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DEC1 and DEC2 Regulate Apoptosis of Human Prostate Adenocarcinoma LNCap Cells

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ABSTRACT

Basic helix-loop-helix (bHLH) transcription factors DEC1 (BHLHE40) and DEC2 (BHLHE41) have been reported to associate with the regulation of apoptosis, cell proliferation, circadian rhythms and malignancy in various cancers. Our previous study suggested that DEC2 inhibited paclitaxel-induced apoptosis in castration-resistant prostate cancer (CRPC) DU145 and PC-3 cells. In the present study, we investigated the roles of DEC1 and DEC2 in human castration-sensitive prostate cancer LNCap cells in response to paclitaxel. Paclitaxel increased the expression of DEC1/DEC2 in LNCap cells. We found that DEC1 siRNA decreased the amount of cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-8, whereas increased that of Bcl-xL. In addition, DEC1 overexpression increased the protein level of cleaved PARP and cleaved caspase-8, whereas decreased Bcl-xL expression. On the contrary, DEC2 siRNA upregulated the amount of cleaved PARP and cleaved caspase-8 but downregulated Bcl-xL regardless of paclitaxel. Corresponding results were obtained in DEC2-overexpressed LNCap cells. These data indicated that DEC1 and DEC2 have pro-apoptotic and anti-apoptotic effects in paclitaxel-induced apoptosis of LNCap cells, respectively.

Keywords: DEC1, DEC2, Paclitaxel, Apoptosis, Prostate cancer, LNCap cells

Abbreviations: DEC1: Differentiated Embryonic Chondrocyte Gene 1; DEC2: Differentiated Embryonic Chondrocyte Gene 2; ADT: Androgen Deprivation Therapy; PARP: poly (ADP-ribose) Polymerase; CRPC: Castration-resistant Prostate Cancer; CSPC: Castration-sensitive Prostate Cancer

INTRODUCTION

Prostatic cancer remains the most common cancer and the second most leading cause of cancer deaths in industrial countries [1]. Androgen deprivation therapy (ADT) beneficially effects on the control of androgen-dependent tumors, however, after a short-term remission, surviving cancer cells often re-growth with increased malignancy [2]. Taxane-based chemotherapy is a recommended therapeutic approach to treat patients recurring from ADT. Paclitaxel, a natural diterpenoid isolated from the stem bark of Taxus, has become a research focus for decades because of its complex structure, unique therapeutic mechanism and excellent anticancer activities [3]. By promoting tubulin assembly and stabilizing microtubules, paclitaxel can inhibit mitosis and finally leads to apoptosis of tumor cells [4]. Paclitaxel has significant effect in a variety of cancers, including several refractory tumors such as ovarian carcinoma, acute myeloid leukemia, castration-resistant prostate cancer (CRPC) [5-7]. Molecular mechanisms of paclitaxel in anti-cancer therapy involve the activation of c-Jun N-terminal kinase (JNK), downregulation of Bcl-2/Bcl-xL, activation of caspases and poly (ADP-ribose) polymerase (PARP) [8-11], and leading to cell growth arrest at the G2/M phase of the cell cycle [12].

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Differentiated embryonic chondrocyte gene (DEC) 1 and DEC2 belong to basic helix-loop-helix (bHLH) transcription factor family, and function as regulators of cell proliferation, circadian rhythm, cancer progression, as well as targets of hypoxia [13-16]. We previously reported that DEC1 and DEC2 differentially regulated apoptosis, *i.e.*, DEC1 promoted, while DEC2 inhibited apoptosis of human breast cancer MCF-7 cells [17]. We also found that DEC2 negatively regulated paclitaxel-induced apoptosis in CRPC cells DU145 and PC-3 [18]. However, the roles of DEC1/DEC2 in apoptosis of CSPC cells are not well known.

The objective of the current study was to investigate the roles of DEC1/DEC2 in paclitaxel-induced apoptosis of LNCap cells. Our results demonstrated that DEC1 and DEC2 have pro-apoptotic and anti-apoptotic effects in paclitaxel-treated LNCap cells, respectively.

MATERIALS AND METHODS

Cell culture: Human prostate cancer LNCap. FGC cells (RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere (95% air, 5% CO_2). Cells were treated with different concentrations of paclitaxel (Sigma-Aldrich, St. Louis, MO, USA) for the indicated period of time.

RNA interference for knockdown of DEC1/DEC2: Short interference RNA (siRNA) for silencing DEC1 or DEC2 was synthesized by Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequences of DEC1, DEC2, and the negative control siRNA have been described previously [19]. 5×10^4 cells were seeded per 35-mm well and transfected with siRNA against DEC1 or DEC2, or negative control siRNA using Lipofectamine RNA iMAX reagent (Invitrogen) according to the manufacturer's protocol. The cells were treated with paclitaxel for 24 h and collected for western blot analysis.

Overexpression of DEC1/DEC2: Human DEC1 or DEC2 expression plasmids (DEC1 pcDNA, DEC2 pcDNA) were kindly gifted from Dr. Katsumi Fujimoto (Hiroshima University, Japan) [14]. 5×10^4 cells were seeded per 35-mm well and cultured for at least 24 h. DEC1 or DEC2 expression plasmid was introduced into the cells with Lipofectamine LTX reagent (Invitrogen) for 18 h. The transfected cells were subsequently incubated with paclitaxel for 24 h and collected for western blot analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR): Triplicate RNA samples from LNCap cells were prepared for RT-qPCR. RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) and was used for reverse transcription according to the manufacturer's instructions. First cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). Quantitative PCR was performed using *Taq* PCR Master Mix Kit (Qiagen). The primer sequences and product sizes of DEC1, DEC2 and GAPDH were described previously [19]. The cDNAs for DEC1, DEC2 and GAPDH were amplified at 27 cycles, 27 cycles and 20 cycles, respectively. The PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels stained with ethidium bromide.

Western blotting: The cells were lysed using M-PER lysis buffer (Thermo Scientific, Waltham, MA, USA), and the bicinchoninic acid (BCA) assay was used for determining the protein concentrations. The lysates (10 μg protein) were subjected to SDS-PAGE, and the separated proteins were transferred to PVDF membranes (Immobilon P, Merck Millipore, Billerica, MA, USA). Western blot bands were detected by Bio-Rad systems (Bio-Rad, Hercules, CA, USA) with the ECL-prime or ECL-select detection systems (GE Healthcare, Wauwatosa, WI, USA).

Cell viability assay: Cells seeded in 96-well plate were cultured with indicated doses of paclitaxel for 24 h. In the case of combination treatment, cells were transfected with pcDNA or DEC2 pcDNA for 18 h on ahead, and then cultured in the medium containing 50 μM of paclitaxel for 24 h. The pcDNA-transfected cells without paclitaxel treatment were used as control. The cell viability was detected with MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay as described previously [20].

Statistical analysis: Each experiment was repeated in triplicate and data are presented as means ± standard deviation. The ordinary one-way ANOVA analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered as statistical significance.

RESULTS

DEC1/DEC2 expression in LNCap cells treated with paclitaxel

Firstly, we analyzed the expression of apoptosis markers and DEC1/DEC2 of LNCap cells in response to paclitaxel. Paclitaxel induced the cleavage of PARP and caspase-8 at the concentration of 10 μM, and reached maximum at 100 μM. When referred to anti-apoptotic factor Bcl-xL, lower doses of paclitaxel failed to decrease Bcl-xL expression, whereas higher doses of paclitaxel (such as 50 μM and 100 μM) inhibited its expression. Additionally, no significant changes of caspase-9 and Bax were observed **(Figure 1A)**. The protein and mRNA levels of DEC1/DEC2 were upregulated in a concentration-dependent manner when treated with paclitaxel for 24 h (**Figure 1, B and C**).

DEC1 promoted apoptosis of LNCap cells treated with paclitaxel

In paclitaxel-untreated LNCap cells, knockdown or overexpression of DEC1 failed to induce the cleavage of PARP and caspase-8 **(Figure 2, A and B)**. However, in the presence of 50 μM of paclitaxel, DEC1

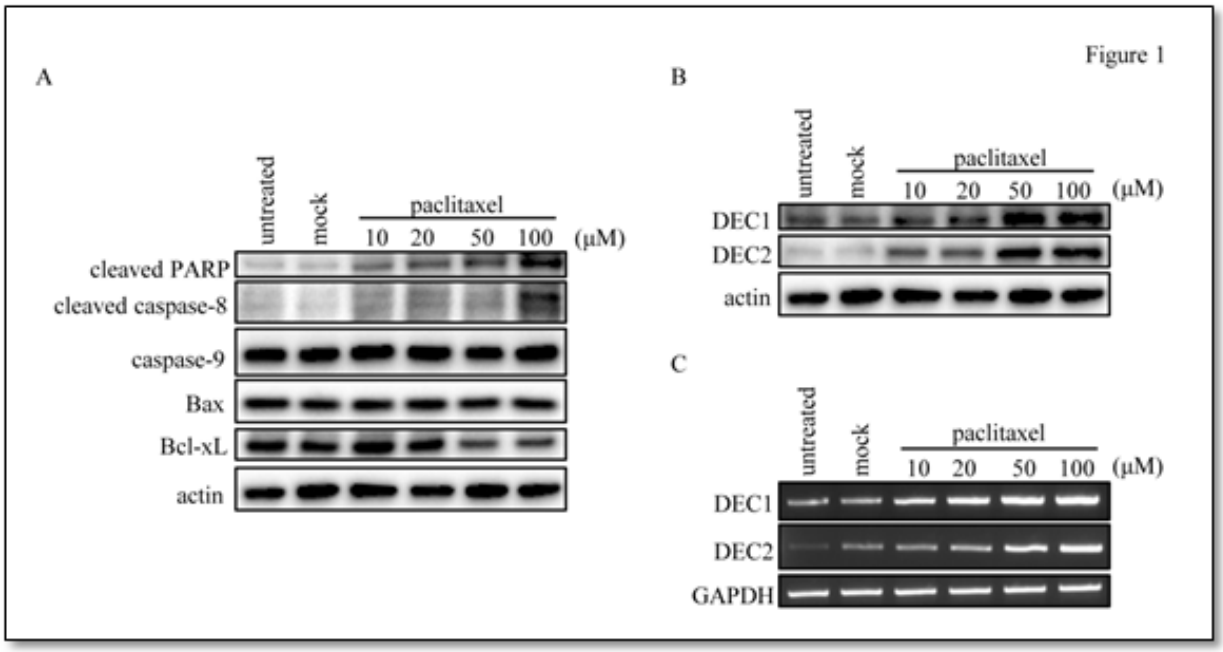

Figure 1. Apoptosis was induced and the expression of DEC1/DEC2 was upregulated by paclitaxel. LNCap cells were untreated or mock treatment (buffer alone) or 10, 20, 50 or 100 μM of paclitaxel for 24 h. Protein was prepared for western blotting analyses of cleaved PARP, cleaved caspase-8, caspase-9, Bax, Bcl-xL, and actin (A), as well as DEC1 and DEC2 (B). RNA was purified for RT-qPCR analyses of DEC1, DEC2 and GAPDH (C).

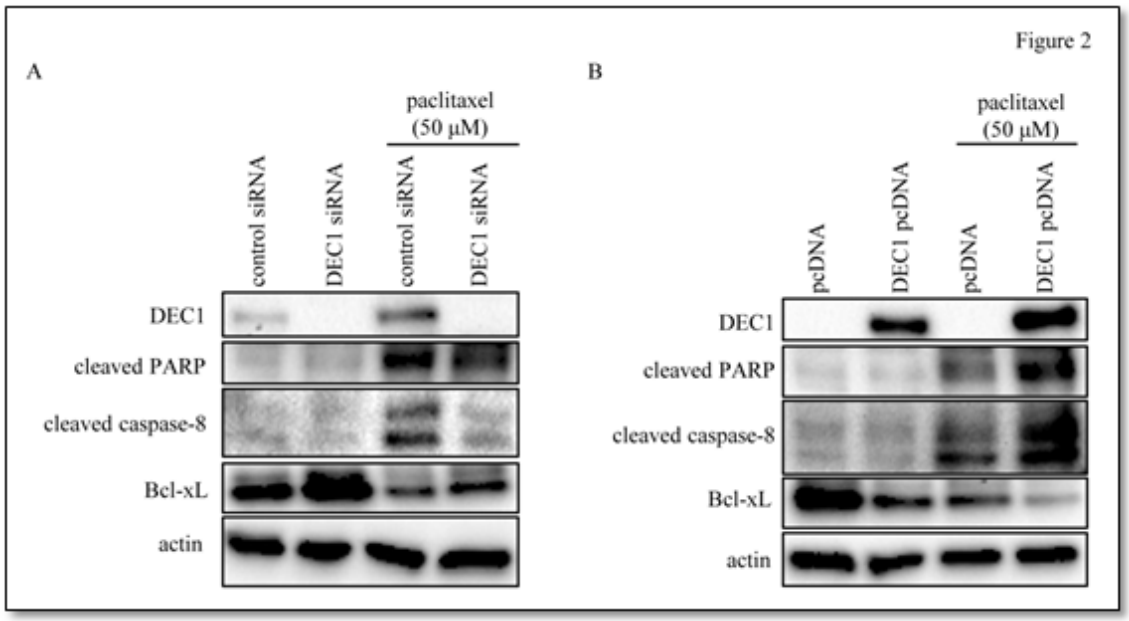

Figure 2. DEC1 positively regulated paclitaxel-induced apoptosis of LNCap cells. (A) Cells were transfected with DEC1 siRNA and incubated for 24 h, followed with or without paclitaxel (50 μM) for another 24 h. Cell lysates were prepared for western blotting analyses of DEC1, cleaved PARP, cleaved caspase-8, Bcl-xL and actin. (B) LNCap cells were transfected with DEC1 pcDNA for 18 h, followed by paclitaxel (50 μM) for another 24 h. Purpose proteins mentioned above were analyzed. Triplicate experiments were performed.

functioned as a positive regulator of apoptosis, that is, the amounts of cleaved PARP and cleaved caspase-8 were decreased by DEC1 siRNA but were increased by DEC1 pcDNA. On the other hand, DEC1 negatively correlated with the anti-apoptotic protein Bcl-xL regardless of paclitaxel treatment **(Figure 2, A and B)**.

DEC2 inhibited apoptosis no matter with paclitaxel in LNCap cells

To analyze the roles of DEC2 in apoptosis of LNCap cells, the siRNA or plasmid of DEC2 was transfected into LNCap cells and similar analyses with those mentioned above were carried out. The amounts of cleaved PARP and cleaved caspase-8 were slightly increased when knockdown of DEC2 only, but were significantly increased when combined with paclitaxel **(Figure 3A)**. Besides, the expression of Bcl-xL, which was decreased by DEC2 knockdown, was further inhibited under paclitaxel treatment. In corresponding with these results, we got opposite expression styles of apoptosis-related factors in DEC2 pcDNA-transfected LNCap cells **(Figure 3B)**.

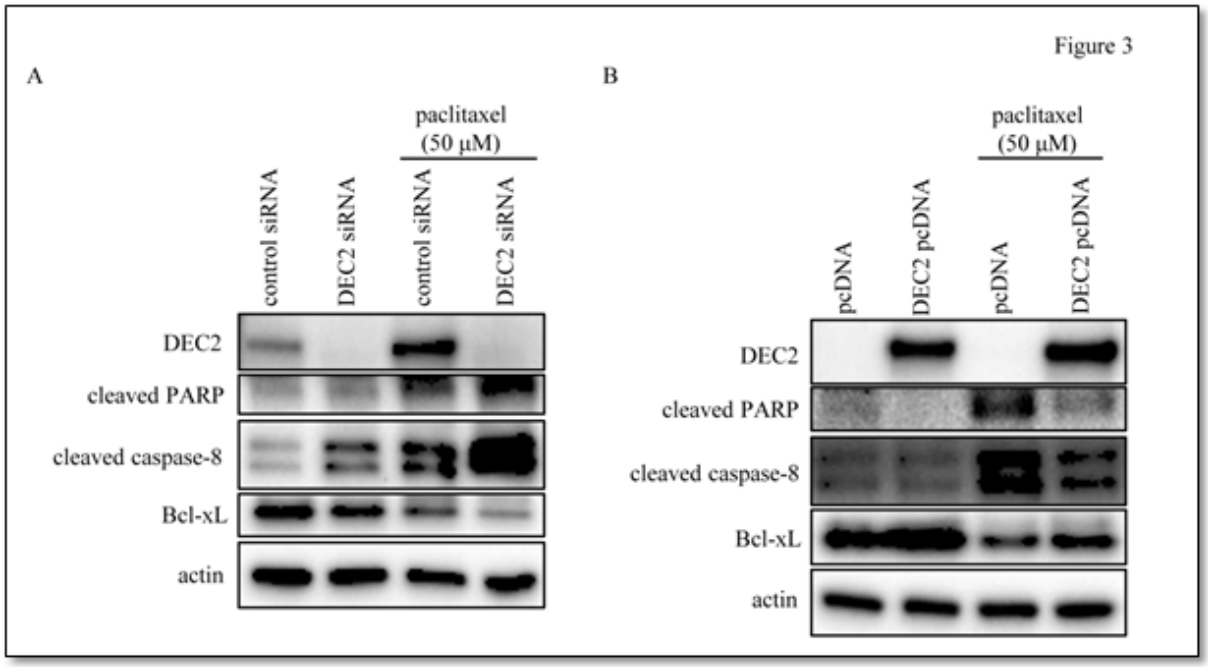

Figure 3. DEC2 negatively regulated paclitaxel-induced apoptosis of LNCap cells. DEC2 siRNA (A) or DEC2 pcDNA (B) were introduced into LNCap cells for 24 h, followed by culturing with paclitaxel (50 μM) for another 24 h. Western blot analyses of DEC2, cleaved PARP, cleaved caspase-8, Bcl-xL and actin were carried out. Triplicate experiments were performed.

DEC2 protected LNCap cells from paclitaxel-induced apoptosis

By using MTS assay, we found that paclitaxel decreased the number of viable cells (**Figure 4A**). Next, we examined whether DEC2 overexpression affected the cell viability in LNCap cells. DEC2 overexpression in the presence of paclitaxel (50 μM) markedly upregulated the cell viability compared with the paclitaxel-treated control. Moreover, DEC2 overexpression without paclitaxel treatment exhibited little effect on the cell viability **(Figure 4B)**.

DISCUSSION

We previously demonstrated the roles of DEC1/DEC2 in paclitaxel-induced apoptosis of human castration-resistant prostate cancer (CRPC) cell lines DU145 and PC-3. Our results exhibited that DEC1 had pro-apoptotic effects, while DEC2 had anti-apoptotic effects on paclitaxel-induced apoptosis in the two cell lines [18]. The present study focused on the DEC functions in human castration-sensitive prostate cancer (CSPC) LNCap cells. The CSPC cells were chosen since a majority of early-stage prostate cancer patients showed androgen dependent growth. Paclitaxel upregulated cleaved PARP at the concentration of 10 μM in LNCap cells, which was lower than those of DU145 or PC-3 cells [18]. Meanwhile, paclitaxel induced DEC1/DEC2 expression in a concentration-dependent manner. To further examine the roles of DEC genes in apoptosis, the siRNAs or the expression plasmids of DEC1 or DEC2 were transiently transfected into the LNCap cells. When LNCap cells were exposed to paclitaxel, DEC1 overexpression augmented paclitaxel-induced apoptosis. In addition, DEC1 negatively related to the anti-apoptotic molecular Bcl-xL independent of paclitaxel. However, the cleavage of caspase-8 and PARP

occurred only in the presence of paclitaxel. DEC1 was proposed to cause apoptosis by combining with other proteins and function as a helper or an enhancer in paclitaxel-induced apoptosis pathway. On the other hand, knockdown of DEC2 by siRNA remarkably caused the cleavage of caspase-8, and paclitaxel furthered this effect.

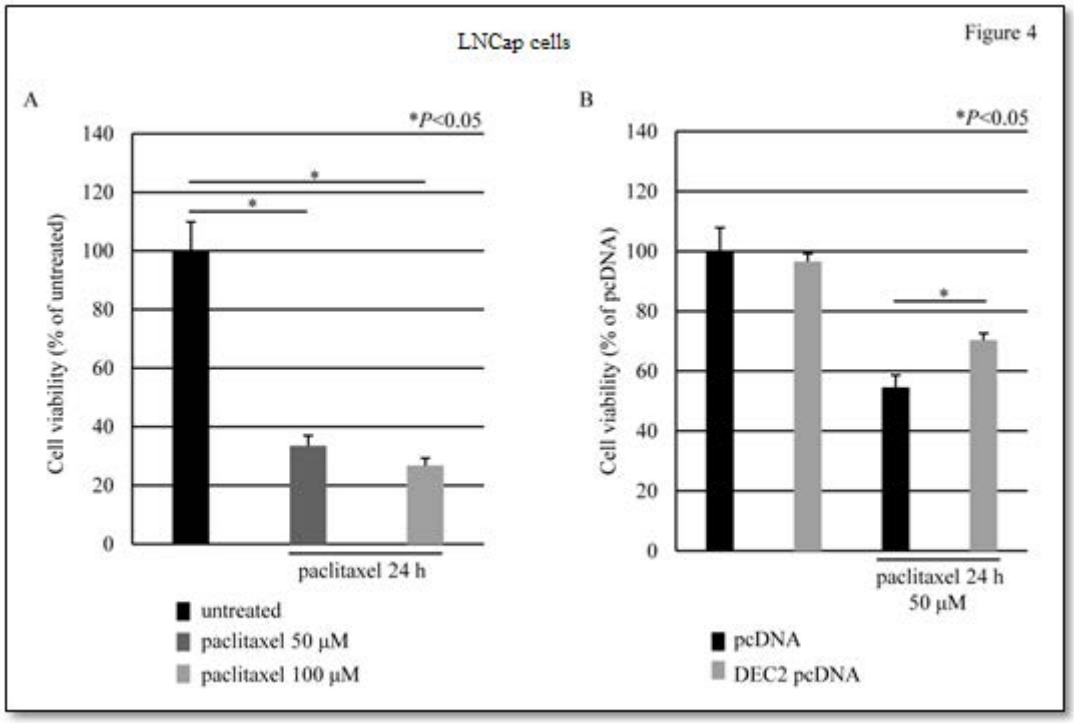

Figure 4. DEC2 protects LNCap cells from paclitaxel-induced apoptosis. (A) LNCap cells cultured in 96-well plate were treated with 50 or 100 μM of paclitaxel for 24 h, and cell viability was examined using the MTS assay. The values are calculated as a percentage of the untreated group. Each value represents the mean ± standard deviation of triplicate experiments (**P*<0.05, compared with untreated). (B) LNCap Cells transfected with DEC2 pcDNA were treated with or without 50 μM of paclitaxel for an additional 24 h. The values are calculated as a percentage of the control. Each value represents the mean ± standard deviation of triplicate experiments (**P*<0.05, compared with pcDNA).

Unlike DEC1, DEC2 positively correlated with Bcl-xL expression in LNCap cells. These data indicated an anti-apoptotic effect of DEC2 in paclitaxel treatment. Although DEC2 suppressed apoptosis induced by paclitaxel, we finally obtained the cleaved caspase-8 and cleaved PARP in paclitaxel-treated LNCap cells. Consideration of these results, we deduced that DEC1 was more effective in paclitaxel-induced apoptosis process than DEC2. The differences in the functions of DEC1 and DEC2 have been concluded in our previous reports [18]. We concluded that DEC2 functioned as an 'anti-apoptotic factor' mainly through regulating Bcl2 family proteins, including the anti-apoptotic subfamily member Bcl-2 [17] and Bcl-xL, as well as the pro-apoptotic member such as Bim [21].

Members of Bcl-2 family play a central role in monitoring cell growth and proliferation and in modulating the genetic programs of the organisms [22]. We examined the expression of several members as those mentioned in previous report [18]. However, Bcl-2 protein could not be detected in LNCap cells (data not shown), and the expression of Bax and Bad (data not shown) kept constant among various kinds of treatment. The mechanism by which DEC2 regulating the Bcl2 family members was needed to be clarified. The distinction of its targets upon different treatment methods should be taken into consideration in our future studies.

We confirmed that DEC2 functioned as an anti-apoptotic factor in human castration-sensitive prostate cancer cell line LNCap. The data of the current study further evidenced our hypothesis that DEC2 would be an 'inhibitor' of apoptosis. Suppressing DEC2 expression may become one of the choices in clinically therapy of prostate cancer as well as other cancers.

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