RESEARCH ARTICLE

DCUN1D1 promotes tumour progress in prostate cancer and its effect on DU145 in vitro

Shuntan Huang[1], Zezhen Liu[2], Funeng Jiang[3], Huichan He[4], Weide Zhong[5]

Abstract

Objective: To compare the expression levels of Defective In Cullin Neddylation 1 Domain Containing 1 oncogene in prostate cancer tissues and normal prostate tissues, to explore its effect on cancerous cells, and to investigate its underlying mechanisms on such cells in vitro.

Methods: The cross-sectional study was conducted at Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics from Jan 03,2017 to Nov 05,2018, and comprised prostate tissue samples on which immunohistochemistry was used to detect the expression of Defective In Cullin Neddylation 1 Domain Containing 1 oncogene. Short hairpin ribonucleic acid expression plasmid targeting the oncogene was constructed and transferred into prostate cance cell line DU145. The roles of the oncogene in prostate cancer progression were confirmed in vitro. The expression of vimentin and epithelial cadherin influenced by the oncogene were detected using Western blot. Data was analysed using SPSS 24.

Results: Of the 80 samples, 3(3.75%) were normal prostate tissues, 7(8.75%) adjacent normal prostate tissues, 20(25%) hyperplasia, and 50(62.5%) prostate cancer tissues. Defective In Cullin Neddylation 1 Domain Containing 1 oncogene expression in prostate cancerous tissues was significantly associated with high Gleason score ($p<0.001$), metastasis ($p<0.05$) and pathological stage ($p<0.001$). The oncogene was found to be an independent prognostic factor for disease-free survival of prostate cancer patients ($p=0.0108$). In vitro analysis confirmed the tumour promotive role of the oncogene through cell proliferation, invasion and migration assays. Its expression was closely correlated with aggressive progression and poor prognosis in prostate cancer patients ($p<0.05$). Vimentin and epithelial cadherin were affected by the oncogene.

Conclusion: Defective In Cullin Neddylation 1 Domain Containing 1 oncogene highly expressed in DU145 and the prostate cancer tissues, which correlated with prognosis.

Keywords: Prostate cancer, DCUN1D1, Vimentin, E-cadherin, EMT. (JPMA 71: 473; 2021)

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Introduction

Prostate cancer (PCa) is among the most common cancers and is the second leading cause of cancer-related death in males in western countries. With economic development and lifestyle changes of the previous decades, morbidity and mortality of PCa have also increased in China.[1,2] Clinical behaviour of PCa varies from indolent tumours with no or little clinical significance to aggressive metastatic and lethal diseases. It is important to explore the molecular biological mechanism of the occurrence and development of PCa in order to decide the optimal therapeutic strategies and predict its prognosis. The Defective In Cullin Neddylation 1 Domain Containing 1 (DCUN1D1) is a new oncogene located on chromosome 3q26.3,[3,4] which is involved in many physiological processes, including cell development, proliferation, differentiation and apoptosis, and is also reported involved in some cancers, like laryngeal carcinoma, glioma, cervical cancer and lung cancer.[5-7] Recent studies suggest that over-expression of DCUN1D1 may accelerate cancer progression.[5-7] However, biological roles of DCUN1D1 in PCa is unclear. The current study was planned to compare DCUN1D1 expression levels in PCa and normal prostate tissues. It was also planned to explore the effect of DCUN1D1 on cancerous cells, and to investigate its underlying mechanisms on such cells in vitro.

[1,5]Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou, China; [2-4]Department of Urology, Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, China.

Correspondence: Wei-De Zhong Email: zhongwd2009@live.cn

Materials and Methods

The cross-sectional study was conducted at Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics from Jan 03,2017 to Nov 05,2018, and comprised prostate tissue samples on which immunohistochemistry (IHC) was used to detect DCUN1D1 expression. To understand DCUN1D1 regulating pathway, vimentin and epithelial (E)-cadherin expression change after DCUN1D1 ribonucleic acid (RNA) interference to assess epithelial-mesenchymal transition (EMT), migration and invasiveness of the cancer cells.

The Cancer Genome Atlas (TCGA) is a publicly available dataset including primary prostate cancer patients with microarray expression data.[8] Clinico-pathological features of prostate cancer associate with many factors such as age, gleason score, pathological stage, metastasis, PSA failure, overall survival.[9,10]

DU145 (BeNa Culture Collection, Beijing, China) cells were cultured in dulbecco,s modified eagle medium (DMEM) (No: 12491-015, Thermo Fisher, Shanghai,China) with 10% foetal bovine serum(FBS) (No: 12484-010, Thermo Fisher, Shanghai,China) and 1% penicillin and streptomycin (No:15140122, Thermo Fisher, Shanghai,China). Cells were cultured with 5% CO_2 at 37℃ in a humidified chamber.

DCUN1D1 over-expression plasmids and control vector were arranged (HYYMed Company, Guangzhou, China) Short hairpin RNA (shRNA) of inhibited DCUN1D1 were synthesised (Sango Biotech, Shanghai, China). DU145 cells were infected with the virus of plasmid. Sequence sh-RNA of DCUN1D1 was: GATCCGCAGATGACATGTCTAATTC TTCCTGTCAGA AATTAGACATGTCATCTGCTTTTTGAATT. Cells were transfected using Lipofectamine 2000 ReagentCat.No:11668019, Invitrogen, US） according to the manufacturer's protocol. For migration, invasion, apoptosis, proliferation and western blot assays, were used after 48 hours of transfection.

Seed DU145 cells were placed in a 96-well plate at a density of 104 cells/well in 100ul of DMEM without FBS. DU145 cells were cultured in a CO2 incubator at 37℃ for 4, 24, 48, 72 hours, and 10μl of Cell Counting Kit-8(CCK-8) solution to each well of the plate was added using a repeating pipettor. The plate was incubated for 1 hour in the incubator, and the absorbance was measured at 450nm using a microplate reader.

Transwell assay was prepared using CytoSelect Cell Migration and Invasion Kit (Cell Biolabs, Inc, San Diego, CA, USA) according to the manufacturer's instructions. First, 5 X 104 DU145 cells in 200 μl medium were placed in upper chambers. The DMEM with 10% foetal bovine serum were placed in lower chambers. Cell was cultured for 48 hours at 37℃. The membranes were fixed with 10% formalin and stained with 0.05% Crystal Violet. The number of cells that migrated through the membranes was counted.

For wound-healing assay, Seed DU145 cells were placed in a 6-well plate at a density of 2X105 cells/well in 2ml DMEM without FBS, and, 24 hours later, a scratch was made with a pipette tip of 1ml. The cells cultured under normal conditions with mitomycin for 24, 30, 48 hours. The plates were washed with phosphate buffered saline (PBS) twice. The cell area from the wound edge was counted.

For IHC, tissue microarrays (TMAs) were arranged (Xian Alenabio Biotech Co, Ltd; Cat No: PR807c). TMA was used to measure DCUN1D1 protein expressions in the tissues. The staining was performed on section using UltraSensitive Streptavidin-Peroxidase kit (MXB NO: KIT-9701/9702/9703, China). Endogenous peroxidase was blocked for 10 minutes at room temperature. The sections were incubated overnight at 4℃ with antibody anti-DCUN1D1 (5ug/ml, No: ab99506, Abcam, USA). After a thorough wash, peroxidise-labelled polymer and substrate chromogen were employed to visualise the staining of the interested protein. The sections were scored by two independent experienced pathologists. The percentage was grouped as: 0 = 0%, 1 = 1–10%, 2 = 11–50% and 3 + >50%). The staining intensity was categorised a: 0 = negative), 1 = weak, 2 = moderate and 3 = strong.

For western blot analysis, the cells were lysed in a lysis buffer and 1% phenylmethanesulfonyl fluoride (PMSF). The total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Whole-cell lysates prepared from these cultures were subjected to western blotting to detect DCUN1D1 (No: ab181233, Abcam, USA); E-Cadherin (No: ab15148, Abcam, USA); and Vimentin (No:BM0135, BOSTER, Wuhan, China). The beta-actin (No: BM5422, BOSTER, Wuhan, China) was used as internal reference. Membranes were probed with antibodies overnight at 4℃. Removing the antibodies and washing with tris buffered saline-tween (TBST) for 10mins three times. It was Incubated with second antibody (Goat Anti-Mouse ImmunoglobulinG (H+L) (IgGH+L) secondary antibody (1:3000, BA1038 dilution), Goat Anti-Rabbit IgG(H+L) secondary antibody (1:3000 dilution, BA1039) for 1 hour. Washing was done three times again. Immunoblotting was performed at three times for each antibody.

Data was analysed using SPSS 24 and GraphPad Prism 7. The relationship between the expression of DCUN1D1 protein and clinical information was derived from the Cancer Genome Atlas (TCGA). Data was presented as mean±standard error of the mean (SEM) and was compared using t test or one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Of the 80 samples, 3(3.75%) were normal prostate tissues, 7(8.75%) adjacent normal prostate tissues, 20(25%) hyperplasia, and 50(62.5%) were PCa tissues. IHC indicated that the expression of DCUN1D1 in PCa tissues was significantly higher than than in para-cancer tissues (p=0.015) (Figure 1).

Expression of DCUN1D1 was significantly higher in those with Gleason score >8 than than in those with Gleason score <8 (p<0.001). Similar results were seen for pathological stage ≥T3A (p<0.001) or having metastasis (p=0.022) (Table). However, messenger RNA (mRNA) level of DCUN1D1 was not obviously correlated with age,

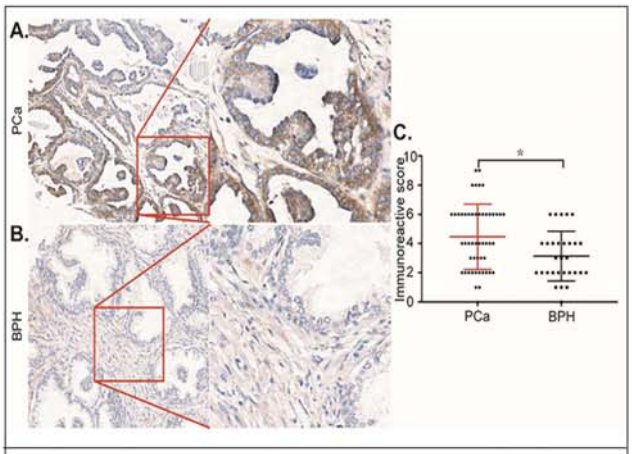

Figure-1: Overexpression DCUN1D1 in prostate cancer tissue. HE staining of 7 pairs of frozen human PCa tissue(A) & benign tissue(B). DCUN1D1 expression is significantly enhanced in both human PCa cells & tissues(C).

Table: Association of DCUN1D1 Expression with clinical pathological features of prostate cancer (TCGA dataset)[20]

Clinical features (numbers)		DUNC1D1	
	Case no.	$\bar{x} \pm s$	p-value
Age(years)			
< 66	354	444.7 ±183.0	0.142
≥ 66	143	473.4±226.8	
Gleason score			
< 8	291	427.6±186.4	< 0.001
≥ 8	206	488.8±206.0	
Pathological stage			
< T3A	186	415.3±180.3	< 0.001
≥ T3A	304	475.7±204.0	
Metastasis			
No	416	443.6±196.1	0.022
Yes	82	487.9±196.0	
PSA failure			
Negative	370	443.6±192.4	0.067
Positive	59	493.2±195.5	
Overall survival			
Negative	187	453.1±198.0	0.862
Positive	10	442.2±140.6	

prostate-specific antigen (PSA) failure and overall survival (OS) (p>0.05). There were significant differences in recurrence-free survival (RFS) between high and low DCUN1D1 expression groups (p=0.0108), showing DCUN1D1 expression as an independent prognostic factor for PCa (Figure 2).

Protein expression of DCUN1D1 in DU145 cell line with over-expression of DCUN1D1 was significantly higher than that in the blank virus vector (NC) group, while there was a reduced trend regarding the expression of DCUN1D1 in DU145 cell line carrying lentivirus vector with DCUN1D1 expression inhibited (Figure 3). Compared with

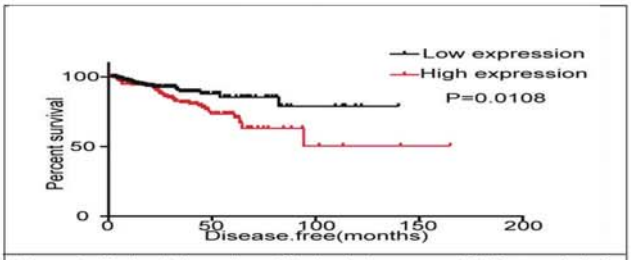

Figure-2: Kaplan-Meier analyses of biochemical recurrence (BCR)-free survival of prostate cancer (PCa) patients based on DCUN1D1 expression in Taylor dataset. The data revealed that there were significant differences in recurrence-free survival between high and low DCUN1D1 expression group(p=0.0108), which showed DCUN1D1 expression were independent prognostic factor for Pca.

Figure-3: DU145 cell line overexpressing, inhibiting DCUN1D1 expression and blank virus vector group (NC group). The results showed that protein expression of DCUN1D1 in DU145 cell line with overexpression of DCUN1D1 was significantly higher than that in NC group, while there was a reduced trend regarding the expression of DCUN1D1 in DU145 cell line carrying lentivirus vector with DCUN1D1 expression inhibited.

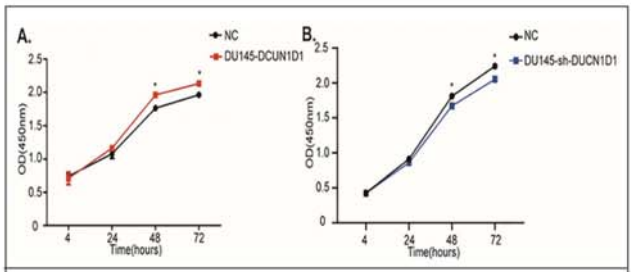

Figure-4: Cell proliferation experiment (CCK8 assay) The results indicated that the proliferation ability of DU145 with overexpressed DCUN1D1 was significantly enhanced (Fig.4A) , which was obviously weakened in DU145 with inhibited expression of DCUN1D1 (Fig.4B) .

corresponding NC group, cell proliferation experiment indicated that the proliferation ability of DU145 with over-expressed DCUN1D1 was significantly enhanced (Figure 4A), which was obviously weakened in DU145 with inhibited expression of DCUN1D1 (Figure 4B). The invasiveness of prostate cancer cell line with DCUN1D1 over-expressed was significantly enhanced (Figure 5A) and was remarkably attenuated in those with the expression of

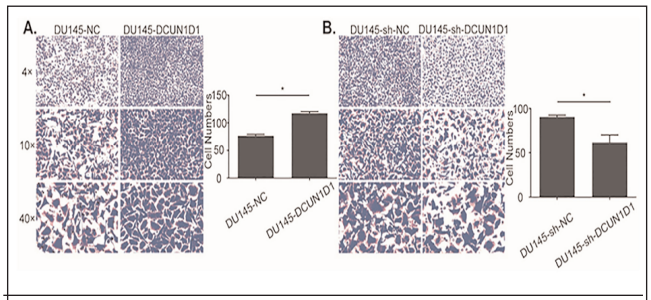

Figure-5: Cell invasion experiment(Transwell assay) .The results showed that the invasiveness of prostate cancer cell line with DCUN1D1 overexpressed was significantly enhanced (Fig.5A) and was remarkably attenuated in those with the expression of DCUN1D1 inhibited (Fig.5B) , which indicated that DCUN1D1 can promote prostate cancer cell invasion.

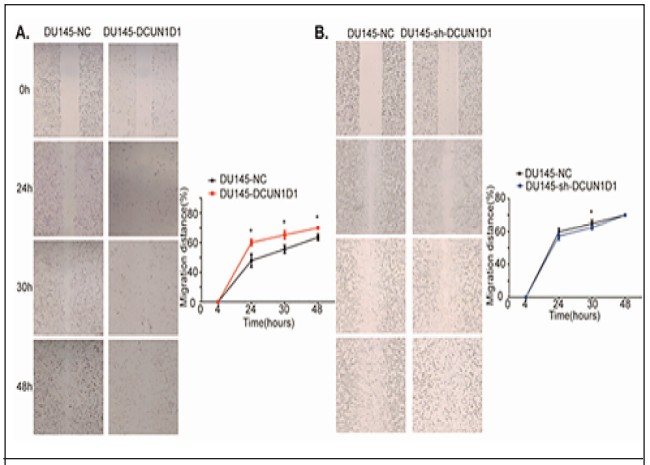

Figure-6: Wound-heal assay. The results showed that the migration ability was enhanced in DU145 cells with overexpression of DCUN1D1(Fig.6A) and decreased in DU145 cells with inhibited expression of DCUN1D1(Fig.6B).

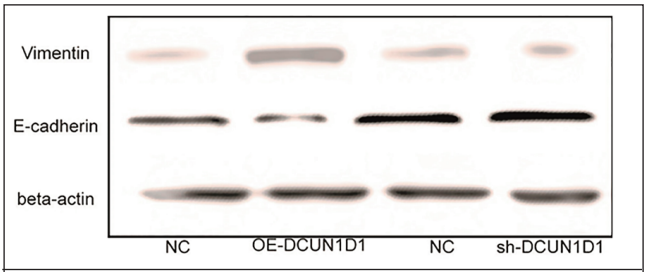

Figure-7: DCUN1D1 regulate E-cadherin、 Vimentin by Western blotting. The results showed that suppression of DCUN1D1 expression cause diminished levels of protein E-cadherin but higher protein Vimentin level .

DCUN1D1 inhibited (Figure 5B), which indicated that DCUN1D1 can promote prostate cancer cell invasion. The migration ability was enhanced in DU145 cells with overexpression of DCUN1D1 (Figure 6A) and decreased in DU145 cells with inhibited expression of DCUN1D1 (Figure 6B).

Suppression of DCUN1D1 expression caused diminished levels of protein E-cadherin but higher protein vimentin level (Figure7).

Discussion

PCa is a common malignant tumour in elderly men, which is the first leading cause of morbidity and the second leading cause of mortality among male malignant tumours in the United States.[11] The incidence of PCa is on the rise with the aging of population. The progression of the primary focus of PCa is relatively slow, while advanced patients lose their chance of operation owing to the metastasis to distant organs. Tumour metastasis is the main cause of death in patients with PCa. In this process, the transformation of cancer cells from androgen-dependent PCa (ADPC) to castration-resistant PCa (CRPC) results in the decrease of therapeutic effect and eventually death of patients1.19.

The incidence of human cancers has been confirmed to be related to the amplification of the genome 1-3 region. Amplification of the q3 region of the genome has been found in quite a variety of tumours. However, the most prominent one is squamous cell carcinoma (SCC) of the mucosal system, which is mainly manifested clinically as the spread of tumour in lung, neck, oesophagus, cervix, etc.[12]

Sarkaria et al. identified that DCUN1D1 (184.1Mb) was one of the potential targets for 3q region amplification during the onset of mucosal SCC in the region of 3q26.3, and demonstrated that SCC-related oncogene exhibited the ability of malignant transformation in vivo and in vitro.[12] DCUN1D1 is a new oncogene located on chromosome 3q26.3, which can amplify along this region in human SCC, causing cell deterioration. The amplification and over-expression of DCUN1D1 gene can lead to poor prognosis of cancer. A study has shown that DCUN1D1 can only proliferate in cancer cells, which can induce tumourigenesis and maintain such malignant lesions, showing an essential role in the occurrence of SCC of mucosal system. The current affirmed that high expression of DCUN1D1 in PCa was positively correlated with pathological stage, Gleason score and metastasis by TCGA dataset. Survival analysis by Kaplan-Meier method demonstrated that average survival time with high expression was shorter than that with low expression. Simultaneously, DCUN1D1 was also reported to be involved in the Hedgehog (Hh) pathway, which could regulate the level of glioma-associated Oncogene 1(Gli1) in the Hh signalling pathway. Gli1 is an important regulator of the Hh pathway and is involved in the malignant transformation of SCC. Current reports have confirmed that DCUN1D1 is a ubiquitin-related protein, which acts as a key

factor in the positive regulation of Cullin protein Neddyliaton in vivo, leading to the activation of Skpl-Cullins-F(SCF) ubiquitin E3 ligase complex, which increases the effect of ubiquitination, leading to a series of diseases, including cancer.[13-15]

In recent decades, EMT has attracted much attention as a new concept to explain the proliferation and metastasis of PCa cells. During the development of tumours, local tumour cells interact with their microenvironment, causing changes in the polarity of cells following EMT, resulting in enhanced mobility, and eventually metastasis to distant organs, such as skeleton and brain. Adhesion molecule E-cadherin/β-catenin complex plays an important role in maintaining epithelial cell adhesion, inhibiting tumour invasion and metastasis, and regulating the level of β-catenin in cytoplasm. Meanwhile, abnormal expression of β-catenin in the nucleus can cause abnormal activation of Wingless/Integrated (Wnt) signalling pathway and expression of a series of downstream target genes. The activation of these target genes is intimately correlated with EMT during the progression of tumour metastasis.[16] Tumour cells gradually "evolve" into stromal-like cells with strong motility ability through EMT process, and then exfoliate from in situ tumours, invade surrounding tissue spaces and vascular system, thereby leading to distant metastasis of tumours.[17] EMT phenomena have common cytological mechanisms, such as the down-regulation of epithelial proteins, mainly E-cadherin and Keratin-8, and up-regulation of interstitial proteins, such as vimentin and fibronectin, all of which are commonly used as markers to detect the occurrence of EMT.[18] The current study found that suppression of DCUN1D1 expression caused diminished levels of vimentin protein, but higher protein E-cadherin level. The intracellular domain of β-catenin and E-cadherin is connected to form the E-cadherin/β-catenin complex, which is crucial in maintaining the adhesion between epithelial cells and regulating the level of β-catenin in the cytoplasm. Abnormal cell membrane distribution of E-cadherin and vimentin in PCa tissues is correlated with Gleason's score.

The current study confirmed that over-expressing DCUN1D1 could down-regulate protein level of E-cadherin and up-regulate protein level of vimentin, which might be one of the mechanisms of DCUN1D1 promoting cancer cell migration and invasion.

Conclusions

There was promising evidence that DCUN1D1 may function as an oncogene in human PCa, which was highly expressed in DU145 and cancerous tissues. This higher expression was correlated with prognosis as well, and its mechanism might be EMT due to the abnormal expression and distribution of E-cadherin and vimentin in PCa cells which may provide a new idea for PCa treatment.

Disclaimer:

(i) The current study is a small part of a larger project of China's National Natural Science Foundation (No. 81641102) on which animal experimentation has been approved by the experimental animal ethics committee of Guangzhou Medical University. The TRIB1 Project (Research on the mechanism of TRIB1 activating IL8R to induce macrophage differentiation and to promote immune escape of prostate cancer NO.81641102) have the Result of the review. We have offered the Committee review. The current study has only analysed gene chips, is not involved with patients and animals. A second approval was thus not considered necessary.

(ii) 1st and 5th author Shun Tan Huang and Wei De Zhong were affiliated with Guangzhou Medical University, Guangzhou, China, at the time of study.

Conflict of interest: None.

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