miR-583 inhibits the proliferation and invasion of prostate cancer cells by targeting JAK1

ZHONGYI LI[1] and JIANHENG CHEN[2]

[1]Department of Urology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009; [2]Department of Urology, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650031, P.R. China

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Abstract. Prostate cancer (PCa) is a leading cause for death in men and the most commonly diagnosed malignancy globally. MicroRNA (miR)-583 expression levels have been discovered to be downregulated in recurrent PCa samples compared with non-recurrent cases. However, the precise functions and pathogenic mechanism of miR-583 in the development of PCa are vague, thus the aim of the present study was to investigate these. The expression levels of miR-583 and Janus kinase 1 (JAK1) in PCa tissues and cell lines were analyzed using reverse transcription-quantitative PCR and western blotting. The protein expression levels of phosphorylated (p)-STAT3 and STAT3 in PCa cell lines were also analyzed using western blotting. The effects of miR-583 and JAK1 on the proliferation and invasion of PCa cell lines cell lines were determined using MTT and Transwell assays, respectively. The binding interaction between miR-583 and the 3'-untranslated region of JAK1 were predicted by TargetScan, and further validated using dual luciferase reporter assays in PCa cell lines. The results revealed that the expression levels of miR-583 were downregulated, while those of JAK1 were upregulated in PCa tissues and cell lines (DU145 and PC3). The transfection with the miR-583 mimic inhibited the proliferation and invasion, as well as downregulating JAK1 and p-STAT3 protein expression levels in DU145 and PC3 cell lines. These effects were partially abolished following the overexpression of JAK1. Moreover, JAK1 was identified to be a target gene for miR-583 in DU145 and PC3 cell lines and the expression levels of miR-583 were revealed to be negatively correlated with JAK1 expression levels in PCa tissues. In conclusion, the findings of the present study suggested that miR-583 may inhibit the proliferation and invasion of PCa cells by targeting JAK1, thus providing a novel therapeutic target for patients with PCa.

Correspondence to: Dr Jianheng Chen, Department of Urology, The First Affiliated Hospital of Kunming Medical University, 295 Xichang Road, Kunming, Yunnan 650031, P.R. China
E-mail: chenjianhengkmmu@126.com

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Introduction

As the most frequently diagnosed malignancy in men ≥65 years old, prostate cancer (PCa) is characterized by high morbidity and mortality rates worldwide (1,2). The current morbidity of PCa is as high as 31.1 per 100,000 in the population, accounting for ~15% of all cancer cases worldwide (3). Several factors have been reported to be involved in the development of PCa, including family history, genetic inheritance and age (4). Radiotherapy, alone or combined with androgen deprivation therapy, represents a standard treatment regimen for patients with PCa (5). However, despite the advancements which have been achieved in local tumor control, a considerable percentage of patients with PCa still undergo tumor recurrence (6); thereafter, these patients quickly progress to an incurable stage of PCa, with the overall survival merely lasting longer than 3-4 months (7).

MicroRNAs (miRNAs/miRs) are a subtype of non-coding RNA of 22 nucleotides in length (8), which have been demonstrated to be crucial post-transcriptional regulators (9). miRNAs have been identified to serve various roles during cancer progression; for example, miRNAs that promote tumorigenesis are termed 'OncomiRs', while miRNAs that suppress tumorigenesis are referred to as tumor suppressors (10,11). Numerous physiological functions, including stemness maintenance, cell apoptosis, proliferation, migration and invasion, are regulated by miRNAs (12). In particular, miR-583 has been discovered to have a role in several types of human disease; for instance, miR-583 was demonstrated to be important for Zheng differentiation in chronic hepatitis B (13); downregulated expression levels of miR-583 were discovered in the sera of patients with congestive heart failure (14); and miR-583 was reported to negatively regulate the differentiation of natural killer cells through silencing IL2 receptor γ (15). In addition, miR-583 expression levels were found to be upregulated in a good recovery group compared with a poor recovery group following human stroke (16). miR-583 was also identified to be involved in circular (circ)RNA_104515 and circ_100291/miRNA interactions in hepatocellular carcinoma (17). Furthermore, in 2017, a meta-analysis

indicated that miR-583 expression levels were downregulated in recurrent PCa samples compared with non-recurrent cases (18). However, the function of miR-583 in PCa has not been reported, which will be explored in the present study. It was concluded that miR-583 may inhibit the proliferation and invasion of PCa cells by targeting JAK1.

Materials and methods

Patient samples. Tumor tissues and matched adjacent normal tissues were obtained from 21 patients with PCa (aged between 57 and 75 years old) who had been treated at The First Affiliated Hospital of Kunming Medical University (Kunming, China) between January 2015 and June 2017. Participants who had undergone surgical procedures for tissue extraction were included in the present study. None of the participants had received radiotherapy or chemotherapy prior to surgery. Written informed consent was provided by all patients. The use of tissues from patients with PCa for scientific research was approved by the ethics committee of The First Affiliated Hospital of Kunming Medical University.

Cell culture. A human normal prostate epithelial cell line (RWPE-1) and 4 human PCa cell lines (LNCaP, C4-2, DU145 and PC3) were purchased from the American Type Culture Collection. All cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37˚C with 5% CO_2.

Cell transfection. The miR-583 mimic (50 nM, 5'-CAUUAC CCUGGAAGGAGAAAC-3') and miR-negative control (NC, 50 nM, 5'-UCGCUUGGUGCAGGUCGGG-3') mimic were purchased from Guangzhou RiboBio Co., Ltd. pcDNA3 NC (empty vector, 2 μg) and pcDNA3-JAK1 (2 μg) overexpression vectors were purchased from Addgene, Inc. DU145 and PC3 cell lines ($3x10^5$ cells/well) were transiently transfected with each transfectant using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After incubation at 37˚C for 48 h, cells were harvested for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from PCa cell lines and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration of extracted RNA in each sample was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript™ RT Master mix (Takara Biotechnology Co., Ltd.) at 37˚C for 15 min and 85˚C for 5 sec. qPCR was subsequently performed using a SYBR Green qPCR Master mix (Takara Biotechnology Co., Ltd.) on a CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) with the following thermocycling conditions: Denaturation at 94˚C for 2 min, followed by 35 cycles of 94˚C for 5 sec and 60˚C for 30 sec. The expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (19). GAPDH and U6 were used as the reference genes for JAK1 and miR-583, respectively. The primers used in the present study were as follows: JAK1 forward, 5'-GTC TTAGACCCCAGCCACAG-3' and reverse, 5'-CCCCTTCCA CAAACTCTTCC-3'; GAPDH forward, 5'-ATGACCCCT TCATTGACCTCA-3' and reverse, 5'-GAGATGATGACC CTTTTGGCT-3'; U6 forward, 5'-TGCGGGTGCTCGCTT CGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGG T-3'; and miR-583 forward, 5'-CATTACCCTGGAAGGAGA AAC-3' and reverse, 5'-GTTTCTCCTTCCAGGGTAATG-3'.

Western blotting. Total protein was extracted from PCa cell lines using RIPA lysis buffer [Roche Diagnostics (Shanghai) Co., Ltd.]. Protein concentration in each sample was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology) and protein (20 μg per lane) was separated via SDS-PAGE on 10% gel. The separated proteins were subsequently transferred onto a PVDF membrane and blocked using 5% non-fat milk at room temperature for 1 h. The membranes were then incubated with the following primary antibodies at 4˚C for 12 h: Anti-JAK1 (cat. no. 3344; 1:1,000), anti-STAT3 (cat. no. 12640; 1:1,000), anti-phosphorylated (p)-STAT3 (cat. no. 9145; 1:1,000) and anti-GAPDH (cat. no. 2118; 1:1,000) (all Cell Signaling Technology, Inc.). Following the primary antibody incubation and washing with TBS with 0.1% Tween-20 three times for 5 min each, the membranes were incubated with a HRP-conjugated anti-rabbit IgG secondary antibody (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) at room temperature for 2 h. GAPDH was used as the internal reference protein. The immunoreactive bands were visualized using ECL reagents (Beyotime Institute of Biotechnology). The densitometric analysis was performed using ImageJ software (version 1.46; National Institutes of Health).

MTT assay. DU145 and PC3 cells ($3x10^3$ cells/well) were seeded into 96-well plates and incubated at 37˚C for 24, 48 or 72 h. Subsequently, 20 μl MTT solution (5 mg/ml) was added/well prior to incubation at 37˚C for 4 h. Following the incubation, the culture medium and MTT solution were replaced with 150 μl DMSO (Sigma-Aldrich; Merck KGaA) to dissolve the purple formazan. Finally, the absorbance at 570 nm was measured using an ELISA microplate reader (Thermo Fisher Scientific, Inc.).

Transwell assay. For the invasion assay, DU145 and PC3 cells ($1x10^4$ cells/well) were plated in serum-free RPMI-1640 medium into the upper chambers of Transwell plates (Corning, Inc.) precoated with Matrigel for 4 h at 37˚C (Corning, Inc.). The lower chamber was filled with 500 μl RPMI-1640 medium supplemented with 20% FBS. Following incubation at 37˚C for 24 h, the invasive DU145 and PC3 cells were fixed with 100% methanol for 15 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. Stained cells were visualized using a light microscope (magnification, x100).

Dual luciferase reporter assay. The binding sites between JAK1 and miR-583 were predicted using the bioinformatics analysis tool, TargetScan 7.1 (http://www.targetscan.org/vert_71/). The wild-type (WT) and mutant (MUT) type of the JAK1 3'-untranslated region (UTR), which was generated using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) were cloned into the pGL3

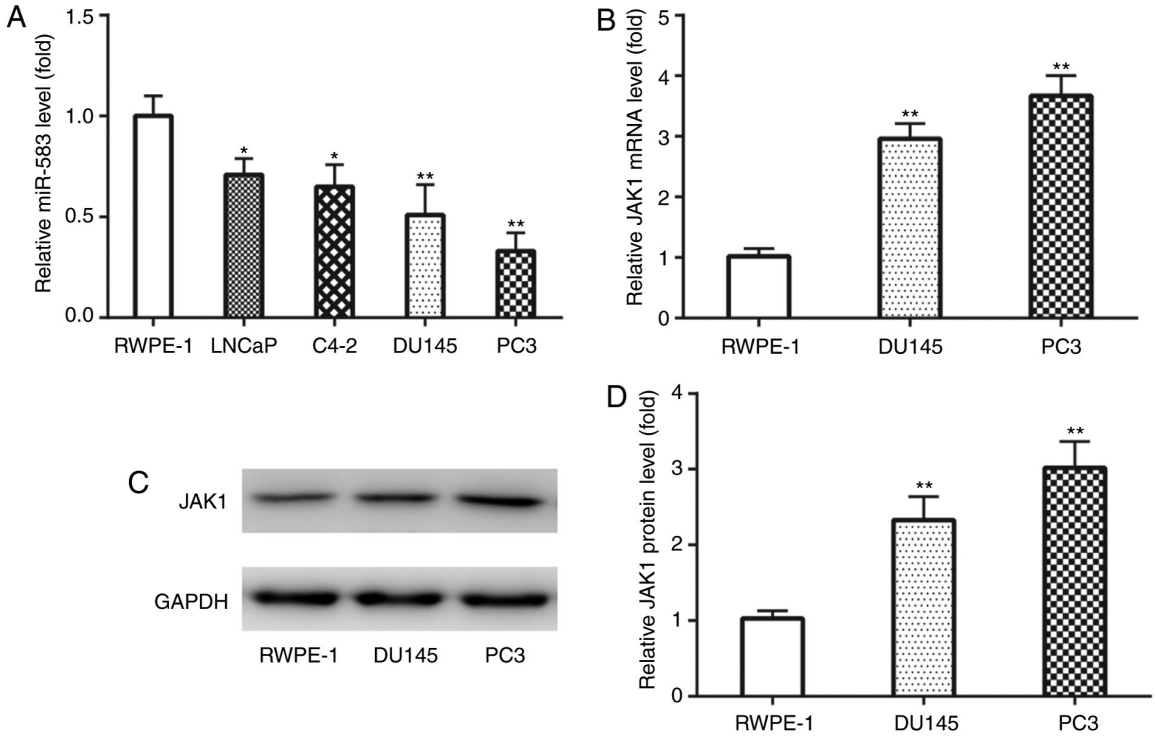

Figure 1. miR-583 expression levels are downregulated, while JAK1 expression levels are upregulated, in prostate cancer cell lines. (A) Compared with the RWPE-1 cell line, miR-583 expression levels were downregulated in LNCaP, C4-2, DU145 and PC3 cell lines. Compared with the RWPE-1 cell line, JAK1 (B) mRNA and (C) protein expression levels were upregulated in DU145 and PC3 cell lines. (D) Semi-quantification of the expression levels presented in part (C). *P<0.05, **P<0.01 vs. RWPE-1. miR, microRNA.

vector (Promega Corporation). The WT (0.4 mg) and MUT (0.4 mg) vectors were co-transfected with the miR-583 mimic (20 nM) or miR-NC mimic (20 nM) into DU145 and PC3 cells ($1x10^4$ cells/well) using Lipofectamine 2000. Following incubation at 37˚C for 48 h, the relative luciferase activity in each group was measured using a Dual Luciferase Reporter assay system (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* (Promega Corporation) luciferase activity.

Statistical analysis. Statistical analysis was performed using GraphPad 6.0 software (GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± SD. An unpaired Student's t-test was used for the comparisons between two groups in the PCa cell lines, whereas a paired Student's t-test was applied for the comparisons between the PCa tissues and the adjacent normal tissues. Statistical differences between ≥3 groups were determined using a one-way ANOVA followed by a Student-Newman-Keuls test or Tukey's post hoc test for multiple comparisons. Pearson's correlation analysis was performed to determine the correlation between miR-583 and JAK1 mRNA expression levels in PCa tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-583 expression levels are downregulated, while JAK1 expression levels are upregulated, in PCa cell lines. The expression levels of miR-583 and JAK1 were first analyzed in PCa cell lines. RT-qPCR analysis revealed that the expression levels of miR-583 were significantly downregulated in LNCaP, C4-2, DU145 and PC3 cell lines compared with the RWPE-1 cell line (Fig. 1A). Since DU145 and PC3 cell lines exhibited markedly lower miR-583 expression levels compared with LNCaP and C4-2 cells, DU145 and PC3 cell lines were used for subsequent experiments.

Conversely, RT-qPCR analysis demonstrated that JAK1 mRNA expression levels were significantly upregulated in DU145 and PC3 cell lines compared with the RWPE-1 cell line (Fig. 1B). Similarly, western blotting also revealed that JAK1 protein expression levels were also significantly upregulated in DU145 and PC3 cell lines compared with the RWPE-1 cell line (Fig. 1C and D). These results indicated the potential underlying involvement of miR-583 and JAK1 in the development of PCa.

miR-583 inhibits the proliferation and invasion of PCa cell lines, which is partially abolished by JAK1. In DU145 and PC3 cell lines, RT-qPCR analysis discovered that miR-583 expression levels were significantly upregulated in the miR-583 mimic group compared with the miR-NC mimic group (Fig. 2A). In addition, RT-qPCR was also used to demonstrate that JAK1 mRNA expression levels were significantly upregulated in the pcDNA3-JAK1 group compared with the pcDNA3 group (Fig. 2B). Similarly, western blotting revealed that JAK1 protein expression levels were significantly upregulated in the pcDNA3-JAK1 group compared with the pcDNA3 group in both cell lines (Fig. 2C and D). These findings indicated the successful transfection of miR-583 mimic and pcDNA-JAK1 into DU145 and PC3 cell lines.

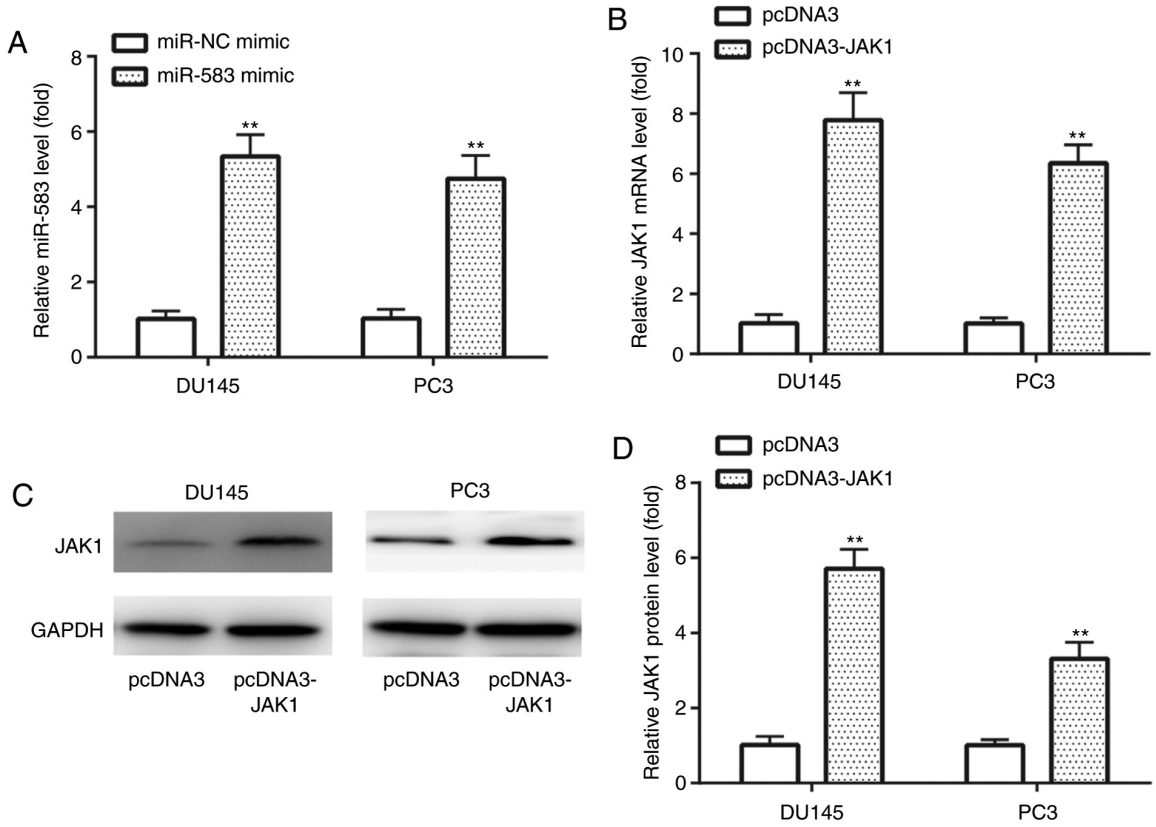

Figure 2. Transfection efficiency of miR-583 mimic and pcDNA3-JAK1 in prostate cancer cell lines. (A) In DU145 and PC3 cell lines, the expression levels of miR-583 were upregulated following the transfection with the miR-583 mimic compared with the miR-NC mimic. JAK1 (B) mRNA and (C) protein expression levels were upregulated following the transfection with pcDNA3-JAK1 compared with pcDNA3. (D) Semi-quantification of the expression levels from part (C). **P<0.01 vs. miR-NC mimic or pcDNA3. miR, microRNA; NC, negative control.

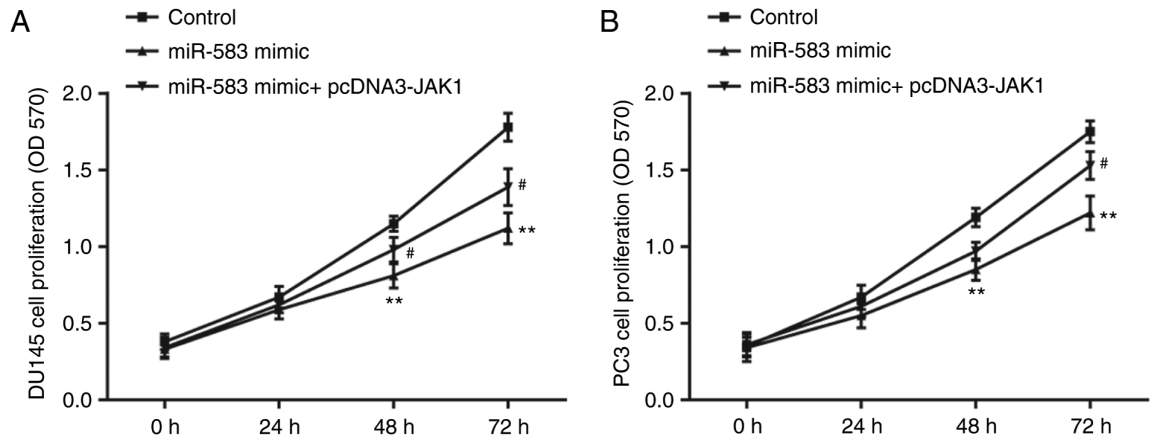

Figure 3. miR-583 mimic inhibits the proliferation of prostate cancer cell lines by targeting JAK1. In (A) DU145 and (B) PC3 cell lines, the proliferative ability was decreased following the transfection with the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), which was then partially reversed by pcDNA3-JAK1. **P<0.01 vs. control; $^{\#}$P<0.05 vs. miR-583 mimic. miR, microRNA; NC, negative control.

In the DU145 cell line, the results of the MTT assay demonstrated that the proliferation was significantly decreased by the miR-583 mimic compared with the control (miR-NC mimic + pcDNA3), which was then subsequently partially reversed by the co-transfection with pcDNA3-JAK1 (Fig. 3A). The results of the MTT assay in the PC3 cell line showed a similar trend in the proliferative ability of each group (Fig. 3B).

Transwell assays demonstrated that DU145 cell invasion was significantly inhibited following the transfection with the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), which was subsequently partially reversed by the co-transfection with pcDNA3-JAK1 (Fig. 4A and B). The PC3 cell invasive ability exhibited similar trends to the DU145 cell line following each transfection (Fig. 4C and D). These findings indicated the potential involvement of miR-583 and JAK1 in the proliferation and invasion of PCa; however, the responsible molecules and the association between miR-583 and JAK1 remains undetermined.

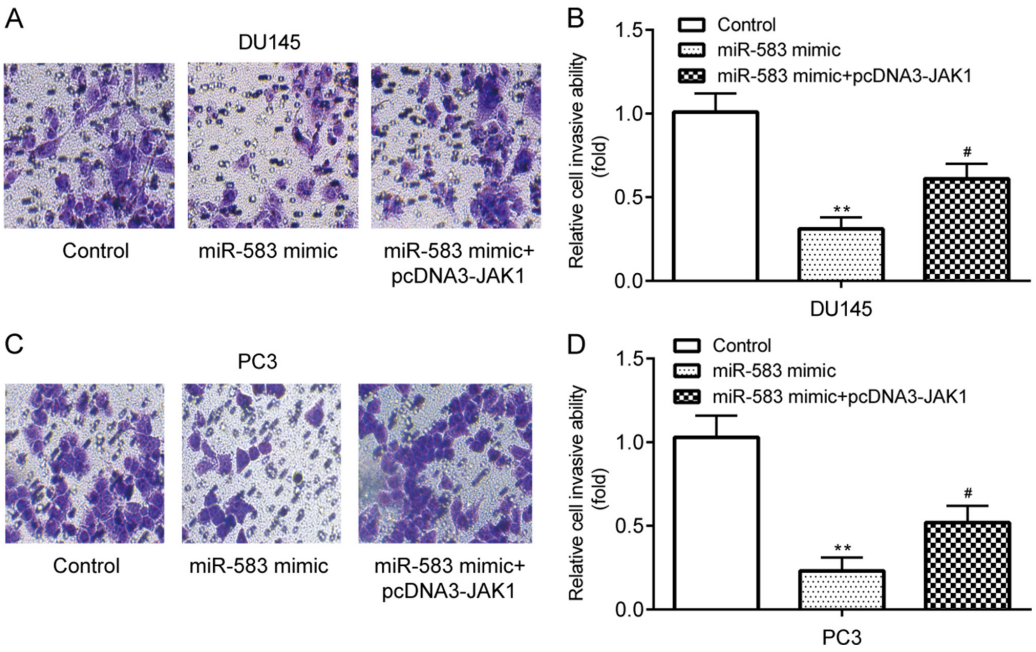

Figure 4. miR-583 mimics inhibit the invasion of PCa cell lines by targeting JAK1. (A) In the DU145 cell line, the invasive ability was decreased following the transfection with the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), and then partially rescued by pcDNA3-JAK1 (magnification, x100). (B) Semi-quantification of the invasive ability from part (A). (C) In the PC3 cell line, the invasive ability was decreased following the transfection with the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), and then partially rescued by pcDNA3-JAK1 (magnification, x100). (D) Semi-quantification of the invasive ability from part (C). **P<0.01 vs. control; $^{\#}$P<0.05 vs. miR-583 mimic. miR, microRNA; NC, negative control.

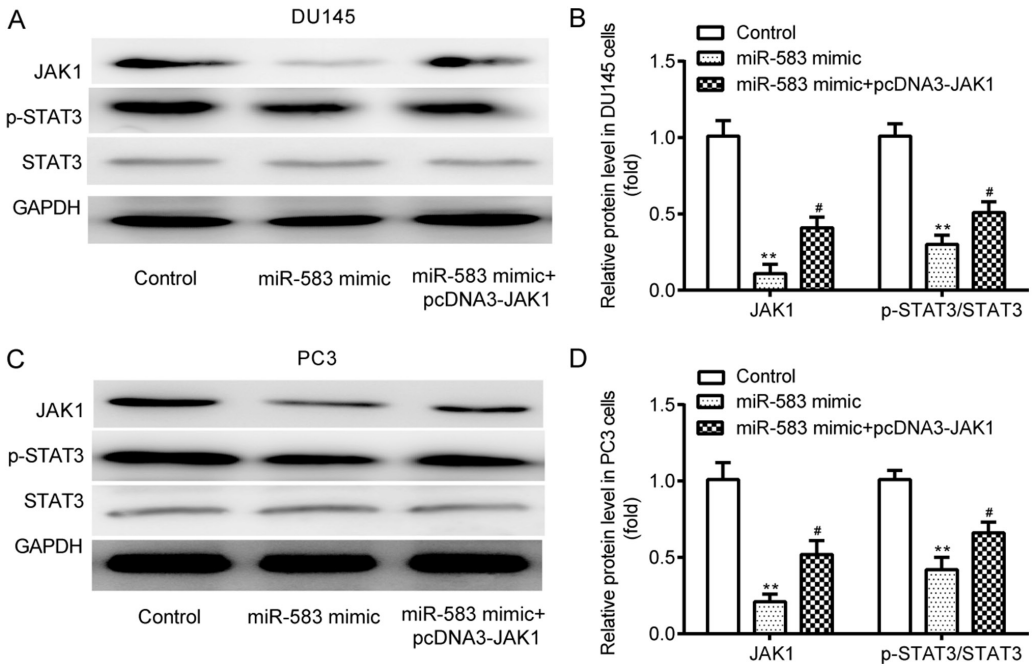

Figure 5. miR-583 mimic downregulates JAK1 and p-STAT3 expression levels by targeting JAK1. (A) In the DU145 cell line, JAK1 and p-STAT3 expression levels were downregulated by the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), which was partially reversed by pcDNA3-JAK1. No significant differences were found in STAT3 expression levels between the 3 groups. (B) Semi-quantification of the expression levels from part (A). (C) In the PC3 cell line, JAK1 and p-STAT3 expression levels were downregulated by the miR-583 mimic compared with the control group (miR-NC mimic + pcDNA3), which was partially reversed by pcDNA3-JAK1. No significant differences were found in STAT3 expression levels between the 3 groups. (D) Semi-quantification of the expression levels from part (C). **P<0.01 vs. control; $^{\#}$P<0.05 vs. miR-583 mimic. miR, microRNA; p-, phosphorylated.

miR-583 downregulates JAK1 and p-STAT3 expression levels, which is partially reversed by JAK1 overexpression. In the DU145 cell line, western blotting revealed that JAK1 and p-STAT3 expression levels were significantly downregulated by the miR-583 mimic compared with the control (miR-NC mimic + pcDNA3); however, these expression levels were subsequently significantly upregulated by the co-transfection with pcDNA3-JAK1 (Fig. 5A and B). No significant differences

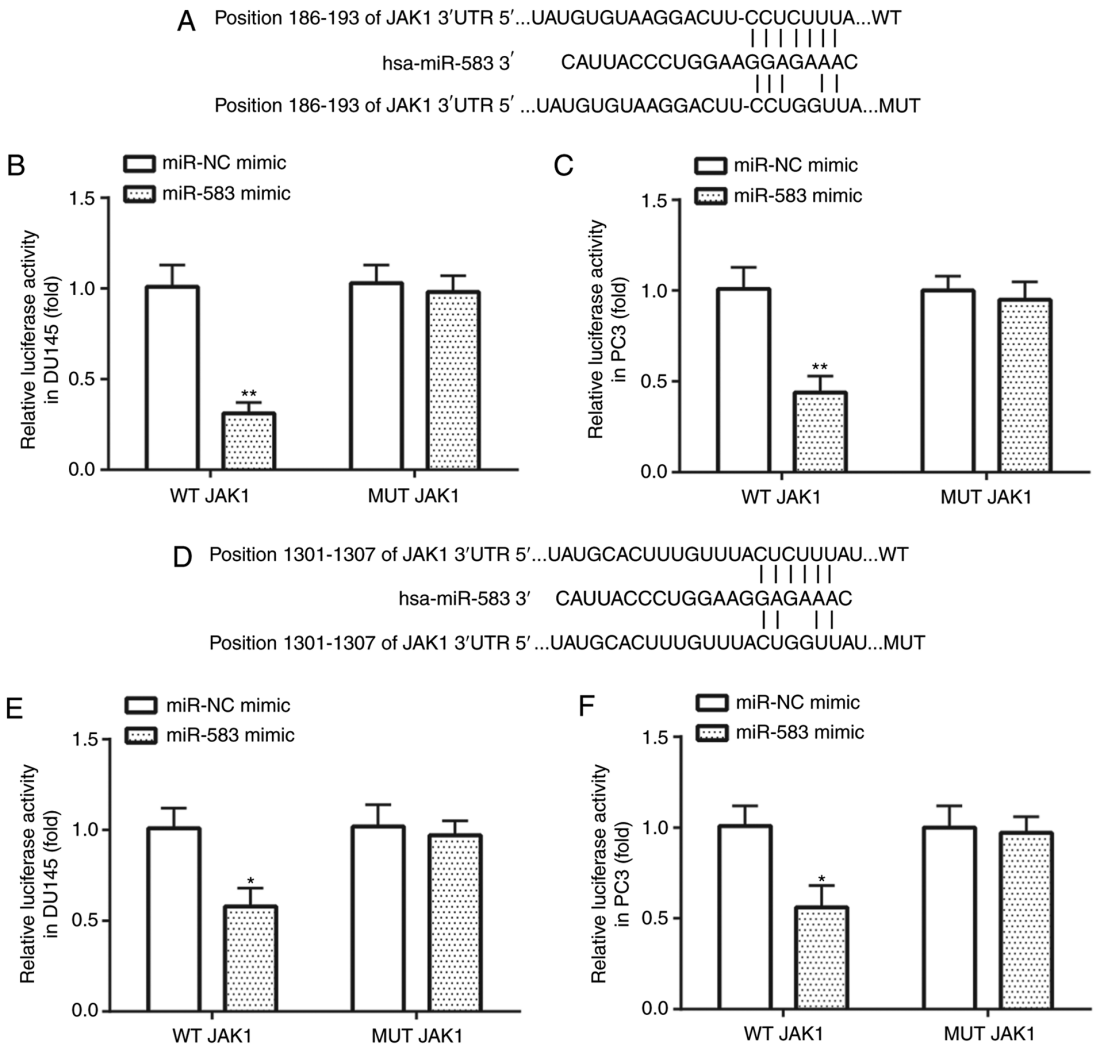

Figure 6. JAK1 is targeted by miR-583 in prostate cancer cell lines. (A) The binding between position 186-193 of the WT JAK1 3'-UTR and miR-583. In (B) DU145 and (C) PC3 cell lines, the transfection with the miR-583 mimic repressed the relative luciferase activity in cells transfected with WT, but not MUT JAK1. (D) The binding between position 1,301-1,307 of the WT JAK1 3'-UTR and miR-583. In (E) DU145 and (F) PC3 cells, the transfection with the miR-583 mimic repressed the relative luciferase activity in cells transfected with WT, but not MUT JAK1. *P<0.05, **P<0.01 vs. miR-NC mimic + WT JAK1. miR, microRNA; NC, negative control; UTR, untranslated region; WT, wild-type; MUT, mutant.

were observed in the expression levels of STAT3 between the 3 groups. Similar trends were obtained in the expression levels of JAK1, p-STAT3 and STAT3 in the PC3 cell line between the three groups (Fig. 5C and D).

JAK1 is a target of miR-583. The complementary binding sequences between position 186-193 of the JAK1 3'-UTR and miR-583 are presented in Fig. 6A. In DU145 (Fig. 6B) and PC3 (Fig. 6C) cells, the binding between position 186-193 of the WT JAK1 3'-UTR and miR-583 was confirmed using dual luciferase reporter assays, exhibiting that the relative luciferase activity in the WT JAK1 vectors co-transfected with miR-583 mimic was significantly lower than that of miR-NC mimic, whereas no significant difference was observed between the MUT JAK1 vectors co-transfected with miR-583 mimic and miR-NC mimic. The complementary binding sequences between position 1,301-1,307 of the WT JAK1 3'-UTR and miR-583 are shown in Fig. 6D. In DU145 (Fig. 6E) and PC3 (Fig. 6F) cells, the binding between position 1,301-1,307 of the WT JAK1 3'-UTR and miR-583 was confirmed using dual luciferase reporter assays, exerting that the relative luciferase activity in the WT JAK1 vectors co-transfected with miR-583 mimic was decreased compared with miR-NC mimic, whereas no significant difference was observed between the MUT JAK1 vectors co-transfected with miR-583 mimic and miR-NC mimic.

miR-583 expression levels are negatively correlated with JAK1 expression levels in PCa tissues. RT-qPCR analysis revealed that miR-583 expression levels were significantly downregulated (Fig. 7A), while JAK1 expression levels were significantly upregulated (Fig. 7B), in PCa tissues compared with the adjacent normal tissues. In addition, in the PCa tissues, a negative correlation was identified between miR-583 and JAK1 expression levels (r=-0.595; P=0.004), with a 95% confidential interval of -0.817 to -0.220 (Fig. 7C).

Discussion

PCa, which is characterized with high morbidity and mortality rates, is the most common type of malignancy to be diagnosed

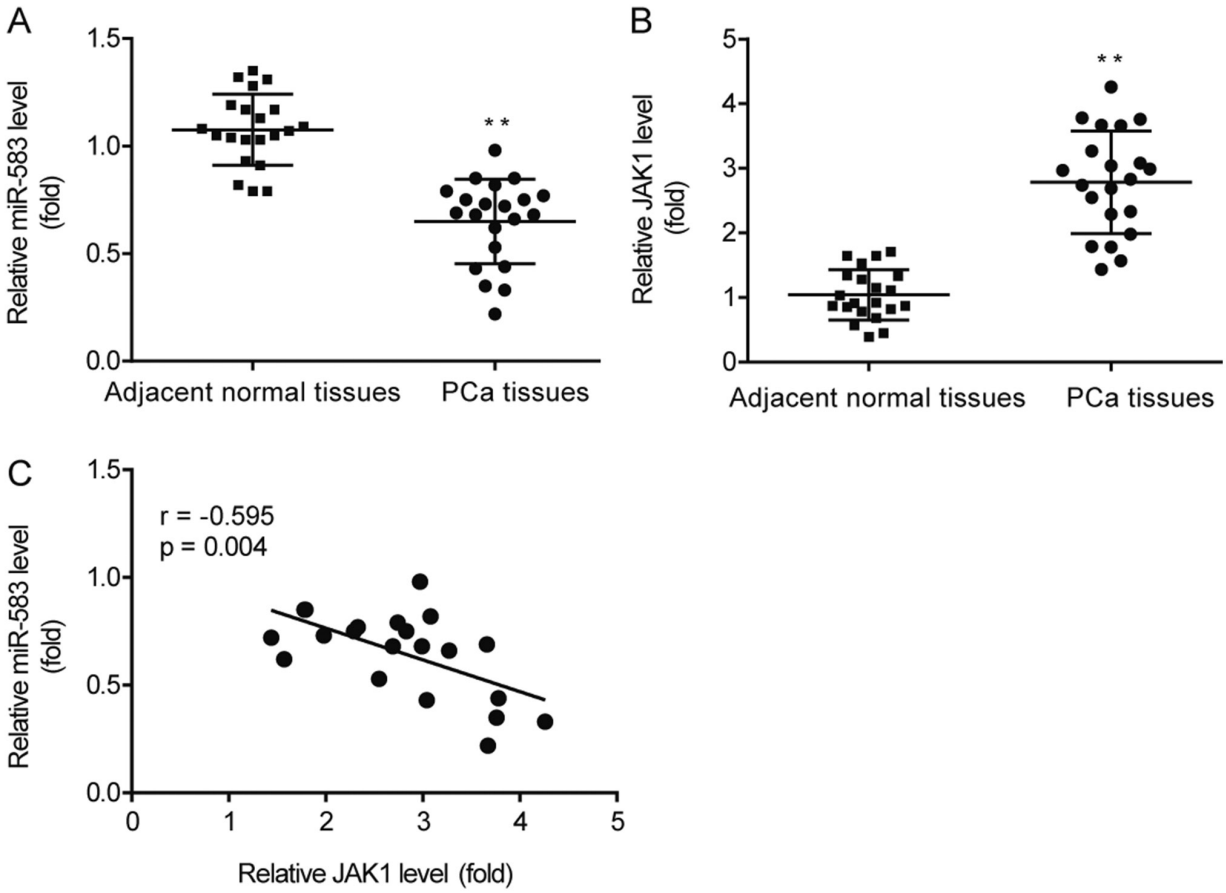

Figure 7. miR-583 expression levels are negatively correlated with JAK1 expression levels in PCa tissues. (A) miR-583 expression levels were downregulated in PCa tissues compared with the adjacent normal tissues. (B) JAK1 expression levels were upregulated in PCa tissues compared with the adjacent normal tissues. (C) A negative correlation was identified between miR-583 and JAK1 expression levels in PCa tissues. **P<0.01 vs. adjacent normal tissues. PCa, prostate cancer; miR, microRNA.

in men (2). Despite the advancements in medical technology, the treatment options available for PCa remain unsatisfactory and tumor recurrence is common in patients with PCa (6), therefore, the overall survival rate is ~3-4 months (7). Therefore, it is of great significance to investigate novel therapeutic targets for PCa.

To date, numerous non-coding RNAs have been demonstrated to have a crucial role in various types of cancer, including PCa (20). For example, miR-215-5p repressed the metastasis of PCa through targeting phosphoglycerate kinase 1 (21); miR-1272 reduced the migration and invasion of PCa by targeting huntingtin interaction protein 1 (22); and the inhibition of miR-4286 inhibited the proliferation and promoted the apoptosis of PCa by targeting spalt like transcription factor 1 (23). As for the role of miR-583 in PCa, to the best of our knowledge, only one meta-analysis report exists reporting its downregulation in recurrent PCa samples compared with non-recurrent cases (18). The findings of the present study revealed that miR-583 expression levels were downregulated in PCa tissues and cell lines, further indicating its potential tumor suppressive role in the development of PCa. However, to the best of our knowledge, until now, the function of miR-583 and the mRNA targets for miR-583 in PCa have not been reported.

Metastasis is the primary cause for PCa-related death (24) and miRNAs have been demonstrated to regulate multiple steps of the metastatic process in PCa by targeting their specific mRNAs (25). Consequently, the roles of oncogenic mRNAs, which have also been associated with the metastasis of PCa, should be further investigated. For instance, miR-448 was discovered to suppress metastasis in pancreatic ductal adenocarcinoma by targeting the JAK1/STAT3 signaling pathway (26); miR-214 inhibited proliferation and invasion in lung cancer by targeting JAK1 (27); and miR-769-5p reduced the migration and invasion of oral squamous cell carcinoma by targeting the JAK1/STAT3 signaling pathway (28). Moreover, the knockdown of JAK1 suppressed the IL-6-induced induction of PCa metastasis (29). However, to the best of our knowledge, no previous studies have determined whether miR-583 functions through regulating JAK1 in PCa. Consistent with the aforementioned reports concerning the potential oncogenetic role of JAK1 during the progression of pancreatic ductal adenocarcinoma, lung cancer, oral squamous cell carcinoma, as well as PCa, the results of the current study also revealed that JAK1 expression levels were upregulated in PCa tissue and cells, which were found to be repressed and targeted by miR-583.

It is commonly known that the JAK/STAT3 signaling pathway plays crucial roles in the progression of PCa. miR-17 has been demonstrated to inhibit PCa cell proliferation and induce PCa cell apoptosis by inactivating the JAK/STAT3 signaling pathway (30). Furthermore. capsazepine has been

reported to inhibit tumor growth and cell survival of PCa by inactivation of JAK/STAT3 signaling (31), and miR-124 can reduce the invasion and proliferation of PCa cells by inhibiting the activation of JAK/STAT3 signaling pathway (32). However, to the best of our knowledge, the relationship between miR-583 and JAK1 in PCa metastases remained unknown until the present study.

The findings of the present study indicated that miR-583 mimic may inhibit PCa proliferation and invasion by targeting JAK1 and inactivating the p-STAT3 signaling pathway, thus providing a potential novel therapeutic target for patients with PCa.

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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

ZL and JC performed the experiments and analyzed the data. JC conceived and designed the study, and supervised and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of tissues from patients with PCa for scientific research was approved by the ethics committee of The First Affiliated Hospital of Kunming Medical University. Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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