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Long noncoding RNA AFAP1-AS1 accelerates the proliferation and metastasis of prostate cancer via inhibiting RBM5 expression

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Abstract. – OBJECTIVE: Recently, the role of long noncoding RNA (lncRNAs) in tumor progression has caught many attentions. In this research, lncRNA AFAP1-AS1 was studied to identify how it functioned in the progression of prostate cancer.

PATIENTS AND METHODS: LncRNA AFAP1-AS1 expression was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in both prostate cancer cells and tissue samples. In addition, to identify the function of AFAP1-AS1 on prostate cancer *in vitro*, cell proliferation, transwell assay, and Matrigel assay were conducted. Furthermore, by performing qRT-PCR and Western blot assay, the underlying mechanism was explored.

RESULTS: The expression level of AFAP1-AS1 was significantly higher in prostate cancer samples than that in corresponding ones. Additionally, the cell proliferation, migration, and invasion capacities were inhibited after AFAP1-AS1 was knocked down in prostate cancer cells. Moreover, the mRNA and protein expressions of RBM5 were upregulated after AFAP1-AS1 was knocked down. Furthermore, the RBM5 expression level was negatively related to AFAP1-AS1 expression level in prostate cancer samples.

CONCLUSIONS: AFAP1-AS1 acts as an oncogene in prostate cancer by enhancing cell metastasis and proliferation via suppressing RBM5, which might be a novel therapeutic strategy in treatment for prostate cancer.

Key Words:
Long noncoding RNA, AFAP1-AS1, Prostate cancer, RBM5.

Introduction

Prostate cancer is a public health issue currently, representing as one of the most common malignancies in men. Prostate cancer accounts for 13% of cancer-related death and has been reported to kill more than 29,000 men in America in 2018[1] (https://seer.cancer.gov/statfacts/html/prost.html). The main treatment for prostate cancer is including surgery or androgen deprivation therapy. Although the diagnosis and therapeutic strategies of prostate cancer are developing greatly in the past decades, the prognosis of these patients is still unsatisfied. The occurrence and recurrence rates of prostate cancer are significantly increasing in both developed and developing countries, which make it the third-leading cause of deaths related to cancer in males all over the world[2-5]. Therefore, it is urgent to find out the underlying mechanism and figure out a new treatment strategy.

Long non-coding RNAs (lncRNAs) are RNA transcripts longer than 200 nucleotides without the ability of coding proteins. Mounting evidence has demonstrated that lncRNAs are emerging as crucial regulators in a variety of biological processes, such as the development of many cancers. For example, through regulating expression of miR-34c and targeting MUC2, lncRNA AF147447 depresses cell growth and cell invasion in gastric cancer infected with *Helicobacter pylori*[6]. Upregulation of lncRNA CRNDE-h has been reported to be closely related to the poor prognosis of colorectal cancer; this may offer a potential biomarker for diagnosis of colorectal cancer[7]. LncRNA MEG3 depended on p53's transcription is downregulated in breast cancer which affects the cell proliferation, invasion, and migration[8]. In addition, overexpression of lncRNA GHET1 facilitates cell proliferation in pancreatic cancer and GHET1 is closely related to tumor node metastasis (TNM) staging and prognosis[9]. However,

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the role lncRNA AFAP1-AS1 played in prostate cancer and its underlying molecular mechanism have not been studied so far.

In our study, we found out that AFAP1-AS1 was highly expressed in prostate cancer tissues. In addition, AFAP1-AS1 promoted the proliferation and metastasis in prostate cancer cells *in vitro*. Moreover, our further experiment explored the underlying mechanism of how AFAP1-AS1 functioned in the development of prostate cancer.

Patients and Methods

Cell Lines and Clinical Samples

A total of 52 prostate cancer patients who received surgery at Weinan City Center Hospital of Shaanxi Province were enrolled for cancer tissues and adjacent normal tissues. Before the operation, the written informed consent was achieved. No radiotherapy or chemotherapy was administered to the patients before the operation. Tissues got from the surgery were stored immediately at -80°C. All tissues were analyzed by an experienced pathologist. This study was approved by the Ethics Committee of Weinan City Center Hospital of Shaanxi Province.

Cell Culture

Human prostate cancer cell lines PC3, LNCaP, DU145, and normal prostate epithelial cell lines P69 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Culture medium consisted of 10% fetal bovine serum (FBS, Life Technologies, Gaithersburg, MD, USA), penicillin as well as Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). Besides, cells were cultured in an incubator, which contained 5% CO_2 and was set at 37°C.

Cell Transfection

After synthesized, shRNA (lentiviral small hairpin RNA) targeting AFAP1-AS1 was cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (BiosettiaInc., San Diego, CA, USA). Empty vector was taken as control. After packaged in 293T cells, they were utilized for transfection of prostate cancer cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the AFAP1-AS1 expression level in these cells was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PCR

The total RNA was separated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs) through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Following are the primers used for qRT-PCR: AFAP1-AS1 primers forward 5′-AGCCTGTTGAATCAGCCAACT-3′, reverse 5′-GGTTCATACCAGCCCTGTCC-3′; GAPDH primers forward 5′-GGGAGCCAAAAGGGTCAT-3′ and reverse 5′-GAGTCCTTCCACGATACCAA-3′. Thermal cycle was as follows: 30 sec at 95˚C, 5 sec for 40 cycles at 95˚C, 35 sec at 60˚C.

Western Blot Analysis

Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after replaced to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Cell Signaling Technology offered us rabbit anti-GAPDH and rabbit anti-RBM5, and goat anti-rabbit secondary antibody (CST, Danvers, MA, USA). The chemiluminescent film was utilized to assess protein expression with Image J software (NIH, Bethesda, MD, USA).

Cell Proliferation Assay

96-well plate was used for the culture of prostate cancer cells (1×10^3 cells/well). Then, we added cell counting kit-8 (CCK-8) (10 μL) into these wells at different times. Microplate reader was used for measuring absorbance at 450 nm (Bio-Rad, Hercules, CA, USA).

Transwell Assay and Matrigel Assay

For transwell assay, 5×10^4 cells in 200 μL serum-free DMEM were transformed to top chamber of an 8 μm pore size insert (Corning, Corning, NY, USA). For Matrigel assay, 5×10^4 cells in 200 μL serum-free DMEM were transformed to top chamber of an 8 μm pore size insert (Corning, Corning, NY, USA) coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). And the bottom chamber was added with DMEM and FBS. 48 h later, the top surface of chambers was immersed for 10 min with precooling methanol after wiped

by cotton swab. Next, they were stained in crystal violet for 30 min. Three fields were used to count the data for invasion membrane.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 was applied to conduct the statistical analysis (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD (standard deviation). The Chi-square test and the Student *t*-text were selected when appropriate. It was considered statistically significant when $p<0.05$.

Results

AFAP1-AS1 Expression Level in Prostate Cancer Tissues and Cells

First, qRT-PCR was conducted for detecting mRNA expression of AFAP1-AS1 in 52 patients' tissues and 3 prostate cancer cell lines. The results of qRT-PCR showed that AFAP1-AS1 was significantly upregulated in tumor tissue samples (Figure 1A). Meanwhile, the expression level of AFAP1-AS1 in prostate cancer cells was higher than that of P69 (normal prostate epithelial cell lines) (Figure 1B).

Knockdown of AFAP1-AS1 Inhibited Cell Proliferation in Prostate Cancer Cells

In our study, we chose PC3 cell lines for the knockdown of AFAP1-AS1. Then, qRT-PCR was utilized for detecting the AFAP1-AS1 expression (Figure 2A). Moreover, we conducted the CCK-8 assay and found that after AFAP1-AS1 was knocked down, the cell growth ability of PC3 cells was significantly repressed (Figure 2B).

Knockdown of AFAP1-AS1 Inhibited Cell Migration and Invasion in Prostate Cancer Cells

The results of the transwell assay revealed that after AFAP1-AS1 was knocked down, the migrated ability of prostate cancer cells was significantly repressed (Figure 3A). The results of the Matrigel assay also revealed that after AFAP1-AS1 was knocked down, the number of invaded cells was remarkably decreased (Figure 3B).

The Interaction Between RBM5 and AFAP1-AS1 in PC3 Prostate Cancer Cells

Then, qRT-PCR data showed that the expression level of RBM5 in prostate cancer cells was higher in AFAP1-AS1 shRNA (shRNA) group compared with empty vector (EV) group (Figure 4A). Western blot assay data showed that after AFAP1-AS1 was knocked down the protein expression of RBM5 was upregulated (Figure 4B).

The Interaction Between RBM5 and AFAP1-AS1 in Prostate Cancer Tissues

We further found that RBM5 expression in prostate cancer tissues was markedly lower than that in adjacent tissues (Figure 5A). The correlation analysis revealed that the negative association was seen between RBM5 expression level

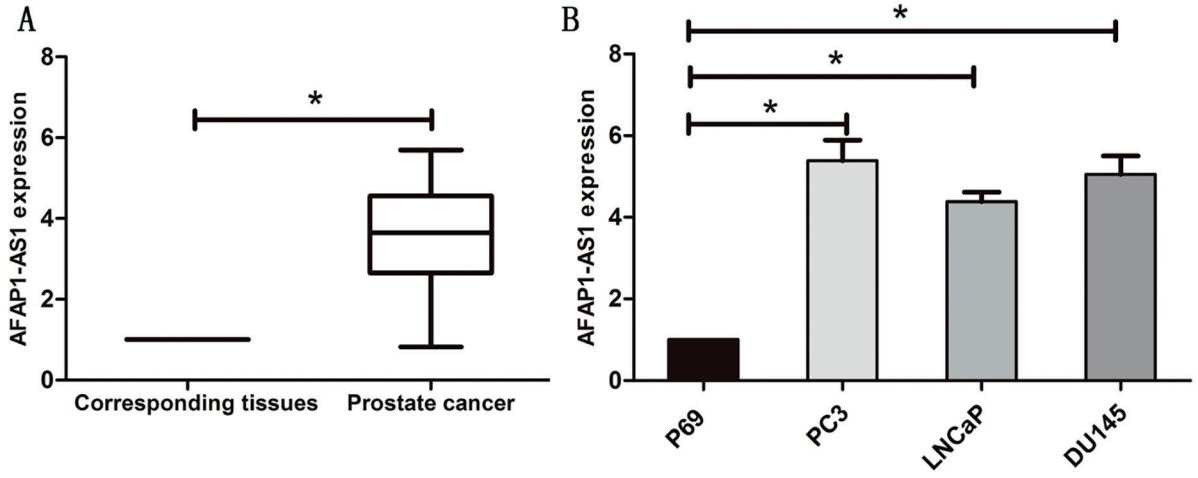

Figure 1. Expression levels of AFAP1-AS1 were increased in prostate cancer tissues and cell lines. ***A,*** AFAP1-AS1 expression was significantly increased in the prostate cancer tissues compared with corresponding tissues. ***B,*** Expression levels of AFAP1-AS1 relative to GAPDH were determined in the human prostate cancer cell lines and P69 (normal prostate epithelial cell lines) by qRT-PCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

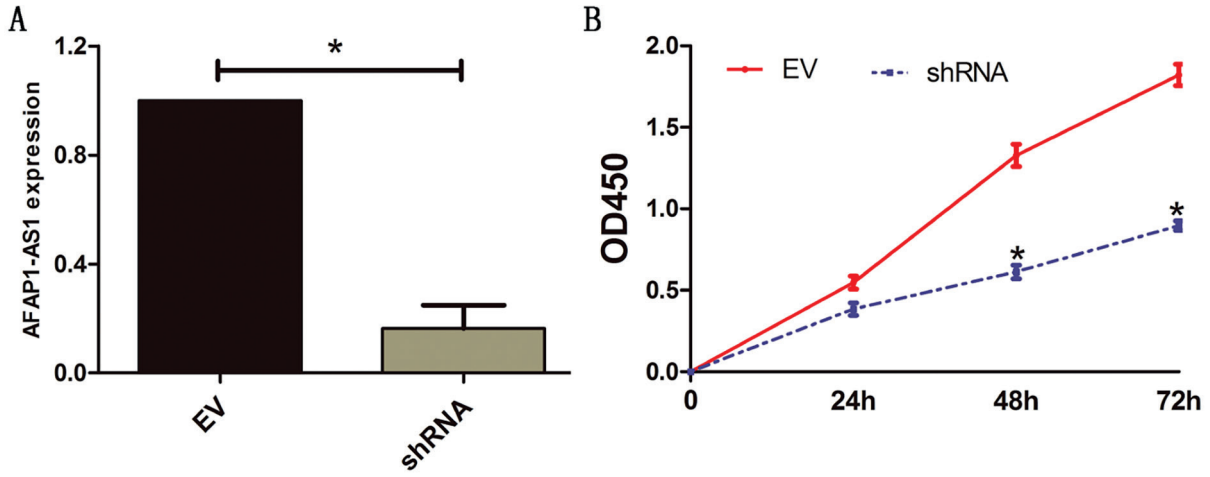

Figure 2. Knockdown of AFAP1-AS1 inhibited the proliferation of PC3 prostate cancer cells. ***A,*** AFAP1-AS1 expression in PC3 prostate cancer cells transduced with AFAP1-AS1 shRNA (shRNA) and the empty vector (EV) was detected by qRT-PCR. GAPDH was used as an internal control. ***B,*** CCK-8 assay showed that knockdown of AFAP1-AS1 significantly inhibited cell proliferation in PC3 prostate cancer cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

and AFAP1-AS1 expression in prostate cancer tissues (Figure 5B).

Discussion

Emerging researches have revealed that lncRNAs functions as critical regulators of tumor progression in prostate cancer. For example, lncRNA SNHG7 promotes the proliferation and cycle progression in prostate cancer cells through miR-503/Cyclin D1 pathway[10]. Repression of lncRNA NEAT1 promotes the development of prostate cancer by disturbing the cell cycle and inhibiting the proliferation of prostate cancer cells[11]. LncRNA ZEB1-AS1 functions as an oncogene in tumorigenesis of prostate cancer through activating ZEB1 and regulating the downstream molecules of ZEB1[12]. LncRNA MALAT1 and HOTAIR have been reported to play the opposite role in transcriptional regulation in prostate cancer cell[13].

Recently, lncRNA AFAP1-AS1 has been revealed to be closely related with several cancers which are originated from the antisense strand at the AFAP1 coding gene locus. For instance, lncRNA AFAP1-AS1 exerts oncogenesis in esophageal squamous cell carcinoma by inhibiting cell apoptosis and promoting cell proliferation[14]. LncRNA AFAP1-AS1 facilitates the proliferation and invasion of gallbladder cancer cells. Meanwhile, upregulation of AFAP1-AS1

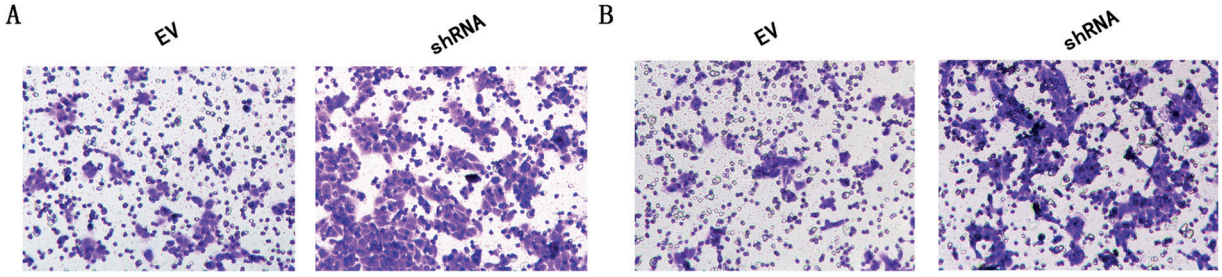

Figure 3. Knockdown of AFAP1-AS1 inhibited the migration and invasion of PC3 prostate cancer cells. ***A,*** Transwell assay showed that knockdown of AFAP1-AS1 significantly decreased the migration of PC3 prostate cancer cells (magnification: 40×). ***B,*** Matrigel assay showed that the number of invaded cells was significantly decreased *via* knockdown of AFAP1-AS1 in PC3 prostate cancer cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

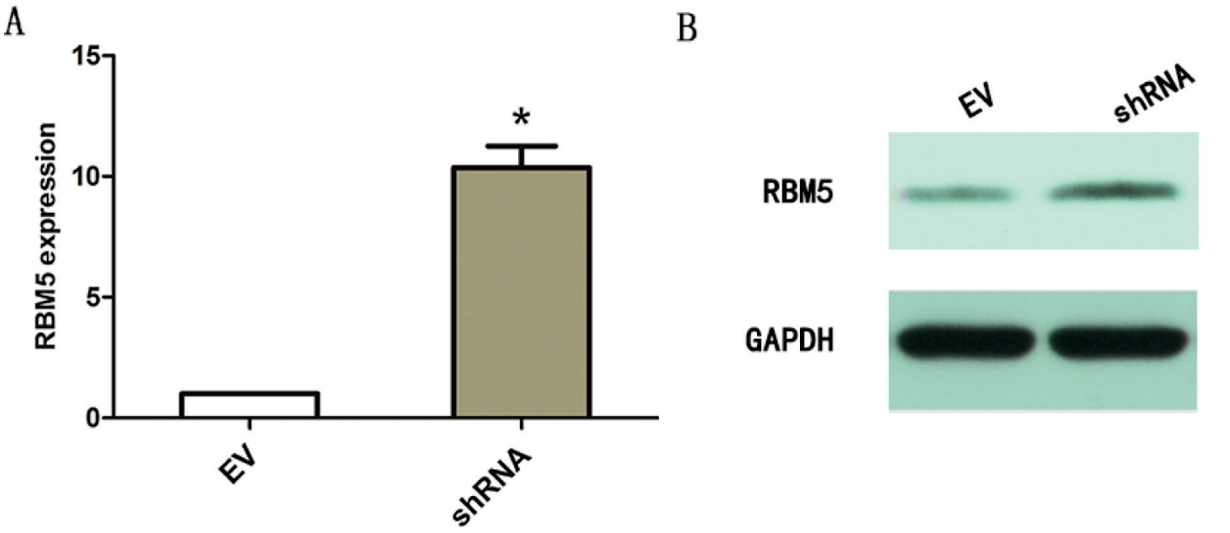

Figure 4. Interaction between AFAP1-AS1 and RBM5 in PC3 prostate cancer cells. ***A,*** qRT-PCR results showed that RBM5 expression was higher in AFAP1-AS1 shRNA (shRNA) compared with the empty vector (EV). ***B,*** Western blot assay revealed that RBM5 protein expression was increased in AFAP1-AS1 shRNA (shRNA) compared with empty vector (EV). The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. **p*<0.05.

is reported to be significantly correlated with the poor prognosis of the patients, which may offer a potential therapeutic target[15]. Upregulation of lncRNA AFAP1-AS1 enhances tumorigenesis of colorectal cancer cells and predicts poor prognosis of patients with colorectal cancer[16]. Moreover, the down-regulation of AFAP1-AS1 depresses the proliferation and induces cell apoptosis in lung adenocarcinoma cells, which could offer a new therapeutic strategy for lung adenocarcinoma[17].

In our research, we found out that lncRNA AFAP1-AS1 was upregulated in both prostate cancer samples and cells. Besides, after AFAP1-AS1 was knocked down, the proliferation and metastasis of prostate cancer cells were found to be inhibited. Above results indicated that AFAP1-AS1 promoted tumorigenesis of prostate cancer and might act as an oncogene.

RNA binding motif 5 (RBM5) is a gene located on the cancer inhibitor region 3p21.3, which participates in the progression of sever-

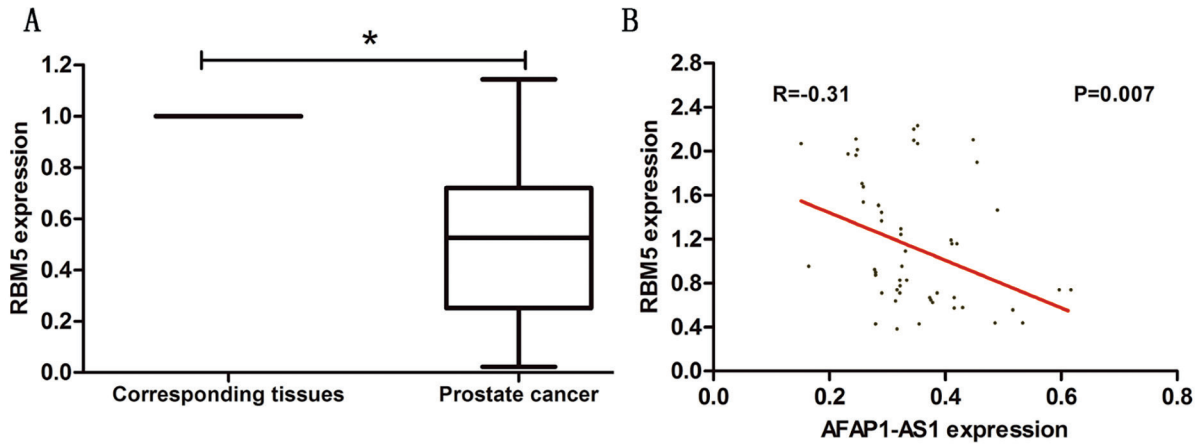

Figure 5. Interaction between AFAP1-AS1 and RBM5 in prostate cancer tissues. ***A,*** RBM5 was significantly downregulated in prostate cancer tissues compared with adjacent tissues. ***B,*** The linear correlation between the expression level of RBM5 and AFAP1-AS1 in prostate cancer tissues. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. **p*<0.05.

al carcinomas[18]. For example, RBM5 depresses tumorigenesis of gliomas by inhibiting Wnt/beta-catenin signaling and inducing cell apoptosis[19]. Through targeting RBM5, miR-483-5p enhances the proliferation and invasion in prostate cancer cells[20]. The mRNA and protein expression level of RBM5 are significantly downregulated in lung adenocarcinoma and non-small cell lung cancer, which can be used as a diagnostic marker for the patients with lung cancer[21,22]. Moreover, downregulation of RBM5 facilitates the progression and metastasis of pancreatic cancer[23].

In the present study, RBM5 expression could be upregulated after knockdown of AFAP1-AS1. Additionally, RBM5 expression in prostate cancer tissues was negatively related with AFAP1-AS1 expression. All those results proved that AFAP1-AS1 might promote tumorigenesis of prostate cancer *via* suppressing RBM5.

Conclusions

We found that AFAP1-AS1 was remarkably upregulated, and AFAP1-AS1 could enhance the invasion and migration in prostate cancer cells by repressing RBM5. Our findings proved that AFAP1-AS1 may contribute to therapy for prostate cancer as a candidate target.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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