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Capn4 expression is modulated by microRNA-520b and exerts an oncogenic role in prostate cancer cells by promoting Wnt/β-catenin signaling

Wei Ren[a,1], Dong Wang[b,1], Chan Li[c], Tao Shu[b], Wei Zhang[b], Xiaoliang Fu[b,*]

[a] Department of Urology, Shaanxi Provincial People's Hospital, The Affiliated Hospital of Xi'an Medical University, The Third Affiliated Hospital of Xi'an Jiaotong University, The Affiliated Hospital of Northwestern Polytechnical University, Xi'an, 710068, China
[b] Department of Urology, Tangdu Hospital, Air Force Military Medical University, Xi'an, 710032, China
[c] Department of Physical Examination, Shaanxi Provincial People's Hospital, The Affiliated Hospital of Xi'an Medical University, The Third Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710068, China

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ABSTRACT

Accumulating evidence reports that Capn4 plays an important role in the development and progression of various malignant cancers. However, whether Capn4 is involved in prostate cancer remains unclear. Therefore, the aim of this study was to investigate the expression, biological function and regulatory mechanism of Capn4 in prostate cancer. Herein, we found that Capn4 was highly expressed in prostate cancer cell lines compared with normal prostate cells. Capn4 gene silencing markedly suppressed the growth, invasion and Wnt/β-catenin signaling of prostate cancer cells, whereas Capn4 overexpression showed an oncogenic effect. Moreover, silencing of β-catenin significantly blocked the oncogenic effect of Capn4 overexpression. Bioinformatics analysis predicted that Capn4 was a potential target gene of microRNA-520b (miR-520b), which has been reported as a tumor suppressive miRNA in various cancers. The dual-luciferase reporter assay confirmed that miR-520b directly bound to the 3′-untranslated region of Capn4. Real-time quantitative PCR and Western blot analysis showed that miR-520b negatively regulated Capn4 expression in prostate cancer cells *in vitro*. Furthermore, we found that miR-520b was significantly downregulated in prostate cancer cell lines and tissues. In addition, miR-520b expression was inversely correlated with Capn4 expression in prostate cancer clinical specimens. Overexpression of miR-520b mimicked the tumor suppressive effect of Capn4 siRNA, whereas inhibition of miR-520b had an oncogenic effect. Restoration of Capn4 significantly blocked the antitumor effect of miR-520b in prostate cancer cells. Overall, our findings demonstrate an oncogenic role of Capn4 in prostate cancer and show that its expression is epigenetically regulated by miR-520b. Our results reveal that suppression of Capn4 by miR-520b inhibits the growth and invasion of prostate cancer cells associated with downregulated Wnt/β-catenin signaling, indicating an important role of the miR-520b/Capn4/Wnt/β-catenin regulation axis in the molecular pathogenesis of prostate cancer. Our study suggests that miR-520b and Capn4 may represent potential and novel therapeutic targets for prostate cancer.

1. Introduction

Prostate cancer is one of the most common malignancies in the urinary system of the male population and is the second leading cause of cancer-related mortality in men [1]. The incidence and mortality rate of prostate cancer in China have increased rapidly in recent years [2]. Although prostate cancer patients with localized or regional disease can be treatable, the majority of prostate cancer patients are diagnosed at a late stage with multiple metastases and therapy resistance, leading to high mortality rates [3,4]. Therefore, the five-year survival rate for those with metastatic prostate cancer remains extremely lower. Therefore, it is of great importance to identify novel targets for early diagnosis and to develop effective clinical management strategies for prostate cancer.

Calpains are a family of calcium-dependent cysteine proteases that catalyze the proteolysis of many specific substrates involved in a variety

Abbreviations: Capn4, calpain small subunit 1; siRNA, interfering RNA; miRNAs, microRNAs; miR-520b, microRNA-520b; mRNA, messenger RNA; 3′-UTR, 3′-untranslated region; FBS, fetal bovine serum; CCK-8, cell counting kit-8; RT-qPCR, real-time quantitative polymerase chain reaction

* Corresponding author at: Department of Urology, Tangdu Hospital, Air Force Military Medical University, No. 1 Xinsi Road, Xi'an, 710032, China.
E-mail address: fuxiaoliangtd@163.com (X. Fu).

[1] These authors contributed equally to this work and shared the first authorship.

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of cellular processes, including apoptosis, cell proliferation, cell cycle, cell migration and invasion [5–8]. Capn4 (also known as calpain small subunit 1, CAPNS1) is a small regulatory subunit of calpains and plays a critical role in regulating the stability and activity of calpains [9,10]. Capn4 has been reported to be involved in various physiological and pathological processes, such as embryonic development, chondrocyte differentiation and myocardial infarction [9,11–13]. The dysregulation of Capn4 is also associated with tumorigenesis in several human tumors [14,15]. High levels of Capn4 expression have been detected in tumor tissues and are correlated with tumor progression and poor prognosis [16–18]. Notably, Capn4 has been demonstrated to regulate the proliferation, migration and invasion of cancer cells by activating various oncogenic signaling pathways, such as Wnt/β-catenin signaling [19,20]. However, the regulatory mechanism of Capn4 in tumorigenesis remains poorly understood.

MicroRNAs (miRNAs) are a type of small, non-coding RNAs that have emerged as critical regulators of gene expression [21]. It has been well-documented that miRNAs regulate gene expression *via* binding to the 3′-untranslated region (UTR) of their target messenger RNAs (mRNAs), leading to either mRNA cleavage or translational repression [22,23]. Therefore, miRNAs inhibit gene expression and function as negative regulators of gene expression. By modulating gene expression, miRNAs are involved in regulating numerous biological functions, including cell proliferation, differentiation and apoptosis [24]. Accumulating evidence has suggested that miRNAs are deregulated during cancer development and progression, functioning as tumor suppressors and oncogenes [25,26]. These miRNAs have potential applications in cancer diagnosis, prognosis and therapy [27,28]. Various miRNAs are dysregulated in prostate cancer and are involved in regulating cancer cell malignant proliferation, metastasis and therapy resistance [29,30]. Therefore, elucidating the miRNA-mediated network underlying prostate cancer pathogenesis may help develop novel and effective miRNA-based therapies for prostate cancer.

Capn4 has been reportedly involved in a variety of cancers, including hepatocellular carcinoma [15], colorectal cancer [31], lung cancer [32] and renal cancer [33]. However, little is known about the role of Capn4 in prostate cancer. In the present study, we demonstrate that Capn4 is highly expressed in prostate cancer cell lines. Functional experiments showed that Capn4 promotes the proliferation, invasion and activation of Wnt/β-catenin signaling in prostate cancer cells *in vitro*. Notably, we identified Capn4 as a target gene of miR-520b. Our results show that miR-520b expression is decreased in prostate cancer tissues and cell lines and is inversely correlated with Capn4 mRNA expression in cancer specimens. Moreover, our results demonstrate that miR-520b inhibits the growth and invasion of prostate cancer cells through targeting Capn4. Taken together, these findings reveal an oncogenic role of Capn4 in prostate cancer and suggest that miR-520b is a critical regulator of Capn4. Our study suggests that the miR-520b/Capn4/Wnt/β-catenin regulation axis may play an important role in the molecular pathogenesis of prostate cancer and serve as a potential target for the development of prostate cancer therapy.

2. Materials and methods

2.1. Cell lines and culture

The prostate cancer cell lines (22Rv1, DU145, PC-3) and the normal prostate epithelium cell line RWPE-1 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the manufacturer's recommended culture methods. In brief, 22Rv1 cells were grown in PMI-1640 Medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. DU145 cells were grown in Eagle's Minimum Essential Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. PC-3 cells were maintained in F-12 K Medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. RWPE-1 cells were cultured in Keratinocyte Serum Free Medium (Gibco) containing 0.05 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor. The 293 T cell line was kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS and 1% penicillin/streptomycin.

2.2. RNA isolation, reverse transcription, and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. For the detection of Capn4 mRNA expression, cDNA was synthesized using Super M-MLV Reverse Transcriptase (BioTeke, Beijing, China) and amplified with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) using the Applied Biosystems 7500 Sequence Detection system. GAPDH was used as the internal control for the normalization of Capn4 expression. For detection of miR-520b expression, cDNA was synthesized with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and amplified with TaqMan MicroRNA Assays Kit (Applied Biosystems). U6 was used as an internal control for normalizing miR-520b expression. The PCR thermal cycle conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative data were obtained using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blot assay

The whole cell lysates were extracted with RIPA buffer (Beyotime Biotechnology, Shanghai, China) and protein concentration was measured using Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Total protein was separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, Boston, MA, USA). The membrane was then blocked with 5.0% non-fat milk at room temperature for 1 h and then incubated with primary antibodies against Capn4 (Abcam, Cambridge, MA, USA), β-catenin (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Abcam, Cambridge, MA, USA) overnight at 4 °C. Afterwards, the membrane was probed with horseradish peroxidase-labeled secondary antibody (Abcam) for 1 h at room temperature and protein band was developed using ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAPDH was used as the loading control.

2.4. Cell transfection

Capn4 siRNAs and negative control (NC) siRNAs were synthesized by Genepharm (Shanghai, China); β-catenin siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transferred into cells according to the manufacturer's instructions. Capn4 shRNA sequences were inserted into pLKO.1 vector (Sigma-Aldrich, St. Louis, MO, USA) to generate Capn4 shRNA expression vector. The ORF of Capn4 was cloned into the pcDNA3.1 vector (Invitrogen) to generate the Capn4 expression vector. The mimics, inhibitor and scrambled control of miR-520b were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The vectors and miRNAs were transfected into cells using RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

2.5. Cell counting kit-8 (CCK-8) assay

Cells were seeded into 96-well plates at 1×10^4 cells/well and cultured overnight. After the indicated treatments, 10 μl of CCK-8 solution (Beyotime Biotechnology) was added to each well and incubated for 2 h at 37 °C. The absorbance at 450 nm was examined using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).