, and si-CCL-5 had inhibitory effects on viability, apoptosis, migration and invasion in DU145 and PC3 cells. In addition, the mRNA expression levels of CCL-5 were negatively correlated with miR-589-5p expression in prostate cancer tissues. These results confirmed that CCL-5 acts as a target gene of miR-589-5p and may serve an important role in the progression and development of prostate cancer.

In conclusion, the present study confirmed that miR-589-5p was downregulated in prostate cancer tissues and cell lines. In addition, it was suggested that miR-589-5p may function as a potential tumor suppressor in prostate cancer to inhibit cell growth and metastasis by targeting CCL-5. Therefore, miR-589-5p may be considered a reliable molecular marker for the diagnosis and prognosis of prostate cancer. This study presented preliminary research on the antitumor effects of miR-589-5p in prostate cancer. At present, it is unclear whether miR-589-5p has anti-tumor effects in other types of cancer and the underlying mechanism requires further investigation; therefore, we aim to further explore the signaling pathways that are involved in CCL-5-mediated regulation of cell viability and metastasis in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BZ conceived and designed the present study. XJ and FM performed the experiments. LJ and ZT acquired and analyzed the data. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Nanjing Medical University. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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