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Original article

DUSP1 is involved in the progression of small cell carcinoma of the prostate

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ABSTRACT

Small cell carcinoma of the prostate (SCCP) is a rare and the most aggressive variant of prostate cancer. There is no effective cure or treatment for SCCP. Therefore, there is an urgent need for new therapy to improve the prognosis of patients with SCCP. DUSP1 is a dual specific phosphatase with an increasingly recognized in tumor biology. Altered expression of DUSP1 induced changes in the expression of genes involved in various biological pathways, including cell-cell signaling and angiogenesis. To understand more about the role of DUSP1 in SCCP, we evaluated the biological function and associated regulatory mechanism of DUSP1. In this study, DUSP1 was significantly down-regulated in human SCCP compared with the non-carcinoma tissues ($P < 0.05$). Overexpression of DUSP1 was found to suppress MAPK signaling and cell proliferation in PC-3 cells. Additionally, silencing of DUSP1 enhanced MAPK signaling and PC-3 cell proliferation. Moreover, it was observed that DUSP1 blocked the phosphorylation of p38 MAPK induced by anisomycin. Taken together, this investigation suggests that DUSP1 is involved in the progression of SCCP and may provide a new therapeutic target for SCCP treatment.

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1. Introduction

Small cell carcinoma of the prostate (SCCP) is an uncommon type of prostate cancer and a high grade malignant neoplasm with neuroendocrine differentiation (Furtado et al., 2011). The morphologic features of small cell including small tumor cells with minimal cytoplasm, nuclear molding, fine chromatin pattern and so on. As other cancers, aberrant gene regulation features significantly in the SCCP.

Dual specificity phosphatase (DUSP) family belongs to phosphatase that can act on tyrosine or serine/tyrosine residues. DUSP2 is able to inactivate Erk2 (Zhang et al., 2005). DUSP1 is a mitogen and stress-inducible dual specificity protein phosphatase. It has been reported that DUSP1/MKP1 can regulate MAPKs activity with an

important role in tumor biology (Moncho-Amor et al., 2011). In addition, DUSP1 level has been found to be decreased in lung squamous carcinoma with cancer progression (Wang et al., 2011). However, the role of DUSP1 is still incompletely understood in the SCCP.

Mitogen-activated protein kinase (MAPK) signaling pathway is a well-known signaling that is evolutionarily conserved kinase modules. It links extracellular signals to the machinery that controls fundamental cellular processes including growth, proliferation, apoptosis, and so on (Dhillon et al., 2007). The mammalian family of MAPKs includes extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38. The core components identified for each of the three major MAPK pathways consist of a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK (Kim and Choi, 2010). P38 MAPK signaling pathway functions in a cell context-specific and cell type specific manner to affect cell proliferation, migration and survival (Wagner and Nebreda, 2009). Dysregulation of p38 MAPK signaling is associated with the development, progression and short survival of different cancer types including breast, bladder, liver, and so on (Dhillon et al., 2007; Koul et al., 2013). However, it is still unclear whether p38 MAPK signaling plays important roles in the SCCP. In this study, we found DUSP1 level was reduced in human prostatic tumors. In addition, DUSP1 overexpression was found to repress

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MAPK signaling and cell proliferation in PC-3 cells. Loss of DUSP1 could reverse the effect of DUSP1 overexpression on PC-3 cells. Moreover, we demonstrated that the phosphorylation of p38 MAPK could be blocked by DUSP1. Our studies investigated the role of DUSP1 in the SCCP, and DUSP1 may be an unexplored target of a novel therapy for the SCCP.

2. Materials and methods

2.1. Cell culture and Materials

Recently, PC-3 cell line has shown specific association of those cases of the SCCP based on the molecular characterization (Furtado et al., 2011). In addition, the PC-3 cell line is also considered to be one of the classical human prostate cancer cell lines for study human prostate cancer (Alimirah et al., 2006). ATCC human prostate cancer cell line PC-3 was cultured as suggested. Briefly, PC-3 cells were maintained in ATCC formulated F12K Medium containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich, St Louis, MO) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

2.2. Quantitative real-time PCR (qRT-PCR)

Total RNA of human tissues or cells were isolated by Trizol reagent (Invitrogen, Carlsbad, CA) as the manufacturer's suggestion. After removal of DNA by DNA-free DNase (Ambion, Austin, TX), residual RNAs were reverse transcribed into cDNA with M-MLV reverse transcriptase (BRL, Gaithersburg, MD). The Taqman qRT-PCR was used for DUSP1 gene expression analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for comparative analyses of gene expression. All specific taqman probes were purchased from Applied Biosystems, (Foster City, CA). All Taqman qRT-PCR studies were performed in triplicate on an ABI 7500 system (Applied Biosystems) and the data were presented as mean ±SE. The amount of each miRNA relative to GAPDH was calculated as previously described (Yin et al., 2015).

2.3. DUSP1 overexpression and knockdown

PC-3 cells were engineered to overexpress DUSP1 by lentiviral system. The lentiviral control vector (Con-OE) contains a non-relevant sequence as a negative control. All the lentiviruses were purchased from Applied Biological Materials Inc (Richmond, BC). The overexpression was performed according to the manufacturer's instructions. For the DUSP1 silencing lentiviral system, short hairpin RNA (shRNA) of DUSP1 lentiviral vector and corresponded lentiviral control vector (Con-KD) as a negative control were purchased System Biosciences (Mountain View, CA). Transfections shRNA lentiviral particles were generated as previously describe (Zhang et al., 2015).

2.4. Western blotting

The 4–20% gradient SDS-polyacrylamide gel (Invitrogen) was used to separate the protein lysis from PC-3 cells. After then, the gel was electrophoretically transferred onto a PVDF membrane (GE) and the membranes were incubated with anti-DUSP1 (Abcam, Cambridge, MA), anti-pp38 (New England Biolabs, Hitchin, Hertfordshire, United Kingdom), anti-p38 (New England Biolabs) primary antibody or GAPDH (Abcam, Cambridge, MA) overnight. After washing with TBST for 5 min, the Horseradish peroxidase (HRP)-conjugated secondary Abs (Bio-Rad, Hercules, CA) were

added the next day. The proteins were visualized by ECL chemiluminescence and exposed to the X-ray film. All the studies were performed in triplicate. Anisomycin (Sigma) was used to induce the p38 MAPK signaling at the indicated concentration.

2.5. Cell proliferation assay

Of 5000 cells were seeded in triplicate in each well of the 96-well tissue culture plates. At indicated time points, a CyQuant assay (Thermo Fisher Scientific, Boston, MA) was performed as the suggestion of manufacture to monitor the growth of the cells. Briefly, the CyQuant solution was prepared immediately before use. 100ul of CyQuant solution was added to the wells after removal of media and incubated in the dark for 45min at room temperature. The plate was read at excitation at 497nm and emission at 520 nm. All studies were performed in 3 independent cell preparation and the data was presented as mean ± SE.

2.6. Dual-luciferase assay

The luciferase reporter assay was performed to detect the MAPK signaling pathway activity as previous description (Zhang et al., 2015). Briefly, cells were seeded in 96-well plate at a density of 2×10^4 cells per well. PC-3 cells overexpressed with DUSP1 or Con-OE transfected with MAPK pathway-luciferase-reporter construct (SABiosciences, Frederick, MD) with lipofectamine 2000. After culture 48h, the cells were harvested for the Dual Luciferase Reporter Assay (Promega). MAPK signaling pathway was also detected in PC-3 cells silenced with DUSP1 or Con-KD by the dual-luciferase reporter assay. Sh-Con was used as a negative vector control.

2.7. Statistics

GRAPHPAD PRISM (version 6.07 for windows, Graphpad Software, La Jolla, CA) was used for statistical analyses. Data are expressed as mean ± SE (n=3 independent cell preparation). Unpaired student t-test was performed to ascertain statistical significance between the two groups. P (probability) less than 0.05 was concerned as significant difference.

3. Results

3.1. DUSP1 mRNA decreased in the tumor tissues

Previous studies showed that DUSP1 was down-regulated in many types of human cancers (Persson et al., 2011; Newie et al., 2014; Schmitt et al., 2015), suggesting a possible significance of DUSP1 in cancer development and/or progression. In this present study, we first examined DUSP1 mRNA level in prostatic tumors and normal tissues. 18 prostatic tumors and 14 noncancerous normal tissues were used to determine DUSP1 expression by qRT-PCR assay. All samples were collected in accordance the ethical guidelines of the First Hospital of Shijiazhuang. GAPDH was used as the endogenous control. We found that DUSP1 mRNA levels in human prostatic tumors were significantly down-regulated compared to the non-cancerous normal tissues ($P < 0.05$) (Fig. 1). It was indicated that down-regulation of DUSP1 may be involved in the progression of SCCP.

3.2. DUSP1 regulates human prostate cancer cell proliferation

We next determined the effects of DUSP1 on human prostate cancer cell line in vitro. We first determined DUSP1 expression levels after infecting PC-3 cells with MOI = 10 of DUSP1 lentivirus

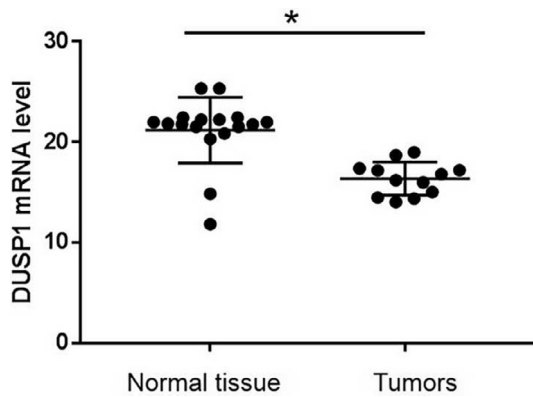


Fig. 1. DUSP1 mRNA level in the prostatic tumors and non-cancerous tissues. * $p < 0.05$ vs Normal tissue.

or Con-OE lentivirus. As shown in Fig. 2, the DUSP1 mRNA and protein expression levels were significantly enhanced after infection compared with Con-OE and wide type (WT) cell groups. The cell proliferation was monitored by CyQuant assay. We observed that DUSP1 overexpression significantly suppressed PC-3 cell proliferation (Fig. 3). To further confirm DUSP1 effect on PC-3 cell proliferation, we next silenced DUSP1 by DUSP1 shRNA lentivirus. As shown in Fig. 4, DUSP1 shRNA significantly reduced the expression of DUSP1 protein expression in PC-3 cells. In addition, DUSP1 shRNA reversed the effects on PC-3 cell proliferation caused by DUSP1 overexpression (Fig. 5).

3.3. DUSP1 regulates MAPK signaling pathway in vitro

We further determined the effect of DUSP1 on MAPK signaling pathway since DUSP1 is one of the important regulators of the cascades of MAPK signaling pathway. Using a simple MAPK dual-luciferase reporter assay, we investigated that DUSP1 overexpression reduced the activity of MAPK signaling compared with Con-OE (Fig. 6A). DUSP1 silencing reversed the effect of DUSP1 overexpression on MAPK signaling activity in the PC-3 cells (Fig. 6B). The luciferase data suggested that DUSP1 regulated the activity of MAPK signaling pathway in the PC-3 cells.

3.4. DUSP1 regulated the phosphorylation of p38

p38 MAPK phosphorylation was found to be regulated by DUSP1 in airway smooth muscle (ASM) cells (Manetsch et al., 2012; Prabhala and Ammit, 2015). DUSP1 has also been reported to inactivate p38 MAPK in the innate immunity (Arthur and Ley,

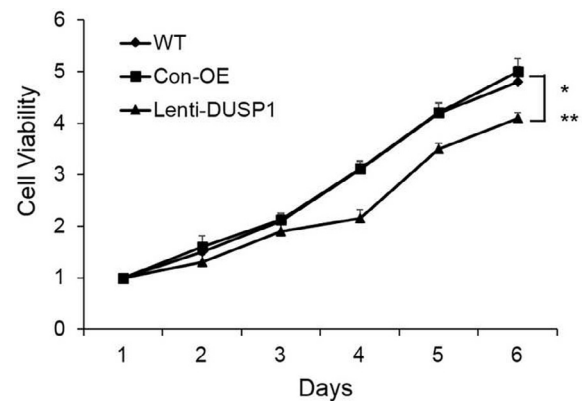


Fig. 3. Effect of DUSP1 overexpression on PC-3 cell growth. PC-3 cell growth was significantly decreased from day 3 after DUSP1 overexpression. Data was mean \pm SE (n = 3). * $P < 0.05$ vs WT; ** $P < 0.05$ vs Con-OE.

2013). Our next step was to determine whether DUSP1 had a similar function on p38 MAPK in the PC-3 cells. To address this, we compared the impact of DUSP1 overexpression on p38 MAPK phosphorylation in PC-3 cells treated with anisomycin, which was the specific p38 MAPK activator. As expected, anisomycin strongly activated p38, but DUSP1 overexpression repressed p38 MAPK phosphorylation induced by anisomycin (Fig. 7).

4. Discussion

SCCP is accounting for less than 1% of all prostate cancers. A wide variety of human tumors contain abnormal expression of DUSP1 and abnormal MAPK signaling associated with various processes such as cell proliferation, differentiation and metabolism. Down-regulation of DUSP1 has been reported in cancer. Currently, DUSP1 has become an attractive regulator gene. However, the roles of DUSP1 in the progression of human SSCP is still largely unknown. In this study, we observed that DUSP1 mRNA was down-regulated in human prostatic tumors compared with normal tissues. It has been reported that DUSP1 was silencing in primary oral squamous cell carcinoma (Khor et al., 2013). And, MAPK signaling depended on increased DUSP1 expression (Kao et al., 2013). Additionally, DUSP1 expression was found to inversely correlate with NF- κ B activity and expression in prostate cancer and promotes apoptosis through a p38 MAPK dependent mechanism (Gil-Araujo et al., 2014). In this present study, we also found that DUSP1 overexpression suppressed MAPK signaling. In addition, DUSP1 overexpression remarkably suppressed PC-3 cell proliferation. DUSP1 silencing reversed the effects of DUSP1 overexpression

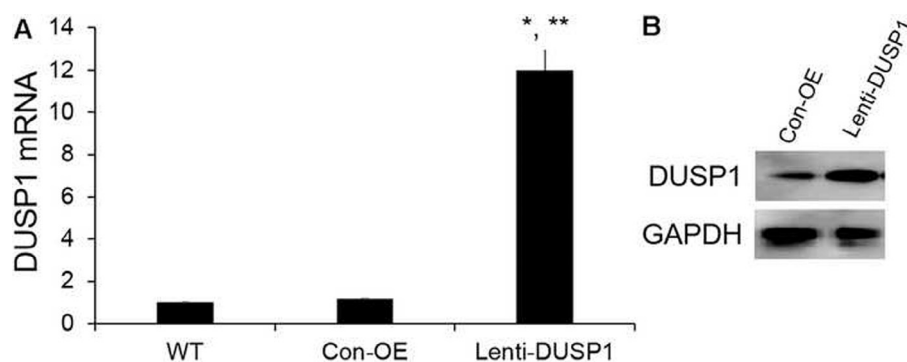


Fig. 2. DUSP1 expression was increased after PC-3 cells overexpressed with DUSP1 by lentiviral system. (A) DUSP1 mRNA expression level was determined by qRT-PCR; (B) DUSP1 protein expression were determined by WB. WT: PC-3 cells without infection. GAPDH was used as an internal control. Results were mean \pm SE (n = 3). * $P < 0.05$ vs WT; ** $P < 0.05$ vs Con-OE.

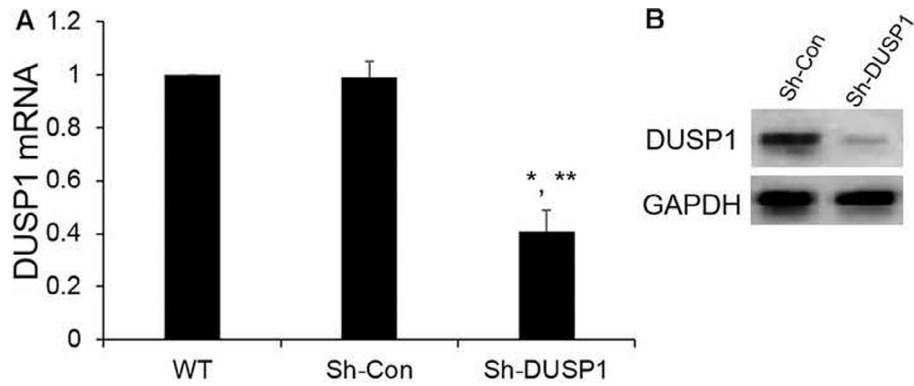


Fig. 4. DUSP1 expression were decreased after PC-3 cells infected DUSP1 silencing shRNA by lentiviral system. (A) DUSP1 mRNA expression level was determined by qRT-PCR; (B) DUSP1 protein expression was determined by WB. WT: PC-3 cells without infection. GAPDH was used as an internal control. Results were mean \pm SE (n = 3). *P < 0.05 vs WT; **P < 0.05 vs Sh-Con.

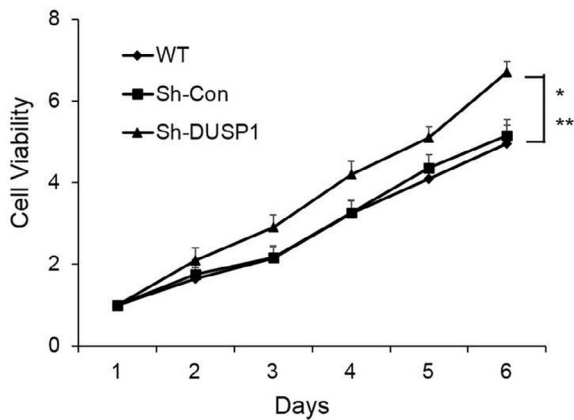


Fig. 5. Effect of DUSP1 silencing on PC-3 cell growth. PC-3 cell growth was significantly increased from day 2 after DUSP1 silencing. Data was mean \pm SE (n = 3). *P < 0.05 vs WT; **P < 0.05 vs Sh-Con.

on PC-3 cells. DUSP1 was also found to regulate the phosphorylation of p38 MAPK in the PC-3 cells. These results suggested that DUSP1 was involved in the progression of the SCCP through p38 MAPK signaling pathway. Our data was derived from a small number of patients. The present study generates hypotheses and further validation in larger datasets is necessary.

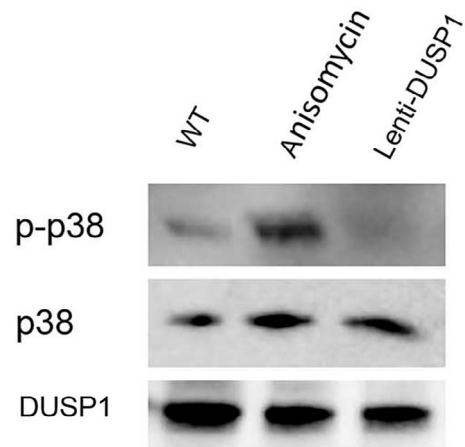


Fig. 7. Effects of anisomycin and DUSP1 overexpression on the level of p-p38 and p38. Western blot was used to analyze p-p38 and p38 proteins in the PC-3 cells. GAPDH was used as the internal control. The data shown was from one representative experiment of 3 independent experiments.

Taken together, DUSP1 expression reduced in prostatic tumors and DUSP1 can regulate PC-3 cell proliferation through p38 MAPK signaling pathway. It indicates that appropriate regulation of DUSP1 has the potential to be used for the therapy of SCCP.

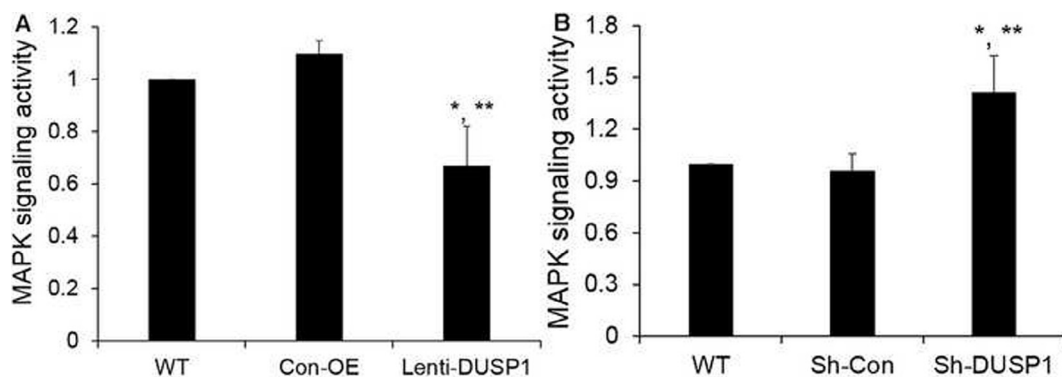


Fig. 6. Effect of DUSP1 on MAPK signaling pathway. (A) PC-3 cells overexpressing DUSP1 were transfected with a MAPK luciferase-reporter construct. (B) PC-3 cells with DUSP1 knockdown were transfected with MAPK luciferase-reporter construct. MAPK activity was normalized to Renilla luciferase activity. Results were mean \pm SE (n = 3). *P < 0.05 vs WT; **P < 0.05 vs Con-OE or Sh-Con.

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