Expression of programmed cell death 5 protein inhibits progression of lung carcinoma *in vitro* and *in vivo* via the mitochondrial apoptotic pathway

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Received November 16, 2013; Accepted April 28, 2014

DOI: 10.3892/mmr.2014.2454

Abstract. Lung cancer is one of the most common and serious types of cancer, and is characterized by uncontrolled cell growth and metastasis from lung tissues to other body parts. The programmed cell death 5 (Pdcd5) protein is known to accelerate apoptosis in different cell types of tumor. The aim of the present study was to explore the role of Pdcd5 in lung carcinoma and to identify the mechanisms underlying the antitumorigenic properties of Pdcd5 in lung cancer. First, we detected and compared the expression of Pdcd5 in healthy and highly differentiated adenocarcinoma lung tissues. The results of histochemical staining and western blot analysis demonstrated that Pdcd5 expression is markedly decreased in highly differentiated lung adenocarcinoma. Next, we used the lung adenocarcinoma cell line A549 to study the effects of Pdc5 expression on proliferation and colony formation. The results revealed that the expression of Pdcd5 significantly inhibits cell proliferation and colony formation in A549 cells. Importantly, Pdcd5 expression induced tumor cell apoptosis, and the apoptotic proteins caspase-3 and -9 were activated. The expression of B-cell lymphoma 2 (Bcl-2) was reduced and that of Bcl2-associated X protein (Bax) was increased, overall suggesting that the intrinsic apoptotic pathway is activated. Furthermore, using a mice xenograft model and vectors for stable expression or silencing of *Pdcd5*, we showed that stable expression of the protein significantly increases the survival rate of mice *in vivo* ($P<0.01$ compared to control). In conclusion, both *in vitro* and *in vivo* experiments demonstrated that Pdcd5 expression inhibits proliferation and induces apoptosis in the A549 cell line, indicating that the Pdcd5 protein may play an important role in the progression of lung cancer. Therefore, Pdcd5 may be a promising target for the therapy of lung carcinoma.

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Key words: programmed cell death 5, lung cancer, apoptosis, caspase

Introduction

Lung cancer is the most common disease worldwide, with high incidence and mortality (1-3). Until 2008, an estimated 1.61 million new cases were reported, representing 12.7% of all new cancer types (4-6). The mortality rate (1.38 million cases) was estimated at 8.2% of the total mortality due to cancer, which renders lung cancer the most common type of cancer associated with mortality. Only in China, lung cancer has been ranked the number one cause of death among people with malignant tumors (7,8). The registered mortality caused by lung cancer has increased by 464.84% in the past 3 decades (9). It has been reported that imbalance between cell proliferation and apoptosis plays a vital role in the development of lung cancer, along with mutations in tumor suppressor genes and oncogenes and inactivation of multiple genes (10-13).

The programmed cell death 5 (*Pdcd5*) gene, formerly designated as TF-1 cell apoptosis-related gene 19 (*TFAR19*), is involved in cell death and is upregulated during apoptosis (14). The gene was first cloned by the Peking University Center for Human Disease Genomics in 1999. It is expressed in more than 50 tissues in adult humans, and is highly expressed in tissues such as heart, kidney, adrenal gland, testis and placenta (15). The Pdcd5 protein translocates rapidly from the cytoplasm to the nucleus and plays an important role in the inhibition of the proteasome-dependent degradation of lysine acetyltransferase 5, which is involved in transcription, DNA-damage response and cell-cycle control. Disorders in the expression of PDCD5 have been associated with tumorigenesis (16,17). Reduced Pdcd5 expression has been reported in several types of tumor and has been associated with the progression and prognosis of cancer. The protein showed potent antitumor activity via the interaction with the histone acetyltransferase Tip60 and the promotion of DNA damage-induced apoptosis (16,18). However, the expression status and clinical significance of Pdcd5 in lung cancer, and whether Pdcd5 can efficiently inhibit the progression of lung carcinomas, have not yet been studied.

In the present study, we compared the expression level of Pdcd5 in lung carcinoma and healthy lung tissues by immunohistochemistry and western blotting. We further explored whether the antitumor activity of Pdcd5 is regulated by the mitochondria-related apoptotic pathway. This study provides new perspectives for the early diagnosis, treatment and prognosis of lung cancer.

Materials and methods

Cell cultures and transfection. A549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mM streptomycin, in an atmosphere containing 5% CO_2. The PCI-neo-Pdcd5 plasmid was kindly provided by Dr Zhigang Liu (General Hospital of Jinan Military Command, Jinan, Shandong, China) and was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Pdcd5 short hairpin RNA (shRNA) lentiviral particles were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Immunohistochemical analysis. Immunohistochemical staining was performed as described earlier (19,20). Briefly, 30 highly differentiated lung adenocarcinoma tissues and 20 healthy lung tissues adajacent to these were fixed in 4% paraformaldehyde for 24 h. The specimens were obtained from the Department of Thoracic Surgery, General Hospital of Jinan Military Command. The subjects and their families provided written informed consent prior to the study. Our study was performed in compliance with the Declaration of Helsinki and we obtained approval for the study from the Ethics Committee of The General Hospital of Jinan Military Command. The tissues were cut from paraffin blocks in 2-5 μm thick sections using a microtome (Microm HM 310, Microm International GmbH, Walldorf, Germany), and were mounted on SuperFrost Plus slides (Carl Roth GmbH, Karlsruhe, Germany). The primary rabbit anti-human polyclonal antibody targeting Pdcd5 (1:100 dilution; Proteintech, Chicago, IL, USA) was incubated overnight in a moist chamber at room temperature. The secondary antibody, goat anti-rabbit, biotinylated anti-IgG (Vector Laboratories Inc., Burlingame, CA, USA) was used at a 1:500 dilution. Paraffin-embeeded stained sections were observed under a light microscope (NAZAR AM5, Germany).

MTT assay. The MTT assay was performed as previously described (21-23). Briefly, A549 cells were placed into 48-well plates. Following cell adherence, the cells were transfected with the PCI-neo-Pdcd5 plasmid or Pdcd5 shRNA lentiviral particles for 24, 48 and 72 h. The proliferation of A549 cells was determined by measuring the optical density (OD) of the samples at 570 nm.

Colony formation assay. For the colony formation assay, cells were seeded in 6-well plates ($2x10^3$ cells/well) and transfected with PCI-neo-Pdcd5 or Pdcd5 shRNA for 24 h. The medium was changed every two days, and the cells were cultured for ten days after transfection of Pdcd5. Surviving colonies (≥50 cells/colony) were fixed with methanol, stained with 1.25% crystal violet and counted under the light microscope ??h after transfection and for a total of ?? h.

Detection of apoptosis by fluorescence-activated cell sorting (FACS). A549 cells were trypsinized, washed three times with cold phosphate-buffered saline, and resuspended in 200 μl binding buffer. Fluorescein isothiocyanate (FITC)-conjugated Annexin V (Biosea Biotechnology Co., Ltd., Beijing, China) was added according to the manufacturer's protocol, to a final concentration of 0.5 μg/ml. Next, 1 μl of 100 μg/ml propidium iodide working solution was added for incubation. Then, cells were incubated for 20 min at room temperature in the dark, and 400 μl of binding buffer (5X Annexin binding buffer; 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4; Life Technologies, MA, USA) was added. The samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. Protein samples were prepared and separated by polyacrylamide gel electrophoresis as previously described (24-26). We used primary antibodies targeting Pdcd5, caspase-3 and -9, Bcl2-associated X protein (Bax), B-cell lymphoma 2 (Bcl2), and β-actin (used as the loading control) at dilutions 1:3,000, 1:5,000, 1:5,000, 1:3,000, 1:5,000 and 1:10,0000, respectively. As a secondary antibody, we used the horseradish peroxidase-conjugated goat anti-mouse anti-IgG. All antibodies were purchased from Santa Cruz Biothechnology, Inc.

In vivo tumor xenograft study. BalB/c mice were purchased from the Experimental Animal Center of Shandong Medical University (Jinan, Shandong, China) and kept in a pathogen-free environment with a 12-h light/dark cycle. All experiments were conducted in conformation to the Guidelines of the Animal Care and Use Committee of the General Hospital of Jinan Military Command. A549 cells ($5x10^5$) were subcutaneously injected into the back of the mice. The mice were randomly divided into three groups (n>5): control (injected with untransfected A549 cells), Pdcd5 shRNA (injected with A549 cells transfected with the Pdcd5 shRNA) and PCI-neo-Pdcd5 (injected with A549 cells transfected with the PCI-neo-Pdcd5). The survival of mice was recorded daily and the survival rate was determined as 100 x (number of survivors/total number of mice).

Statistical analysis. All the experiments were performed and repeated at least three times. The data were analyzed by the SPSS statistical package 11.5 (IBM, Armonk, NY, USA). The data were expressed as the mean ± standard error of the mean. $P<0.01$ and $P<0.05$ denote significantly statistical differences.

Results

Immunohistochemical detection of Pdcd5. Formalin-fixed, paraffin-embedded specimens from 30 highly differentiated lung carcinoma and 20 healthy tissues were analyzed by immunohistochemistry in order to detect the protein expression of Pdcd5. As shown in Fig. 1, positive staining for Pdcd5 in healthy tissues was mainly observed in the cytoplasm,

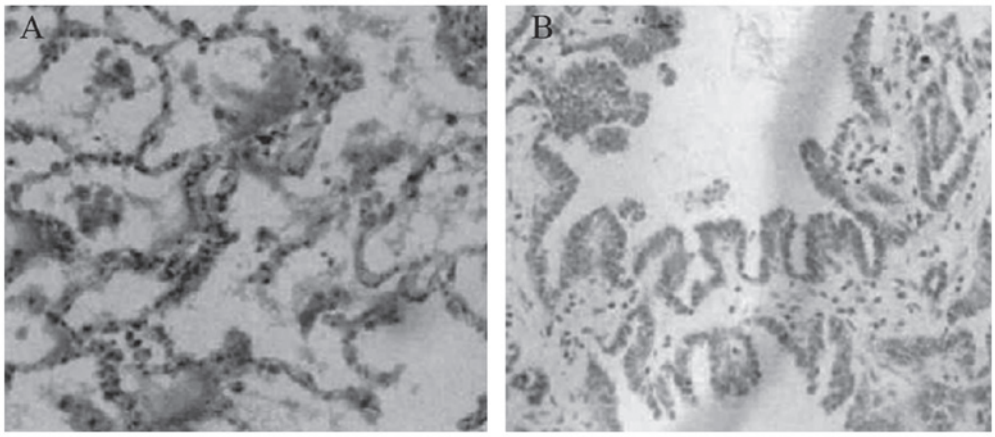

Figure 1. Paraffin-embedded samples of (A) healthy lung tissues and (B) highly differentiated lung adenocarcinoma tissues, analyzed by histochemical staining of the programmed cell death 5 (Pdcd5) protein and observed under a light microscope (magnification, x200). In healthy tissues, Pdcd5 is expressed in the cytoplasm, with some positive staining also detected in the nucleus. The cytoplasmic expression of Pdcd5 is decreased in adenocarcinoma tissues.

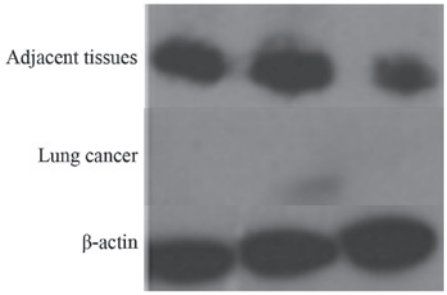

Figure 2. Western blot analysis of programmed cell death 5 (Pdcd5) in adjacent tissues and lung cancer samples. β-actin was used as the loading control. The blot is representative of three independent experiments.

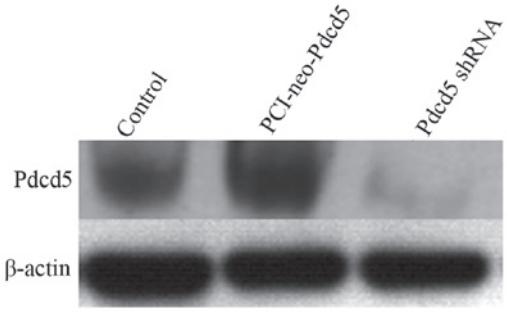

Figure 3. Western blotting aiming to analyze the efficiency of overexpression and silencing of the programmed cell death 5 (*Pdcd5*) gene, using A549 cell transfection with the PCI-neo-Pdcd5 and Pdcd5 short hairpin RNA (shRNA), respectively. The expression of Pdcd5 was detected by western blotting 48 h post-transfection, with β-actin was used as the loading control.

uniformly distributed, and in some cases, in the nucleus. By contrast, decreased immunoreactivity for Pdcd5 was observed in lung carcinoma tissues.

Western blot analysis of Pdcd5 expression. Next, we compared the expression level of Pdcd5 between lung carcinoma and healthy tissues by western blotting. As shown in Fig. 2, the results of three independent experiments showed that Pdcd5 expression is markedly decreased in lung cancer tissues compared to healthy ones.

The efficiency of overexpression and interference of Pdcd5. We next used the lung adenocarcinoma cell line A549 as a cell model to study the effects of *Pdcd5* gene overexpression and silencing at the protein level by western blotting. *Pdcd5* overexpression was achieved by transfecting A549 cells with the PCI-neo-Pdcd5 plasmid, and gene silencing by using a *Pdcd5*-specific shRNA. As shown in Fig. 3, the protein expression of Pdcd5 was markedly reduced in cells transfected with the shRNA, while a slight increase in the Pdcd5 level was observed in cells transfected with the PCI-neo-Pdcd5 plasmid.

Overexpression of Pdcd5 inhibits proliferation in the lung cancer cell line A549. The effects of Pdcd5 on cell viability and proliferation of lung cancer cells were assessed by the MTT and colony formation assays, respectively. As shown in Fig. 4, when Pdcd5 was overexpressed, a significant and time-dependent increase in A549 cell death was observed compared to untransfected cells ($P<0.01$). In addition, the number of colonies was significantly decreased in PCI-neo-Pdcd5-transfected cells in the colony formation assay. Taken together, these results indicate that overexpression of Pdcd5 significantly inhibits A549 cell proliferation and that Pdcd5 may act as a potential tumor suppressor.

Overexpression of Pdcd5 induces apoptosis of the lung cancer cell line A549. In order to examine whether the inhibition of proliferation in A549 cells overexpressing Pdcd5 is related to cell apoptosis, FACS analysis was performed. A549 cells transfected with PCI-neo-Pdcd5 or Pdcd5 shRNA were subjected to dual labeling with Annexin V-FITC and propidium iodide (PI). As shown in Fig. 4C, the apoptotic rate was significantly higher in the PCI-neo-Pdcd5 group (25.8%) compared with the control (3.6%) ($P<0.01$).

Activated caspase-3 and-9, increased Bax and decreased Bcl-2 levels in Pdcd5-overexpressing cells. To further explore the mechanism by which expression of Pdcd5 induces apop-

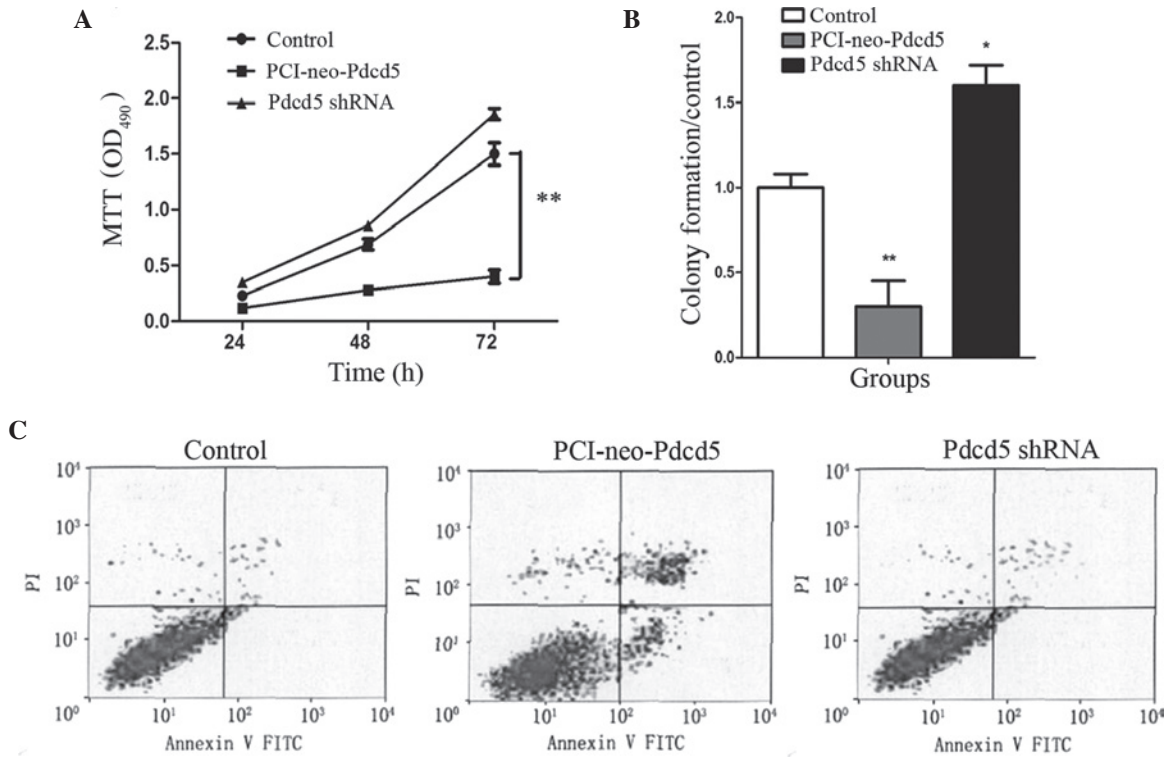

Figure 4. Overexpression of the programmed cell death 5 (Pdcd5) protein induces cell death, inhibits colony formation and promotes apoptosis in the lung cancer cell line A549. (A) MTT assay. A549 cells were transfected with PCI-neo-Pdcd5, Pdcd5 short hairpin RNA (shRNA) or were not transfected (control). After 24, 48 and 72 h, the optical density at 490 nm (OD_{490}) was measured. **P<0.01, compared to the control. (B) Colony formation assay 48 h after ???. **P<0.01, *P<0.05, compared to the control. (C) Apoptosis measured by flow cytometry. The cells of the three groups were stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI). All experiments were repeated at least three times.

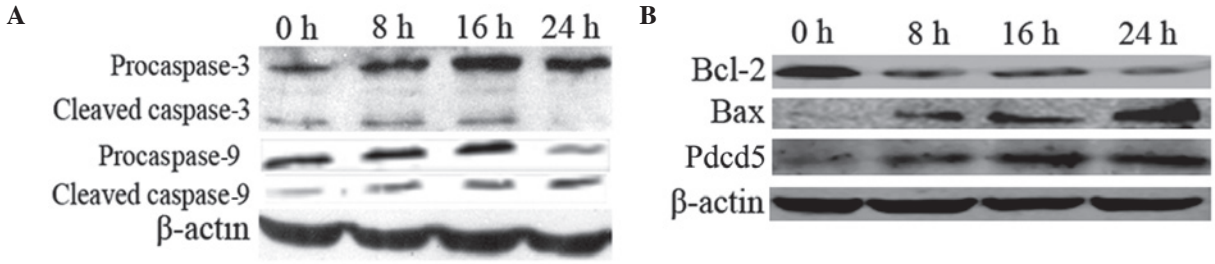

Figure 5. Western blot analysis of the expression of apoptosis-related proteins in A549 cells transfected with PCI-neo-Pdcd5 after 8, 16 and 24 h. β-actin was used as a loading control. Expression of (A) pro- and cleaved caspase-3 and -9 and of (B) B-cell lymphoma 2 (Bcl-2), Bcl2-associated X protein (Bax) and programmed cell death 5 (Pdcd5).

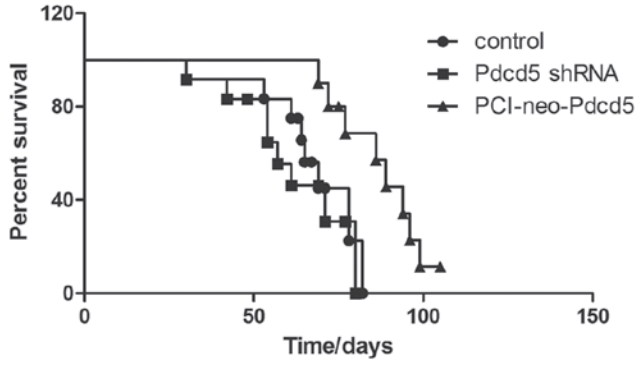

Figure 6. Survival rate of BalB/c mice following lethal challenge with lung carcinoma cells (A549 line). The mice were subcutaneously injected with $5x10^5$ cells in their back. The survival rate was determined as 100 x (number of survivors/total number of mice). Each group contained >8 mice. shRNA, short hairpin RNA; control, mice injected with untransfected A549 cells.

tosis, the levels of caspase-3, caspase-9 and Bcl-2 family proteins were examined by western blot analysis. As shown in Fig. 5A, both procaspase-3 and -9 were cleaved into their characteristic active forms, the relative level of which showed a time-dependent increase, suggesting that the intrinsic mitochondrial apoptotic pathway was activated. Moreover, the level of the Bcl-2 protein was decreased and that of Bax was increased along with the increase in the Pdcd5 level in PCI-neo-Pdcd5-transfected cells (Fig. 5B).

Overexpression of Pdcd5 exhibits antitumor activity in a xenograft nude mouse model. In order to examine the ability of Pdcd5 to induce apoptosis *in vivo*, a nude mice xenograft model was established, and survival of mice injected with different types of A549 cells was calculated. As shown in Fig. 6, stable expression of Pdcd5 significantly (P<0.01) increased the

survival rate of mice compared to Pdcd5 silencing or normal expression (control group).

Discussion

Lung cancer is one of the most common malignant tumor types in China. The incidence and mortality rates are rising every year. Imbalance between cell proliferation and apoptosis plays a vital role in the development of lung cancer, which prompted us to focus on the Pdcd5 protein. Pdcd5 is an apoptosis-regulated programmed cell death protein, first cloned in 1999 by Liu *et al* (14). The gene is widely expressed in various tissues, except for the hematopoietic system, and locates on chromosome 19q12-q1311 (27). The protein is composed of 125 amino acids, including 6 exons and 5 introns. Different expression levels of Pdc5 have been reported in various diseases, with reduced expression in leukemia (28), gastrointestinal stromal tumors (29), astrocytic gliomas (30) and prostate cancer (31). In the present study, the immunohistochemical analysis clearly showed positive staining of Pdcd5 in healthy lung tissues, mostly in the cell cytoplasm, and reduced staining in lung carcinoma tissues. This result was consistent with western blot analysis.

The PCI-neo-Pdcd5 plasmid was transfected into the human lung cancer cell line A549 to induce overexpression of Pdcd5. Consequently, apoptosis was induced in cancer cells, as detected by the MTT assay and flow cytometry analysis. It has been reported that Pdcd5 enhances cisplatin-induced apoptosis in chondrosarcomas (32,33), which is consistent with findings from the present study. During the progression of apoptosis, the expression level of anti-apoptotic and proapoptotic proteins is tightly regulated. Here, the levels of Bcl-2 family proteins were detected by western blot analysis. The expression of Bax was increased and that of Bcl-2 was decreased after 24 h of transfection with the PCI-neo-Pdcd5 plasmid. The ratio of Bax/Bcl-2 was thus increased, and apoptosis is expected to be promoted in such conditions. In addition, the caspase-3 and -9 were activated in A549 cells overexpressing Pdcd5, suggesting that Pdcd5 expression may activate the mitochondria-related apoptotic pathway.

In summary, our study analyzed the expression and clinical significance of Pdcd5 in lung cancer, but also provided evidence for the mechanism of PDCD5-induced cell apoptosis, showing that the mitochondria-related apoptotic signaling pathway may play an important role in the process. However, the exact molecular events of DCD5-induced cell apoptosis need to be explored in future studies. The present study indicated that Pdcd5 may be a useful target for the therapy of lung cancer.

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