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Long noncoding RNA PANDAR promotes progression and predicts poor prognosis via upregulating ROCK1 in prostate cancer

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Abstract. – OBJECTIVE: Recent researches have proved that long noncoding RNAs (lncRNAs) play an important role in tumorigenesis. In this research, lncRNA PANDAR was explored to identify the role it played in the development of prostate cancer and how it achieves.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) was utilized to detect PANDAR expression in both prostate cancer tissue samples and cells. Moreover, the associations between expression level of PANDAR and patients' disease-free survival rate were studied respectively. Then wound healing assay and transwell assay were conducted. Furthermore, RT-qPCR and Western blot analysis were used to explore the underlying mechanism.

RESULTS: By comparison with PANDAR expression in adjacent tissues, PANDAR expression level was significantly higher in prostate cancer samples, which was closely associated with patients' disease-free survival. Moreover, after PANDAR was knocked down, cell migration and cell invasion capacity of prostate cancer cells were enhanced *in vitro*. In addition, after overexpressing PANDAR, the mRNA and protein expression of ROCK1 was upregulated, respectively. Furthermore, it was found that ROCK1 expression was positively correlated to PANDAR expression in prostate cancer tissues.

CONCLUSIONS: Results above suggest that PANDAR could enhance cell migration and invasion of prostate cancer by upregulating ROCK1, which may offer a potential therapeutic target in prostate cancer.

Key words:
Long noncoding RNA, PANDAR, Prostate cancer, R...

Introduction

Prostate cancer is one of the most frequent malignancies in the males. It is reported that more than 220,0... cases were diagnosed of prostate cancer in An... in 2015 (http://seer.cancer.gov/faststats. Acc... January 20, 2016). Mo... patients newly diagnosed of prostate c...er can be treated with surgery or androgen ...rivation ther... if they are still at early stage[1]. ...ever, prosta... cancer is still one of the lead-i... uses of c...logic death in males in the wo... re, recent research have found out that ... morbidity and mortality of pros-...tate cancer are significantly rising in developing ...s[3,4]. Therefore, it is urgent to find out the ...ng mechanism and differentiate more aggressive tumors.

Long non-coding RNAs (lncRNAs) are one subgroup of non-coding RNAs, which do not encode proteins. However, numerous evidences have proved that lncRNAs play an important part in the development of malignant tumors. For instance, lncRNA PlncRNA-1 acts as an oncogene in the progression of colorectal cancer cell by regulation of PI3K/Akt signaling pathway[5]. Through negatively regulating miR-488, lncRNA PRNCR1 promotes tumor progression in non-small cell lung cancer[6]. Overexpression of long noncoding RNA CRNDE-h is associated with poor prognosis of colorectal cancer, which might be used as a novel serum-based biomarker for diagnosis of colorectal cancer[7]. The progression of triple-negative breast cancer could be significantly inhibited after knockdown of lncRNA snaR, which may offer a potential treatment modality[8]. In addition, lncRNA CCAT2 promotes proliferation and metastasis of intrahepatic cholangiocarcinoma and predicts a poor prognosis of the patients[9]. However, the function of lncRNA PANDAR in prostate cancer remains unexplored so far.

In this study, we found out that the expression of PANDAR was remarkably higher in prostate

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cancer tissues. Moreover, PANDAR promoted the migration and invasion of prostate cancer cell *in vitro*. Moreover, our further experiment explored the underlying mechanism how PANDAR functioned in prostate cancer development.

Patients and Methods

Cell Lines and Clinical Samples

PC3, LNCaP, DU145 and 22Rv1 human prostate cancer cells and P69 (normal human prostate epithelial cell lines) were offered by the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Culture medium consisted of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) as well as penicillin. Besides, cells were cultured in an incubator containing 5% CO_2 at 37°C.

50 prostate cancer patients were received surgery at The First Affiliated Hospital of Jinzhou Medical University. Their tissue samples got from the surgery were stored immediately at –80°C. All tissues were analyzed and confirmed by a [illegible] rienced pathologist. The Research Ethics [illegible]-mittee of The First Affiliated Hospital of Jin[illegible] Medical University ratified this study and writ[illegible] informed consent was given by th[illegible]ients.

Cell Transfection

Then pLenti-EF1a-F[illegible]F2A-P[illegible]tor was used for cloning [illegible] PANDAR (Biosetti[illegible]., San[illegible], CA, USA). After synthesized [illegible]NDAR lei[illegible]es (PANDAR) and the [illegible]ector were p[illegible]ged by 293T cells, [illegible]n we[illegible]n used for transfection in pro[illegible]te cancer ce[illegible] h later, Real-time quantita[illegible] polymerase ch[illegible]action (RT-qPCR) [illegible] used [illegible] detect PA[illegible]DAR expression leve[illegible] these [illegible].

Wound [illegible]ling A[illegible]y

[illegible]er tr[illegible]re[illegible]o 6-well plates, cells were cu[illegible]d in D[illegible]o's Modified Eagle's Medium[illegible]MEM) me[illegible]ium overnight. After scratched w[illegible], cells were cultured in serum-free [illegible]becco's [illegible]odified Eagle's Medium (DMEM). [illegible]und closure was counted after 72 h.

Tra[illegible]well Assay

For detecting the migrating ability of these treated cells, 5×10^4 cells in 200 µL of serum-free DMEM were transformed to top chamber of an 8 µm pore size insert (Millipore, Billerica, MA, USA). And Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (F[illegible] added to the bottom chamber. 48 h l[illegible], the t[illegible] surface of chambers was immer[illegible]or 10 min with precooling methanol after [illegible]by cotton swab. Following were stain[illegible]in crys[illegible]let for 30 min.

For detecting the in[illegible]ng ability o[illegible] treated cells, 5×10^4 ce[illegible] 200 [illegible] serum-[illegible] DMEM were transf[illegible]l to t[illegible]chambe[illegible] of an 8 µm pore size i[illegible]t ([illegible], Bille[illegible] MA, USA), which [illegible]forwa[illegible]ted [illegible] 50 µg Matrigel (BD[illegible]sciences, F[illegible]Lakes, NJ, USA). An[illegible]'s Modifie[illegible]gle's Medium (DM[illegible]) and [illegible]ovine serum (FBS) were added to the bottom c[illegible]er. 48 h later, the top sur[illegible]chambers wa[illegible]mersed for 10 min w[illegible]precooling methanol after wiped by cotton [illegible]b. Following[illegible]ere stain in crystal violet for [illegible]in. The data[illegible]s counted in three fields from m[illegible]ion and i[illegible]ion membrane.

RNA E[illegible]ion and RT-qPCR

TRIzol reagent (Invitrogen, Carlsbad, CA, [illegible]as utilized for separating the total RNA. [illegible] reverse Transcription Kit, the total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs) (TaKaRa Biotechnology Co., Ltd., Dalian, China). Following are the primers using for RT-qPCR: PANDAR primers forward: 5′-CTGTTAAGGTGGTGGCATTG-3′, reverse: 5′-GGAGGCTCATACTGGCTGAT-3′; GAPDH primers forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse: 5′-TGATGGCATGGACTGTGGTCATTCA-3′. Thermal cycle was as follows: 95°C for 30 s at, 5 s for 40 cycles, 60°C for 35 s.

Western Blot Analysis

Protein was extracted from cells by Reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) protein assay kit was used for quantifying protein concentrations (TaKaRa, Dalian, China). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate proteins. They were replaced to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated with antibodies. Rabbit anti-GAPDH and rabbit anti-ROCK1 were provided by Cell Signaling Technology (CST, Danvers, MA, USA), as well as goat anti-rab-

bit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for assessment of protein expression.

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SD (Standard Deviation). Student *t*-text and Kaplan-Meier method were performed to the data. It was considered of statistically significance when $p<0.05$.

Results

PANDAR Expression Level in Prostate Cancer Tissues and Cells

Firstly, PANDAR expression was detected *via* RT-qPCR in 50 patients' tissues and 4 prostate cancer cell lines. As a result, PANDAR was significantly upregulated in tumor tissue samples (Figure 1A). PANDAR expression level of prostate cancer cells was higher than that of P69 (normal human prostate epithelial cell lines) (Figure 1B).

The Interaction Between Expression of PANDAR and Disease-Free Survival of Prostate Cancer Patients

Patients' survival after surgery was analyzed through Kaplan–Meier method. According to the median expression, divided prostate cancer patients into two groups: high-PANDAR and low-PANDAR. Kaplan-Meier analysis showed that prostate cancer patients in high-PANDAR group had a poorer disease-free survival when compared with the low-PANDAR group (Figure 2).

Overexpression of PANDAR Promoted Cell Migration and Invasion Prostate Cancer Cells

In this study, we chose PC3 and DU145 prostate cancer cell lines for the overexpression of PANDAR. Then, PANDAR expression was detected by RT-qPCR (Figure 3A). Moreover, results of wound healing assay showed that overexpression of PANDAR enhanced the ability of migration in prostate cancer cells significantly (Figure 3B). The outcome of Transwell assay also revealed that the number of migrated and invaded cells was remarkably increased when PANDAR was overexpressed in prostate cancer cells, respectively (Figure 3C-D).

Interaction between ROCK1 and PANDAR in Prostate Cancer

Previous studies suggested ROCK1 acted as an oncogene in many cancers including prostate cancer. To explore the interaction between ROCK1 and PANDAR, we conducted RT-qPCR and found that expression level of ROCK1 in prostate cancer cells was significantly higher in PANDAR lentiviruses (PANDAR) group when compared with the ROCK1 level in empty vector group (Figure 4A). The result of western blot assay also showed that after PANDAR was overexpressed, ROCK1 could be upregulated at protein level (Figure 4B). Furthermore, we found that ROCK1 expression

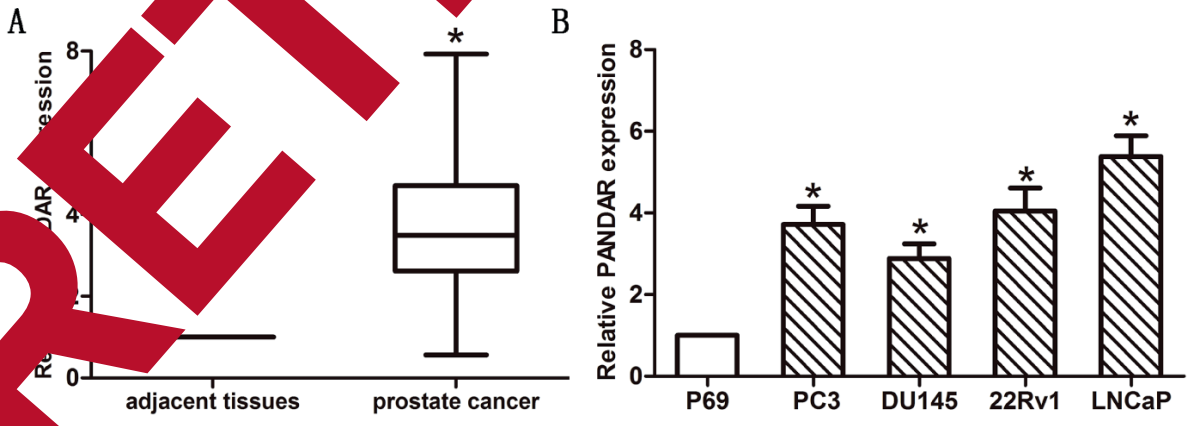

Figure 1. Expression levels of PANDAR were increased in prostate cancer tissues and cell lines. ***A,*** PANDAR expression was significantly increased in the prostate cancer tissues compared with adjacent tissues. ***B,*** Expression levels of PANDAR relative to GAPDH were determined in the human prostate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

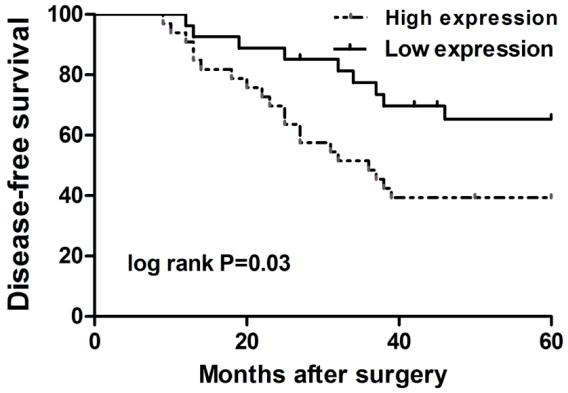

Figure 2. Interaction between PANDAR expression and disease-free survival of prostate cancer patients. High level of PANDAR was associated with poorer disease-free survival of prostate cancer patients.

in prostate cancer tissues was remarkably higher when compared with that of adjacent tissues (Figure 4C). Correlation analysis demonstrated that ROCK1 expression level was posi[tively] related to PANDAR expression in c[ancer] tissu[es] (Figure 4D).

Discuss[ion]

Evidence indicated [that] lncR[NAs] serve [as] crucial regulatory [roles] th[e] velopm[ent] of prostate cancers [by] ach[anging] cell prol[iferation], apoptosis, and [invas]ation. [For] ampl[e], downregulation of lnc[RNA] PVT1 supp[resses] prolifer-ation and [invasion] of prostate [cancer] cells by regulating [the] exp[ression] and phosphorylation of p38[10]. LncRNA Z[FAS1] acts as an onco-

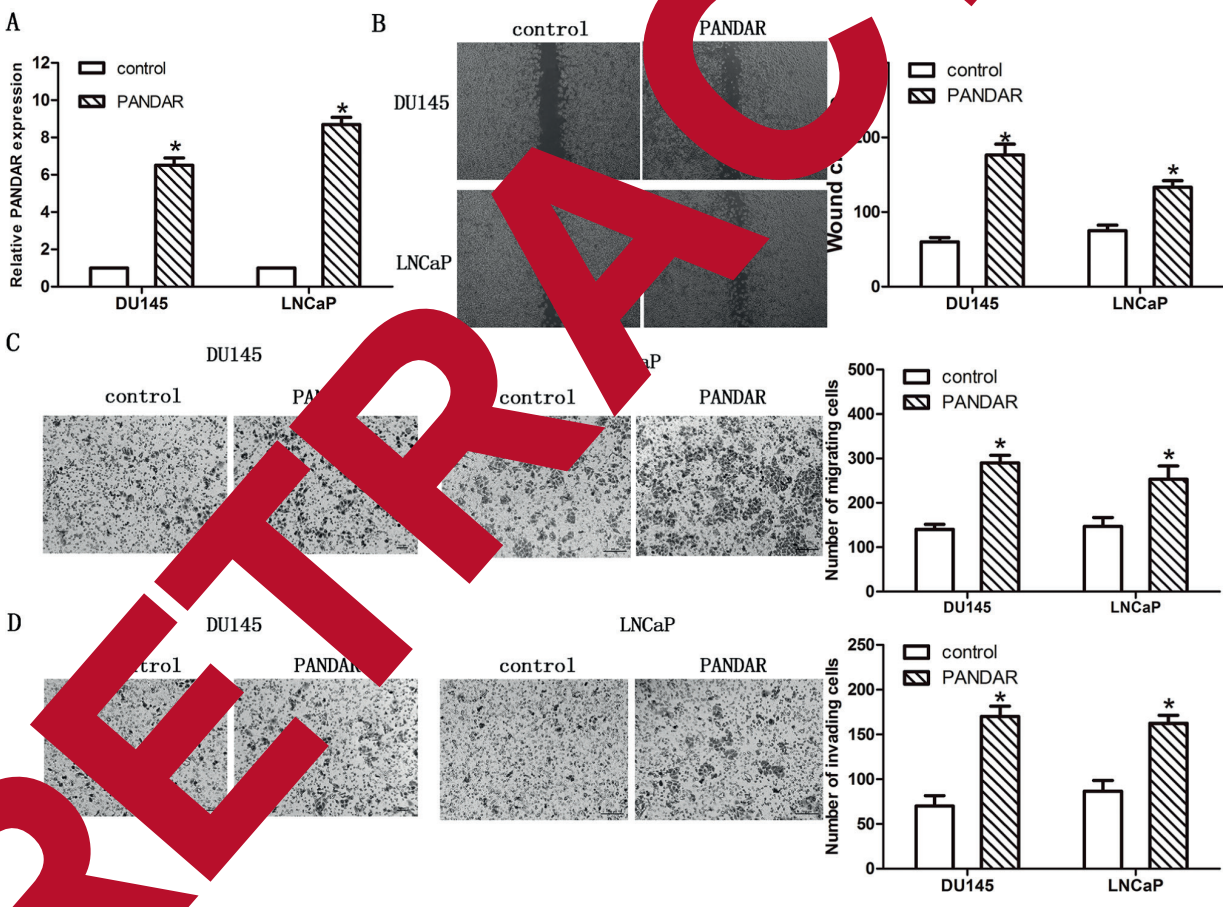

Figure 3. Overexpression of PANDAR promoted prostate cancer cell migration and invasion. ***A,*** PANDAR expression in prostate cancer cells transduced with PANDAR lentiviruses (PANDAR) and the empty vector was detected by RT-qPCR. GAPDH was used as an internal control. ***B,*** Wound healing assay showed that overexpression of PANDAR significantly increased cell migration in prostate cancer cells. ***C,*** Transwell assay showed that number of migrated cells was significantly increased *via* overexpression of PANDAR in prostate cancer cells. ***D,*** Transwell assay showed that number of invaded cells was significantly increased *via* overexpression of PANDAR in 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.

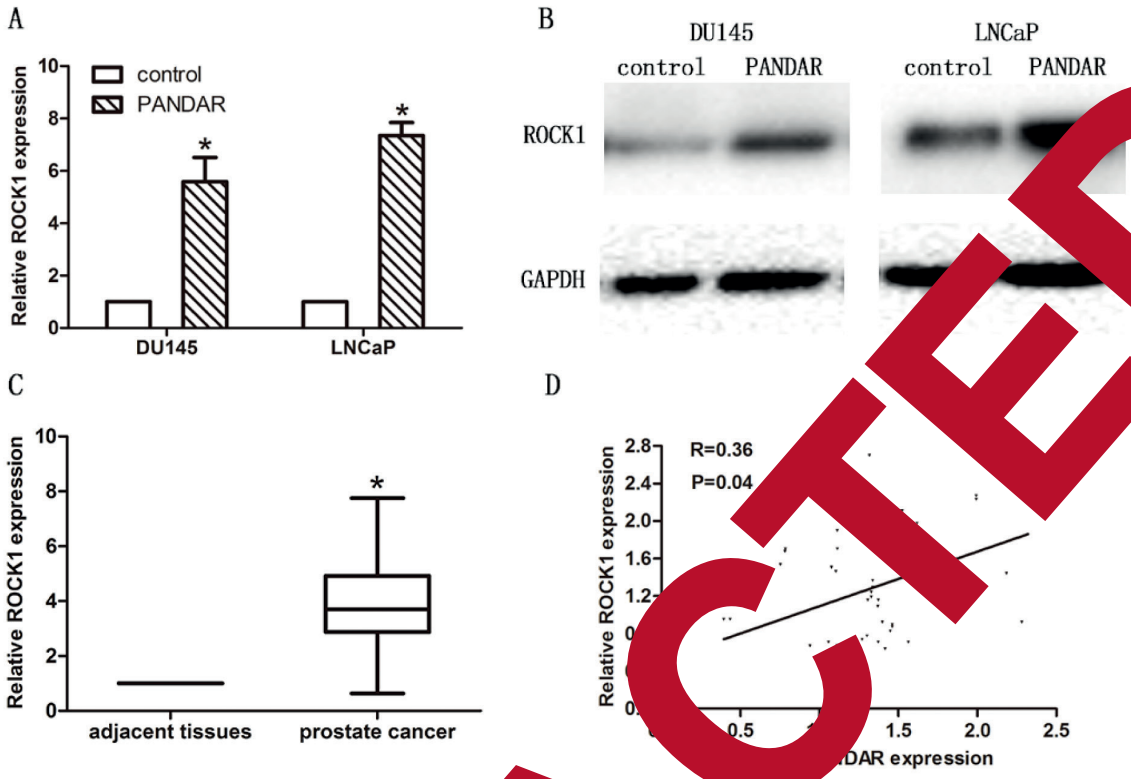

Figure 4. Interaction between PANDAR and RO CR results showed that ROCK1 expression was increased in PANDAR lentiviruses (PANDAR) compared wi emp . Western blot assay revealed that ROCK1 protein expression was increased in PANDAR lentiviruses (P AR) n the empty vector. ***C,*** ROCK1 was significantly upregulated in prostate cancer tissues compared with a ues. ***D,*** The linear correlation between the expression level of ROCK1 and PANDAR in prostate cancer tissues. The epresent the average of three independent experiments Data are presented as the mean ± standard e mean. *p

gene in prostate cancer tivatin nd regulating the downst am of Z. LncRNA SChLAP1 ntribu he develop-ment of aggressi rostate ca y antago-nizing the fun he SWI/SN nplex[12]. In addition, NA CR enhances the in-vasion of state cance ugh inhibiting the expressi of TIMP2/3 etically which may l potent l interventi n for preventing pro cance etastasis[13]. Moreover, by acti-vati ncRNA CSEAT facilitates the develop f pros cancer, which may offer ntia target[14].

DAR coding RNA that is 1506bp in l th, locate at the chromosome 6p21.2 po-si , Hung *et al*[15] reported that PAN-R inhibited cell apoptosis by regulating the lear transcription factor Y. Recently, lncRNA DAR is found to take part in the progression of ious cancers. For example, overexpres-sion of lncRNA PANDAR promotes cell prolif-eration in cervical cancer, which is also related to the poor prognosis[16]. On the other hand, low expression of lncRNA PANDAR represses cell apoptosis in non-small cell lung cancer through regulating Bcl-2 and predicts a poor prognosis of NSCLC[17]. Upregulation of lncRNA PANDAR is associated with poor clinical outcome of AML, suggesting a potential biomarker for predicting the prognose[18]. Moreover, expression of lncRNA PANDAR predicts adverse phenotypes in retino-blastoma by regulating cell growth and apopto-sis[19]. In this study, we found that PANDAR was upregulated both in prostate cancer samples and cells. Besides, the close relationship was seen between patients' prognosis and expression lev-el of PANDAR. Furthermore, overexpression of PANDAR promoted cell migration and invasion in prostate cancer. Above results indicated that PANDAR promoted tumorigenesis of prostate cancer and might act as an oncogene.

Rho-associated kinase 1 (ROCK1), a protein serine/threonine kinase, has been reported to participate in a variety of biological and patho-

logical processes, including cell motility, tumor metastasis and epithelial-to -mesenchymal transition (EMT)[20]. For instance, Mst1 regulates cell apoptosis in NSCLC by ROCK1/Factin pathways induced mitochondrial damage[21]. Silencing of URG11 represses the cell proliferation and EMT in benign prostatic hyperplasia cells through RhoA/ROCK1 pathway[22]. LncRNA LOC441178 inhibits cell invasion and cell migration in oral squamous carcinoma *via* targeting ROCK1[23]. In the present study, we firstly discovered the interaction between ROCK1 and PANDAR. The results showed that the expression level of ROCK1 could be upregulated after overexpression of PANDAR. Furthermore, ROCK1 expression in prostate cancer tissues was positively related to PANDAR expression. All the results above suggested that PANDAR might promote tumorigenesis of prostate cancer through upregulating ROCK1.

Conclusions

We identified that PANDAR was remar[illegible]ly upregulated and was negatively related[illegible] ease-free survival of prostate cancer pa[illegible]s. Besides, PANDAR could facilitate cell migra[illegible] and invasion in prostate cancer through upre[illegible] lating ROCK1. These findings s[illegible] that PA[illegible] DAR may contribute to thera[illegible]r p[illegible]ate cancer as a candidate target.

Conflict of Inter[illegible]

The Authors decla[illegible] have no confl[illegible] interests.

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