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Original Article

miR-448 inhibits proliferation and induces apoptosis in prostate cancer cells by modulating cyclin-dependent kinase 19 expression

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Abstract: Background: MicroRNAs (miRNAs), as a class of non-coding RNAs, are involved in prostate cancer (PCa) progression by performing a post-transcriptional regulatory mechanism. However, the precise mechanisms of miRNAs have not been completely clarified in the carcinogenesis of PCa. The present study aimed to investigate the potential functions of miR-448 and its downstream target cyclin-dependent kinase 19 (CDK19) in proliferation and apoptosis of PCa cell lines. Methods: Fifty-two pairs of tumor tissues and corresponding non-tumorous tissues were obtained from PCa patients undergone surgical operation. Human LNCaP and 22RV1 cells treated with pre-miR-Con or pre-miR-448, and sh-Con or sh-CDK19. The CCK-8 and flow cytometry were performed to measure the cell viability and apoptosis in PCa cell lines. The expression levels of gene and protein were assayed by qRT-PCR and western blotting, respectively. The targeted genes of miR-448 were predicted by a bioinformatics algorithm and confirmed by a luciferase reporter assay. Results: Expression levels of miR-448 were found to bereduced, and CDK19 levels were elevated in PCa tissues. miR-448 low expression or CDK19 high expression was associated with poor survival prognosis in PCa patients. Expression levels of miR-448 were inversely correlated with CDK19 in PCa tissues. Moreover, overexpression of miR-448 or inhibition of CDK19 led to retardation of cell growth and acceleration of apoptosis in PCa cell. Furthermore, CDK19 was validated as a direct target of miR-448. Conclusion: miR-448 might facilitate PCa cell death, through a molecular mechanism, at least partially, due to suppression of CDK19 expression.

Keywords: Prostate cancer, miR-448, CK19, apoptosis, post-transcriptional regulation

Introduction

Prostate cancer (PCa) is a frequently occurring urinary system malignant tumor with a high incidence and recurrence rate in men [1]. According to cancer statistics in China, 2015, 60,300 cases will be newly diagnosed and 26,600 deaths occur annually, suggesting that the mortality rate of PCa patients is approximately 44% [2]. In the initiating stage of PCa, androgen deprivation therapy (ADT) and surgical castration are effective therapeutic methods to prevent progression of PCa [3]. Unfortunately, hormone-refractory PCa (HRPC) or castration-resistant PCa (CRPC) emerges after 18-24 months treatment and results in PCa recurrence, poor prognosis and low survival rate [4]. Thus, it is imperative to investigate the pathogenesis of PCa.

MicroRNAs (miRNAs) are a type of non-coding RNAs with a length of approximately 18-25 nucleotides and involve in messenger RNA (mRNA) translation by targeting to its 3'-untranslated regions (3'-UTRs) [5]. Previous studies have proven that miRNAs are implicated in the progression of cancer as tumor promoters or suppressors and may function as novel predictors of cancers diagnosis [6-8]. Comprehensive miRNA profiling analysis reveals that numerous miRNAs are dysregulated in PCa, including miR-26b, miR-130a, miR-141, and miR-194 [9-12]. Previous studies reveal that miR-448 functions as a tumor suppressor in various cancers, such as breast cancer, acute lymphoblastic leukemia, ovarian cancer and non-small cell lung cancer [13-17]. However, there is no relevant evaluation report on the effects of miR-448 in the carcinogenesis of PCa.

Cyclin-dependent kinase 19 (CDK19) is a kind of kinase that encodes a protein and reversibly interacts with the Mediator complex as part of a four-subunit called the kinase module [18]. CDK19 is reported to be expressed in a diverse range of tissues including fetal eye and fetal brain [19]. Recently, CDK19 is also widely expressed in tumor tissues and drives epithelial-to-mesenchymal transition (EMT) and tumor cell invasion [20]. A recent study uncovers that CDK19 shows a significant high expression in primary PCa and is associated with increased aggressiveness and shorter disease-free survival, reflecting that CDK19 may be a novel therapeutic target in advanced PCa [21]. Both genetic and pharmacological inhibition of CDK19 exerts anti-proliferative and pro-apoptotic activity in PCa cells [21, 22]. In this study, miR-448 expression was decreased in PCa tumor tissues and cell lines and as a post-transcriptional regulator targeted to inhibit CDK19 expression. Overexpression of miR-448 suppressed proliferation and induced apoptosis in PCa cells by modulating CDK19 expression. Overall, these findings indicated that miR-448/CDK19 signaling implied in the progression of PCa, which might provide a novel therapeutic strategy for the treatment of PCa.

Material and methods

Tissue sample collection

Fifty-two pairs of tumor tissues and corresponding non-tumorous tissues were obtained from PCa patients with surgically resected in the Department of Urology of Tiantai People's Hospital (Tiantai, China), Department of Urology, Ninghai Hospital (Ninghai, China) and Zhejiang Province People's Hospital (Hangzhou, China) between Jan 2009 and Jan 2013. All of the patients were recruited according to the histopathological evaluation without radiotherapy or chemotherapy before surgical operation. All of the tissues were immediately stored at liquid nitrogen after surgical operation. Informed consent forms were obtained from the patients. This study was permitted by the Ethics Committee of the Tiantai People's Hospital (Tiantai, China), Ninghai Hospital (Ninghai, China) and Zhejiang Province People's Hospital (Hangzhou, China).

Cell culture

Human normal prostate epithelial RWPE-1 cell and four PCa cell lines (LNCaP, 22RV1, PC3 and DU145) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (Thermo Scientific HyClone, Beijing, China), 5% CO_2, 95% air atmosphere in a humidified incubator (Thermo Fisher Scientifc, Inc., Waltham, MA, USA).

Cell transfection and plasmid constructs

Pre-miR-Con and pre-miR-448 were synthesized by RiboBio (Guangzhou, China) and transfected with into LNCaP and 22RV1 cells with a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientifc, Inc., Waltham, MA, USA) for 48 h at 37°C according to the manufacturer's protocol.

Short hairpin RNA (shRNA) was designed to specifically target CDK19 using shRNA design tools (http://rnaidesigner.thermofisher.com/rnaiexpress/). Using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the designed shRNA was targeted only the CDK19. Sh-CDK19 and sh-Con were synthesized by GenePharma (Shanghai, China).

Cell viability detection by CCK-8

Human LNCaP and 22RV1 cells (1×10^4) were seeded in the 96-well plate for 24 hours, 48 hours and 72 hours transfected with pre-miR-Con or pre-miR-448. Cells viability was measured using CCK-8 Cell Proliferation/Viability Assay Kit (Dojindo Japan). Absorbance was recorded at 450 nm using Elx800 Reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Flow cytometry for apoptosis

Human LNCaP and 22RV1 cells (1×10^4) treated with pre-miR-Con or pre-miR-448, and sh-Con or sh-CDK19, apoptosis was monitored using Annexin V-FITC/PI apoptosis detection kit (Carlsbad, Calif, USA) according to the manufacturer's protocol. Apoptotic cell proportion was analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA) and calculated by CELL Quest 3.0 software (BD Biosciences).

Luciferase reporter gene assay

The wild-type (WT) or mutant-type (MT) 3'-UTR of CDK19 were inserted into the multiple cloning sites of the luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Sci-

entific, Inc.). The luciferase activity was measured using the Luciferase Reporter® Assay System (Promega, USA) on a Luminoskan™ Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (QRT-PCR)

Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Total RNA (2 μg) was used to synthesize cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). miR-448 was detected using TaqMan® MicroRNA assay (Applied Biosystems, Foster City, USA) followed by manufacturer's instructions. U6 snRNA was used as an endogenous control. qRT-PCR was performed by Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.) with the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) for detection of CDK19 mRNA expression. Relative expression levels of CDK19 mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method [23] and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were shown as follows: CDK19 forward 5'-GAGCATGACTTGTGGCATATT-3' and reverse 5'-TGGATACCATCAAGAATCTGGT-3'; GAPDH forward 5'-ACAGGGGAGGTGATAGCATT-3' and reverse 5'-GACCAAAAGCCTTCATACATCTC-3'.

Western blotting

Protein was extracted using radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China). The procedures of Western blotting were performed as described previously [24]. The primary antibody of CDK19 (cat. no. E-AB-13137; dilution: 1:1000) was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Subsequently, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (cat. no. sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 hours and visualized by chemiluminescence (Thermo Fisher Scientific, Inc.). Signals were analyzed with Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA). β-actin (cat. no. sc-130065; dilution: 1:2,000; Santa Cruz Biotechnology) was used to as the control antibody.

Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics Version 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used to analyze two-group differences. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. Overall survival was calculated using the Kaplan-Meier method with the log-rank test applied for comparison. Spearman's rank analysis was used to identify the correlation between the expression levels of miR-448 and CDK19 in PCa tissues. *P* value less than 0.05 was considered as a significant statistical difference.

Results

miR-448 expression was down-regulated in PCa tissues and cell lines

First, qRT-PCR assay was performed to detect miR-448 expression in fifty-two pairs of PCa tissues and corresponding non-tumorous tissues. The results demonstrate that expression of miR-448 was significantly lower in PCa tissues than in the corresponding non-tumorous tissues (**Figure 1A**). In addition, 46 of these cases (88.5%) exhibited down-regulation of miR-448 expression in PCa tissues (**Figure 1B**). To investigate the association between miR-448 and overall survival prognosis in PCa patients, Kaplan-Meier analysis was performed in 52 PCa patients, which were divided into 2 groups: high expression group (n = 26) and low expression group (n = 26) according to the Log_2 fold change ≥ 1. PCa patients with miR-448 low expression showed a significant poorer survival prognosis than that of in high expression group (**Figure 1C**). Furthermore, miR-448 expression in human normal prostate epithelial cell line, RWPE-1, and four PCa cell lines, LNCaP, 22RV1, PC3 and DU145, were analyzed by qRT-PCR assay which revealed that expression levels of miR-448 are significantly decreased in PCa cell lines, except DU145 cell, compared with RWPE-1 cells (**Figure 1D**).

Overexpressed miR-448 inhibited proliferation and induced apoptosis in PCa cells

To determine the role of miR-448 on cell proliferation and apoptosis in PCa cells, pre-miR-

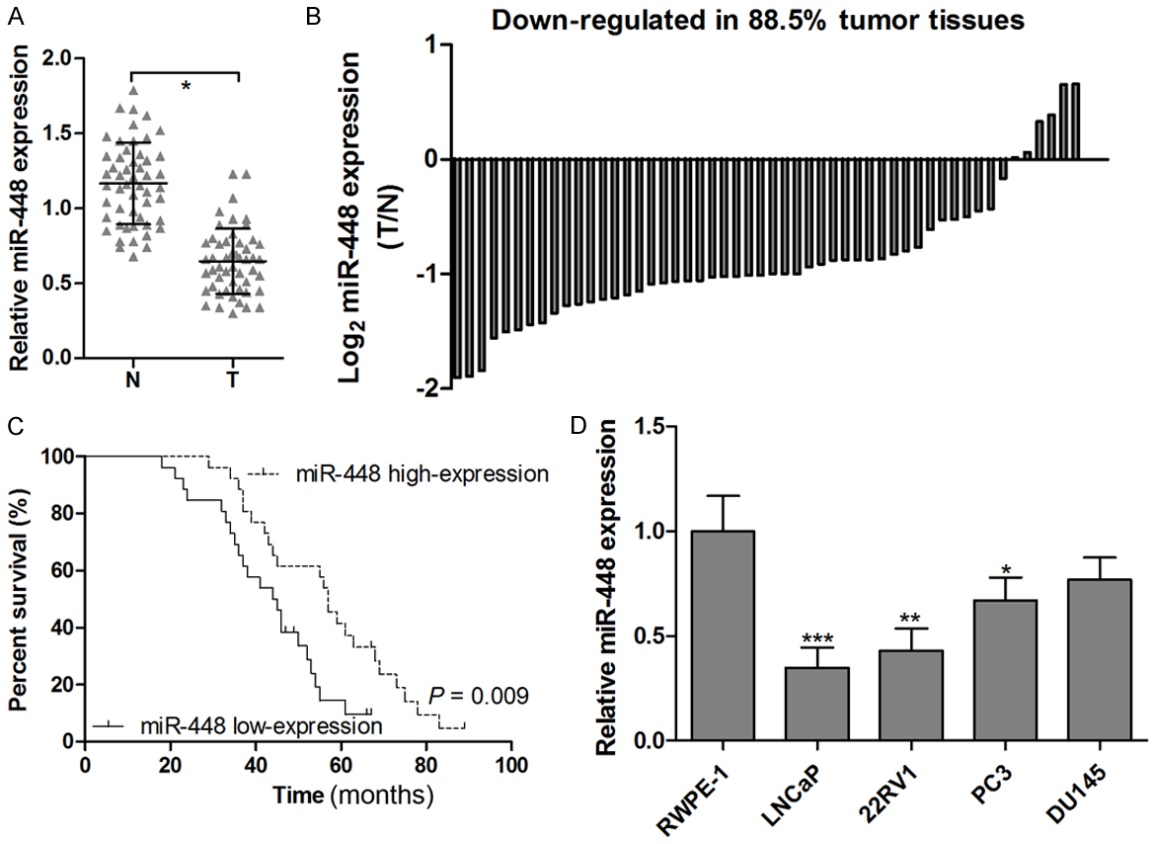

Figure 1. miR-448 expression was down-regulated in PCa tissues and cell lines. The expression levels of miR-448 in 52 pairs of PCa tissues and corresponding non-tumorous tissues were detected by QRT-PCR assays (A and B). Kaplan-Meier survival curve was used to evaluate whether miR-448 expression was associated with overall survival in PCa patients (C). Expression levels of miR-448 in human normal prostate epithelial cell line, RWPE-1, and four PCa cell lines, LNCaP, 22RV1, PC3 and DU145, were detected by QRT-PCR assays (D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group.

Con and pre-miR-448 were transfected into LNCaP and 22RV1 cells. Expression levels of miR-448 were dramatically elevated in LNCaP and 22RV1 cells after transfected with pre-miR-448 as compared to that of in the pre-miR-Con group (**Figure 2A**). The growth rate of LNCaP and 22RV1 cells was markedly reduced after transfected with pre-miR-448 at 48 h and 72 h (**Figure 2B**). Moreover, overexpression of miR-448 increased the apoptotic proportion of LNCaP and 22RV1 cells (**Figure 2C** and **2D**).

CDK19 was a direct target of miR-448 in PCa cells

To investigate miR-448-regulated potential molecular targets, bioinformatics database TargetScan (http://www.targetscan.org/) was used to predict potential binding sites of miR-448 and target genes. miR-448 was found to bind to the 3'-UTR of CDK19 (**Figure 3A**), which had been reported as a potential therapeutic target of PCa [21, 22]. Next, a luciferase report assay was performed to verify whether contained the conserved binding sites between miR-448 and 3'-UTR of CDK19. The experimental measurements showed that the luciferase activity was significantly weakened in LNCaP and 22RV1 cells contained WT 3'-UTR of CDK19 and co-transfected with pre-miR-448 (**Figure 3B**). However, in LNCaP and 22RV1 cells contained MT 3'-UTR of CDK19, the luciferase activity had no significant difference after transfected pre-miR-448 or pre-miR-Con (**Figure 3C**). Interestingly, both mRNA and protein expression of CDK19 were repressed in LNCaP (**Figure 3D**) and 22RV1 (**Figure 3E**) cells after transfected with pre-miR-448.

CDK19 expression was up-regulated in PCa tissues and associated with poor prognosis

mRNA expression of CDK19 was detected in fifty-two pairs of PCa tissues and correspond-

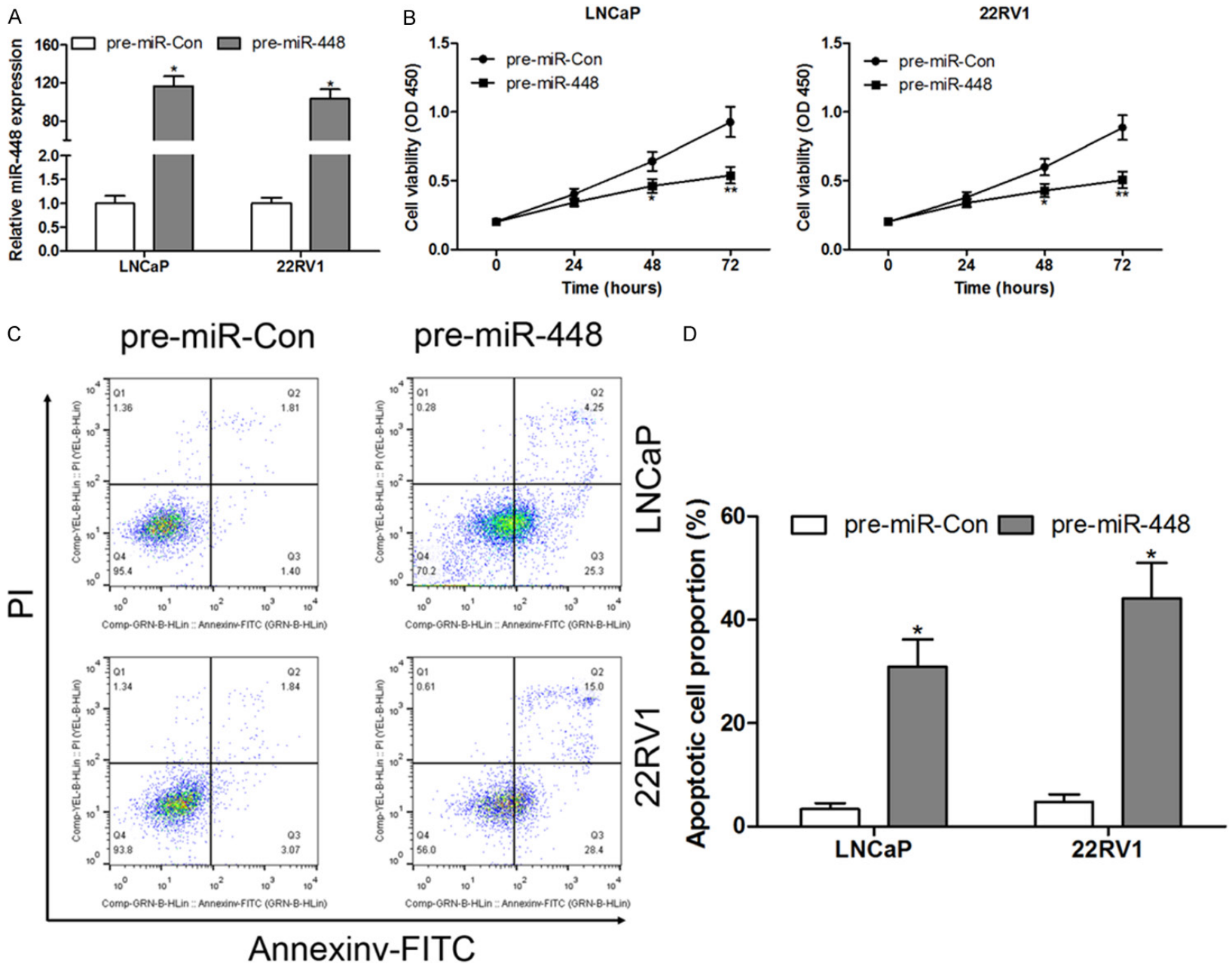
A
pre-miR-Con
pre-miR-448
Relative miR-448 expression
LNCaP
22RV1
B
LNCaP
22RV1
pre-miR-Con
pre-miR-448
Cell viability (OD 450)
Time (hours)
C
pre-miR-Con
pre-miR-448
PI
Annexinv-FITC
LNCaP
22RV1
Q1
Q2
Q3
Q4
1.36
1.81
95.4
1.40
0.28
4.25
70.2
25.3
1.34
1.84
93.8
3.07
0.61
15.0
56.0
28.4
D
Apoptotic cell proportion (%)
pre-miR-Con
pre-miR-448
LNCaP
22RV1

Figure 2. Overexpressed miR-448 inhibited proliferation and induced apoptosis in PCa cells. After transfected with pre-miR-Con and pre-niR-448 into LNCaP and 22RV1 cells for 48 hours, the expression levels of miR-448 were detected by qRT-PCR assay (A); cell viability was measured by CCK8 assay (B); cell apoptosis was performed by flow cytometry (C and D). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the pre-miR-Con group.

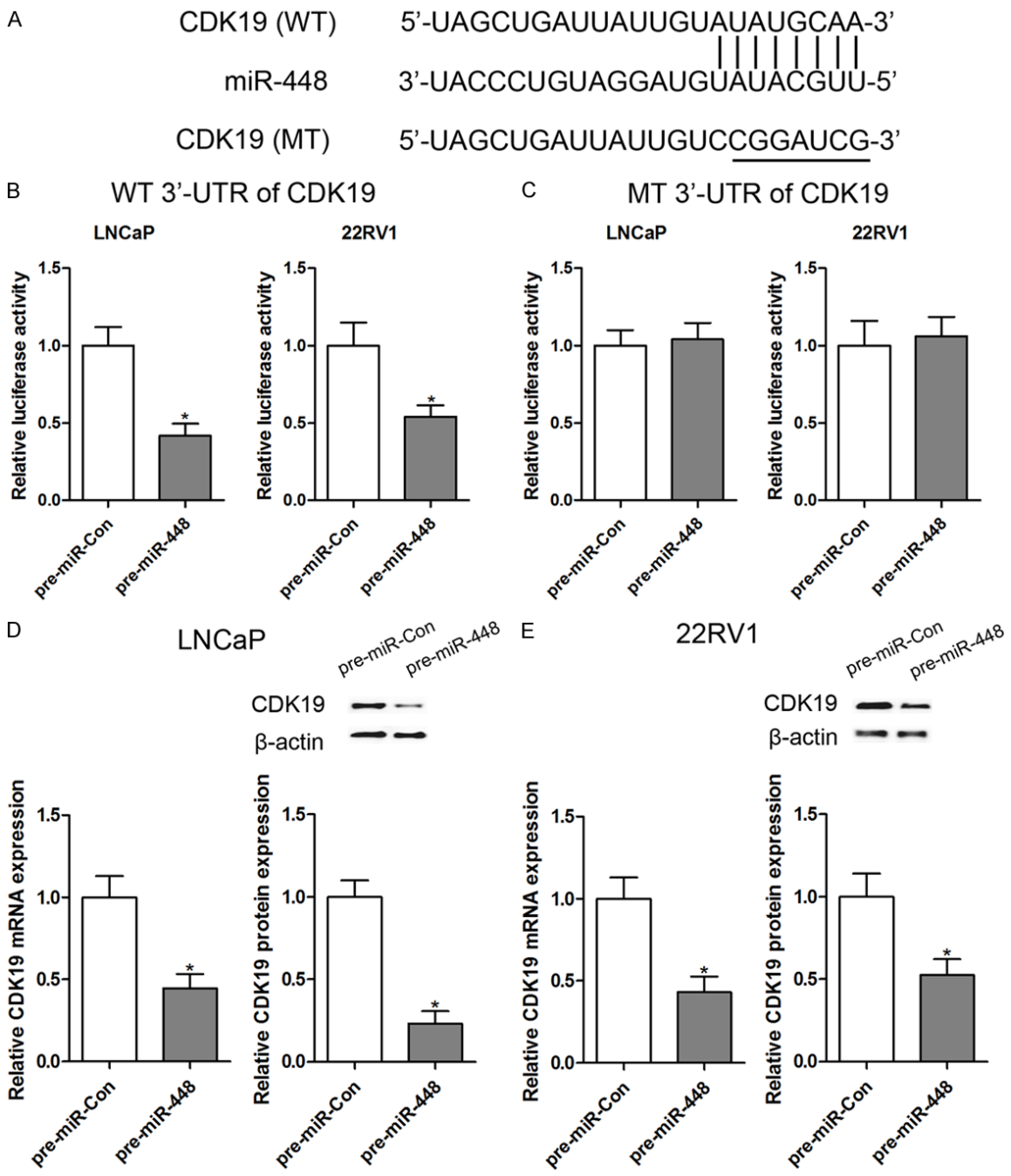

Figure 3. CDK19 was a direct target of miR-448. The putative binding sites between miR-448 and the 3'-UTR of CDK19 were predicted by on-line prediction software (A). WT (B) or MUT (C) 3'-UTR of CDK19 was co-transfected with pre-miR-448 or pre-miR-Con into 5 LNCaP and 22RV1 cells for 48 h, luciferase activity assay was performed. After transfection with pre-miR-Con and pre-niR-448 into LNCaP (D) and 22RV1 (E) cells for 48 hours, the mRNA and protein expression of CDK19 were analyzed by qRT-PCR and Western blotting, respectively. $^{*}P < 0.05$ compared with the pre-miR-Con group.

ing non-tumorous tissues, and qRT-PCR assays showed a significant up-regulation of CDK19 mRNA expression in PCa tissues compared with that in the non-tumorous tissues (**Figure**

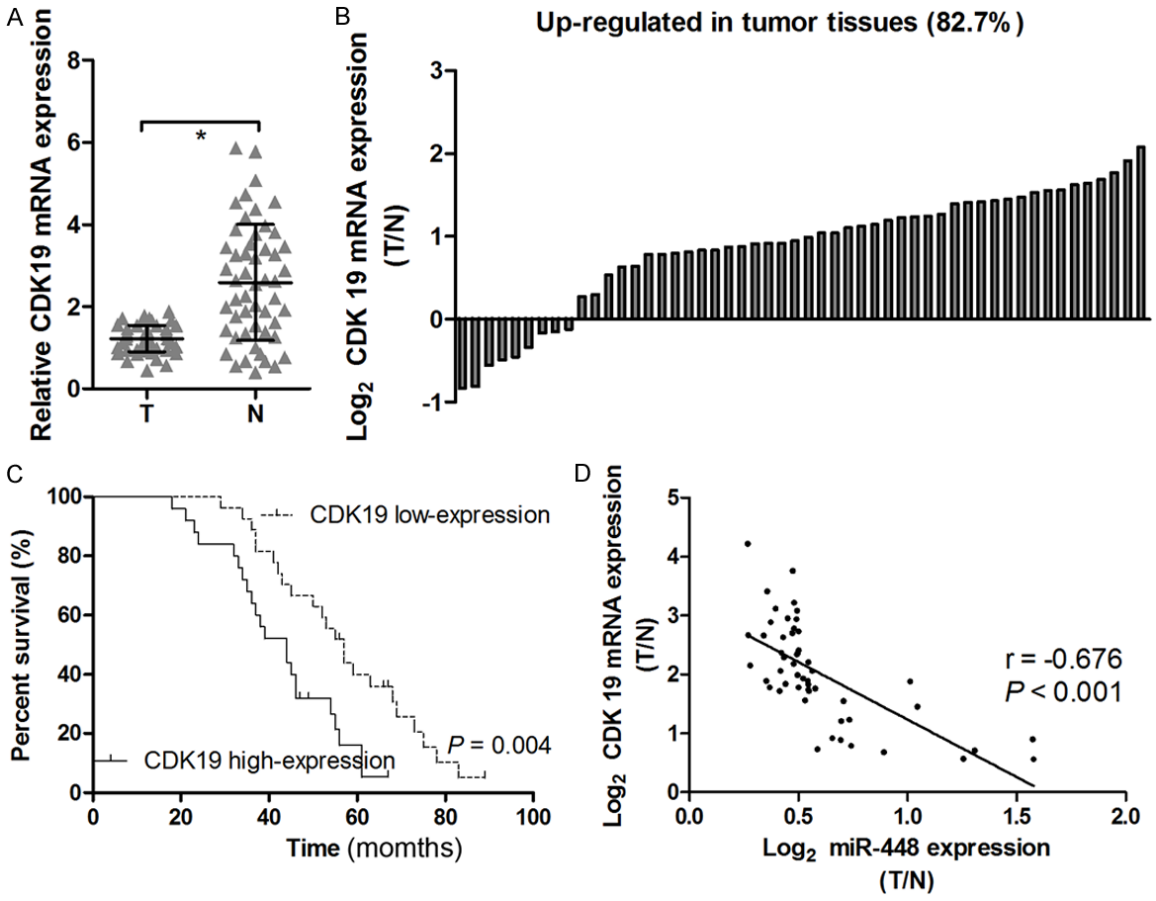

Figure 4. CDK19 expression was up-regulated in PCa tissues and associated with poor prognosis. The mRNA expression levels of CDK19 in 52 pairs of PCa tissues and corresponding non-tumorous tissues were detected by qRT-PCR assays (A and B). Kaplan-Meier survival curve was used to evaluate whether CDK19 mRNA expression was associated with overall survival in PCa patients (C). Spearman's rank correlation analysis was used to evaluate the association between CDK19 and miR-448 in PCa tissues (D). $^{*}P < 0.05$ compared with the control group.

4A). In fact, 43 of these cases (82.7%) exhibited up-regulation of CDK19 mRNA expression in PCa tissues (**Figure 4B**). To investigate the association between CDK19 and overall survival prognosis in PCa patients, Kaplan-Meier analysis was performed in 52 PCa patients, which were divided into 2 groups: high expression group (n = 25) and low expression group (n = 27) according to the Log_2 fold change ≥ 1. PCa patients with CDK19 high expression showed a significant poorer survival prognosis than that of in low expression group (**Figure 4C**). Furthermore, Spearman's rank correlation analysis was used to evaluate the association between CDK19 and miR-448 in PCa tissues, and the results found a significant negative correlation between CDK19 and miR-448 in fifty-two pairs of PCa tissues (r = -0.676, $P < 0.001$; **Figure 4D**).

Inhibition of CDK19 inhibited proliferation and induced apoptosis in PCa cells

To determine the effect of CDK19 on cell proliferation and apoptosis in vitro, sh-Con or sh-CDK19 was transfected into LNCaP and 22RV1 cells. The results show that the mRNA expression of CDK19 was significantly reduced in LNCaP and 22RV1 cells after transfected with sh-CDK19 as compared to sh-Con group (**Figure 5A**). Moreover, LNCaP and 22RV1 cells transfected with sh-CDK19 markedly inhibited PCa cells proliferation (**Figure 5B**) and induced apoptosis (**Figure 5C** and **5D**) compared with the sh-Con group.

Discussion

In the present study, critical roles of miR-448 and its downstream target CDK19 in the tumor-

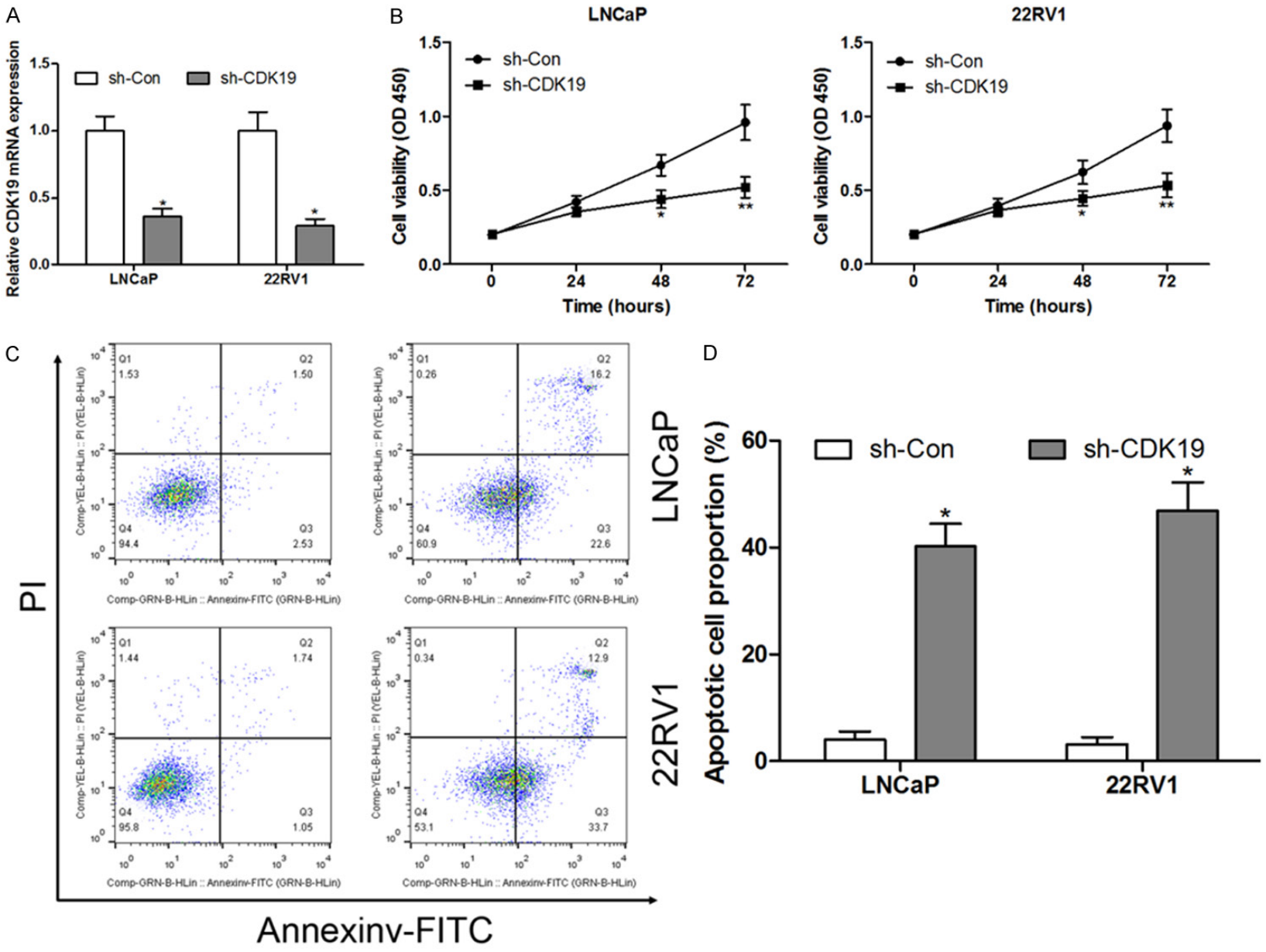
A
Relative CDK19 mRNA expression
sh-Con
sh-CDK19
LNCaP
22RV1
B
LNCaP
22RV1
Cell viability (OD 450)
Time (hours)
C
PI
Annexinv-FITC
LNCaP
22RV1
D
Apoptotic cell proportion (%)

Figure 5. CDK19 knockout inhibited proliferation and induced apoptosis in PCa cells. After transfected with sh-Con and sh-CDK19 into LNCaP and 22RV1 cells for 48 hours, the mRNA expression levels of CDK19 were detected by qRT-PCR assays (A); cell viability was measured by CCK8 assay (B); cell apoptosis was performed by flow cytometry (C and D). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sh-Con group.

igenesis of PCa were uncovered. Expression levels of miR-448 were reduced, and CDK19 levels were elevated in PCa tissues. miR-448 low expression or CDK19 high expression was associated with poor survival prognosis in PCa patients. Interestingly, the expression levels of miR-448 were inversely correlated with CDK19 in PCa tissues. Moreover, overexpression of miR-448 or inhibition of CDK19 led to retardation of cell growth and acceleration of apoptosis in PCa cell. Furthermore, CDK19 was validated as a direct target of miR-448. Taken together, these findings indicate that miR-448 is a tumor suppressor gene that is able to suppress CDK19 expression in the development of PCa.

Previous studies have shown that miR-448 as a post-transcriptional regulator postpones cancer progression by targeting multiple genes. For example, miR-448 is frequently down-regulated following chemotherapy, and miR-448-regulated special AT-rich sequence-binding protein-1 contributes to chemotherapy-induced EMT of breast cancer cells [25]. Down-regulation of miR-448 induces EMT and promotes hepatocellular carcinoma cell invasion by targeting Rho-associated coiled-coil-containing protein kinase 2 [26]. In addition, overexpressed miR-448 suppresses cell proliferation, migration, and invasion in ovarian cancer cells via inhibiting C-X-C motif chemokine 12 expression [16]. It was interesting to note that miR-448 showed a significant regulatory effect on CDK19 expression in LNCaP and 22RV1 cells and exerted an antineoplastic activity in PCa cells by inhibiting proliferation and inducing apoptosis *in vitro*. Mechanistic investigations disclose that miRNA is estimated to target multiple genes, in turn, one gene can be controlled by many distinct miRNAs [27, 28]. In this study, although other miRNAs cannot be absolutely excluded, miR-448 induced growth inhibition and apoptosis in PCa cells, at least partially, through the down-regulation of CDK19.

CDK19 and its paralog CDK8 have been shown previously to associate with the transcriptional Mediator complex and act as co-regulators of several transcription factors, including nuclear factor kappa B, signal transducer and activator of transcription family members and transcription factor II-I [29-31]. CDK8 as an oncogene contributes to the progression of several cancers, such as colorectal cancer [32], breast cancer [33] and melanoma [34]. However, the functions of CDK19 have been reported in only a few studies and have not been completely elaborated in the progression of cancer [20, 29]. Previous studies show that CDK19 inhibitors and CDK19 knockdown exert anti-proliferative and pro-apoptotic activity and decrease migration and invasion in PCa cells [21, 22]. Consistent with these results [21, 22], the findings reported here also show that inhibition of CDK19 with shRNA promoted growth inhibition and apoptosis in PCa cells. More importantly, CDK19 was identified as a direct target could be regulated by post-transcriptional regulator miR-448, which signal axis might be associated with the progression of PCa.

In a previous study, CDK19 is a direct target of miR-383, suppression of miR-383 induces an increase in CDK19 expression, resulting in the improvement of mesenchymal stem cells therapeutic effect in the treatment of spinal cord injury in rat [35]. In addition, miR-18a inhibits pancreatic progenitor cell proliferation, at least partially, by targeting CDK19 [36]. CDK19 may be partly responsible for the pro-proliferative effect in colorectal cancer cells, while miR-18a mimics reverse this effect by inhibiting CDK19 expression [37]. The results of this study also found that CDK19 was closely related with PCa cell proliferation and apoptosis, CDK19 knockout induced growth inhibition and apoptosis in PCa cells. Moreover, CDK19 was a direct target of miR-448. These findings suggest that CDK19 could be regulated by multiple miRNAs in different physiological and pathological conditions.

In conclusion, a novel role of miR-448 in suppression of cell proliferation and induction of apoptosis in PCa cells was discovered by targeting CDK19. miR-448/CDK19 signaling might provide a possible therapeutic target for the treatment of PCa.

Disclosure of conflict of interest

None.

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