Distinct roles of different fragments of PDCD4 in regulating the metastatic behavior of B16 melanoma cells

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Abstract. Melanoma is an aggressive cutaneous malignancy. In this study, we demonstrated that the levels of the *programmed cell death 4* (PDCD4) protein and mRNA were lower in tumor tissues compared with normal tissues. In order to further investigate the effects of PDCD4 and its fragments in B16 melanoma cells, we established B16 clones with expression of different PDCD4 fragments. Intact PDCD4, PDCD4Δ164-469 and PDCD4Δ327-440 expression, respectively, decreased proliferation and migration and increased apoptosis in B16 cells *in vitro*. We found that intact PDCD4, PDCD4Δ164-469 or PDCD4Δ327-440 can inhibit the activity of MMP-2 and the expression of CXCR4. However, PDCD4Δ164-275 showed no effects on B16 cells. These results may prove helpful for the development of novel therapies for melanoma treatment.

Introduction

Melanoma is an aggressive cutaneous malignancy accounting for just 4% of skin cancers but resulting in 80% of all skin-cancer related deaths (1). Melanoma may be induced by continuous proliferation of the melanocytes and dysregulation of the epidermal melanin unit (2). Melanocyte growth is controlled by the surrounding keratinocytes by intercellular communication through cell-cell adhesion molecules, cell-matrix adhesion, and gap junctional intercellular communication (3). A previous study has confirmed that downregulation of *programmed cell death 4* (PDCD4) leads to the downregulation of E-cadherin and urokinase-type plasminogen activator receptor, which leads to degradation of extracellular matrix and invasion (4).

PDCD4 was first identified by differential display that is upregulated upon in apoptosis-induced murine cell lines (5). The human *PDCD4* gene is localized in chromosome 10q24 (6). PDCD4 protein can suppress tumor promotion and progression to carcinomas in cultured cells and transgenic mice (7,8). As shown in Fig. 1, full length human PDCD4 contains an N-terminal putative RNA-binding domain (residues 1-140) and two tandem MA3 domains, designated as nMA3 (residues 164-275) and cMA3 (residues 327-440) (9). The two MA3 domains have very similar structure and eIF4A-binding surfaces (10). The molecular basis for PDCD4-mediated suppression has been linked to its high affinity MA-3 domains (11,12). NMR binding analysis has shown that both MA3 domains of PDCD4 interacted with eIF4A and prevented translation (10,11). However, another study has demonstrated that the cMA3 domain alone is sufficient for the inhibition of RNA helicase and translation (13). PDCD4 expression is significantly downregulated in various human cancers such as lung cancer (14), hepatocellular carcinoma (15), breast carcinoma (16), colorectal cancer (17) and gastric cancer (18). PDCD4 can induce tumor cell to apoptosis, inhibit tumor angiogenesis and increase sensitivity of tumor cells to antitumor drugs or radiotherapy (19,20). Recent studies showed the indirect relationship between PDCD4 and melanoma cells (21-23), however, to our knowledge, little is known yet about the individual role of cMA3 or nMA3 of PDCD4 in B16 melanoma cells.

In our study, we showed that cMA3 domain of PDCD4 caused profound suppression of cell proliferation in B16 melanoma cells. In contrast to the role of cMA3, our findings showed that the antitumor effects of nMA3 were weak relatively in B16 melanoma cells. The cMA3 domain of PDCD4 may serve as an effective drug independently.

Materials and methods

Cell lines and culture. The human melanoma cell line, B16, was obtained from the American Type Culture Collection (ATCC, Bethesda, MD). Cell lines were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained in a humidified incubator with 5% CO_2 at 37˚C.

Subjects. Total specimens of 21 patients with melanoma were obtained from the Department of Plastic Surgery, The First Affiliated Hospital of China Medical University (Jan. 2001 to Dec. 2010). None of the patients underwent radiotherapy or chemotherapy before operation. This study was in compliance with the Helsinki Declaration, all patients gave written

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informed consent for participation and the procedure was approved by Our University Ethics Committee.

Immunohistochemical staining. All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were deparaffinized in xylene. Endogenous peroxidase was blocked with 3% hydrogen peroxide in deionized water for 20 min. Antigen retrieval was carried out in citrate buffer (10 mM, pH 6.0) for 30 min at 95˚C. Section were immunostained with polyclone antibody to PDCD4 (sc-27123; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution or CXCR4 (PA1237; Boster Biological Technology, Fremont, CA) at 1:50 dilution for 60 min at 37˚C, followed by biotinylated secondary antibody for 30 min, subsequently reacted with by HRP for 30 min. For visualization, hydrogen peroxide-activated diamino benzidine (DAB) was applied. Washes (50-min) in PBS were carried out between each step. Tissue sections were lightly counterstained with hematoxylin, dehydrated through graded alcohols, cleared with xylene and mounted in mounting medium. Normal tissue was used as a control. Sections treated without primary antibodies were used as negative controls.

SDS-PAGE and immunoblotting. Proteins (45 μg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corp., Billerica, MA). Western blotting was performed using primary antibodies: PDCD4 (sc-27123; Santa Cruz Biotechnology), CXCR4 (PA1237; Boster Biological Technology) and β-actin (sc-47778; Santa Cruz Biotechnology). Incubation with antibodies was performed in 1.5% BSA in TBS, 0.1% Tween. Detection of the immune complexes was performed with the ECL western blotting detection system (Amersham Biosciences, Piscataway, NJ).

Construction of PDCD4 cDNA expression vector. For the preparation of truncated PDCD4 proteins, the relevant sequences were amplified from full-length *PDCD4* (GenBank accession no. NM_014456.3) by PCR using primers that included designed restriction sites (Table I). PDCD4 and the truncated forms created in this study are presented in Fig. 1. cDNAs of the truncated PDCD4 forms were obtained by PCR, then digested with the relevant restriction enzymes and ligated into pcDNA3.1. Transfection of plasmids into B16 cells was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time PCR and RT-PCR. Total tissue and cellular RNA was isolated using TRIzol reagent (Invitrogen). cDNA was then synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The level of tissular *PDCD4* mRNA was quantitated by real-time PCR (ABI PRISM 7500) using power SYBR® Green PCR Master Mix (Takara Dalian, Dalian, China) and specific primers for *PDCD4* and *GAPDH*. The following primer sets were used: *PDCD4* sense, 5'-GTATGATGTGGAGGAGGTGGAT-3'; antisense: 5'-CCCTCCAATGCTAAGGATACTG-3'; *GAPDH* sense, 5'-GAAGGTGAAGGTCGGAGT-3'; antisense: 5'-CATGGGTGGAATCATATTGGAA-3'. The real-time PCR conditions were as follows: one cycle at 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 1 min. Each reaction was repeated independently three times in triplicate. Results for the quantity of mRNA were expressed as the number of copies of *PDCD4* mRNA per copy of *GAPDH* mRNA.

PCR amplification of cellular cDNA was performed in 15-μl mixtures. The primers are summarized in Table I. The RT-PCR conditions were: one cycle at 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 72˚C for 45 sec, 58˚C for 45 sec and final extension at 72˚C for 10 min. Finally, products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining and a UV imaging system (UVP, Upland, CA).

Immunofluorescence. Transfected cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized in 1% Triton X-100 for 5 min and blocked with 5% bovine serum albumin in PBS containing 0.5% Triton X-100 for 1 h. Intact PDCD4 (aa 1-469), truncated 1 (aa 164-469) and truncated 3 (aa 327-440) were detected using anti-PDCD4 (sc-27123; Santa Cruz Biotechnology). Truncated 2 (aa 164-275) was detected using anti-PDCD4 (sc-376430; Santa Cruz Biotechnology). Cells were washed with PBS and incubated with appropriate secondary fluorophore-conjugated antibody for 1 h at room temperature, washed with PBS and mounted using Prolong Anti-fade (Sigma-Aldrich, Carlsbad, CA). Secondary antibody used for detection of intact PDCD4, truncated 1 and truncated 3 was Alexa Fluor® 488 donkey anti-goat IgG (H+L). Alexa Fluor® 488 goat anti-mouse IgG (H+L) was used to detect truncated 2. Cells were examined and captured using an Olympus CX71 fluorescence microscope (Olympus, Tokyo, Japan).

Cell viability assay. Viability of transfected cells was determined using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium-bromide (MTT) assay (Sigma). Cells were plated in 96-well plates (1,500 cells per well) and incubated under normal culture conditions. After 24 h, the cells were treated with 0.5 mg/ml MTT for 4 h and lysed with dimethyl sulfoxide (DMSO). Absorbance rates were measured at 550-560 nm using a microplate reader (Bio-Rad, Hercules, CA).

Measurement of apoptotic cell death. Annexin V-FITC/PI double staining assays were performed to detect apoptosis. Following the manufacturer's instructions (Apoptosis Detection kit, KeyGEN, Nanjing, China), cells were trypsinized, washed twice with cold PBS, then resuspended in 200 μl binding buffer. Annexin V-FITC was added to a final concentration of 0.5 μg/ml. In addition samples were incubated at room temperature in the dark. After 20 min, 300 μl binding buffer containing 0.5 μg/ml PI was added and samples were immediately analyzed on a FACSCalibur flow cytometer (Becton-Dickinson Medical Devices, Shanghai, China). Cells in the stage of early apoptosis were defined as $FITC^+/PI^-$ cells.

Migration assay. Migration was assessed in Boyden chambers containing polycarbonate filters with 8-μm pore size (Costar, Bodenheim, Germany). The lower compartment was filled in DMEM with 10% FBS used as a chemoattractant and the filter was placed above. Cells were harvested by trypsinization

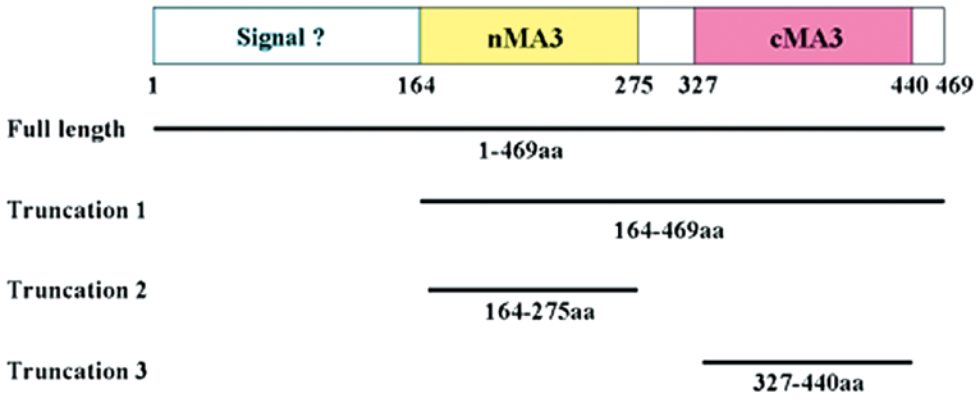

Figure 1. Schematic diagram of the functional regions of PDCD4 protein. The PDCD4 is formed with mMA3 (orange) and cMA3 (magenta) domains. Nuclear localization signal was not confirmed.

Table I. Primers used to generate intact and truncated forms of PDCD4.

Region of PDCD4 amplified	Primers (5'-3')	Product (bp)
aa 1-469 (intact)	F: *AAGCTT* TACCTATATCTTTTACTCGT R: GAATTC CTAGTAGCTCTCAGGTTTAA	1,407
aa 164-469 (truncated 1)	F: *AAGCTT* GTTCGTTTTCGGTTT R: GAATTC CTAGTAGCTCTCAGGTTTAA	918
aa 164-275 (truncated 2)	F: *AAGCTT* GTTCGTTTTCGGTTT R: GAATTC ACATAAGATTCCATC TCCAA	336
aa 327-440 (truncated 3)	F: *AAGCTT* ACAATTTCTCTAACTATACG R: GAATTC GGAAATTATTCCAGCCTGAA	339

Italics indicates *Hin*dIII restriction sites; underlined indicates *Eco*RI restriction sites. F, forward. R, reverse.

and resuspended in DMEM without FBS. Cell suspensions (600 μl) at a density of $3x10^4$ cells/ml were placed in the upper compartment of the chambers. After incubation at 37˚C for 4 h filters were removed, cells were fixed, stained and counted. Each condition was assayed in triplicate and assays were repeated at least twice.

Gelatin zymography. Fifty micrograms of protein was applied to 10% polyacrylamide gels with 1% gelatin incorporated as a substrate for gelatinolytic proteases. After running the gel the SDS was removed by washing twice in 2.5% Triton X-100 for 30 min. The gels were incubated overnight in zymography development buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM NaN_3 and 5 mM $CaCl_2$. After development the gels were stained for 3 h in 45% methanol/10% glacial acetic acid containing 1% (w/v). Coomassie Blue R-250 and subsequently partially destained with the same solution without dye. The gelatinolytic activity of each MMP was qualitatively evaluated as a clear band against the blue stained gelatin background.

Flow cytometry analysis. The CXCR4 or CXCR7 cell surface expression was measured and quantified using the fluorescent-antibody (24). Cells were labeled with APC-anti-CXCR4 monoclonal antibody (#555976, BD Pharmingen™, Baltimore, MD). Mouse anti-CXCR7 (AF4227, R&D Systems China, Shanghai, China) and PE-conjugated goat anti-mouse antibody (#115-116-146, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used for the detection of CXCR7. Expression was measured by flow cytometry using a FACSCalibur machine (BD Biosciences). Receptor expression was evaluated by the mean channel fluorescence values.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). Differences between groups were analyzed using Student's t-test for continuous variables. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS, Inc.) and significance was established at $P<0.05$.

Results

PDCD4 expression in human melanoma specimens. The level of PDCD4 protein in cancer tissue was significantly lower than that in normal tissue ($P<0.05$, Fig. 2B). To determine whether *PDCD4* mRNA was also reduced, real-time PCR analysis was performed. Results show that the level of PDCD4 mRNA expression was coincident with the level of protein expression ($P<0.05$, Fig. 2A). Furthermore, the immunohistochemical staining results showed PDCD4 proteins were located both in nucleus and cytoplasm (Fig. 3A).

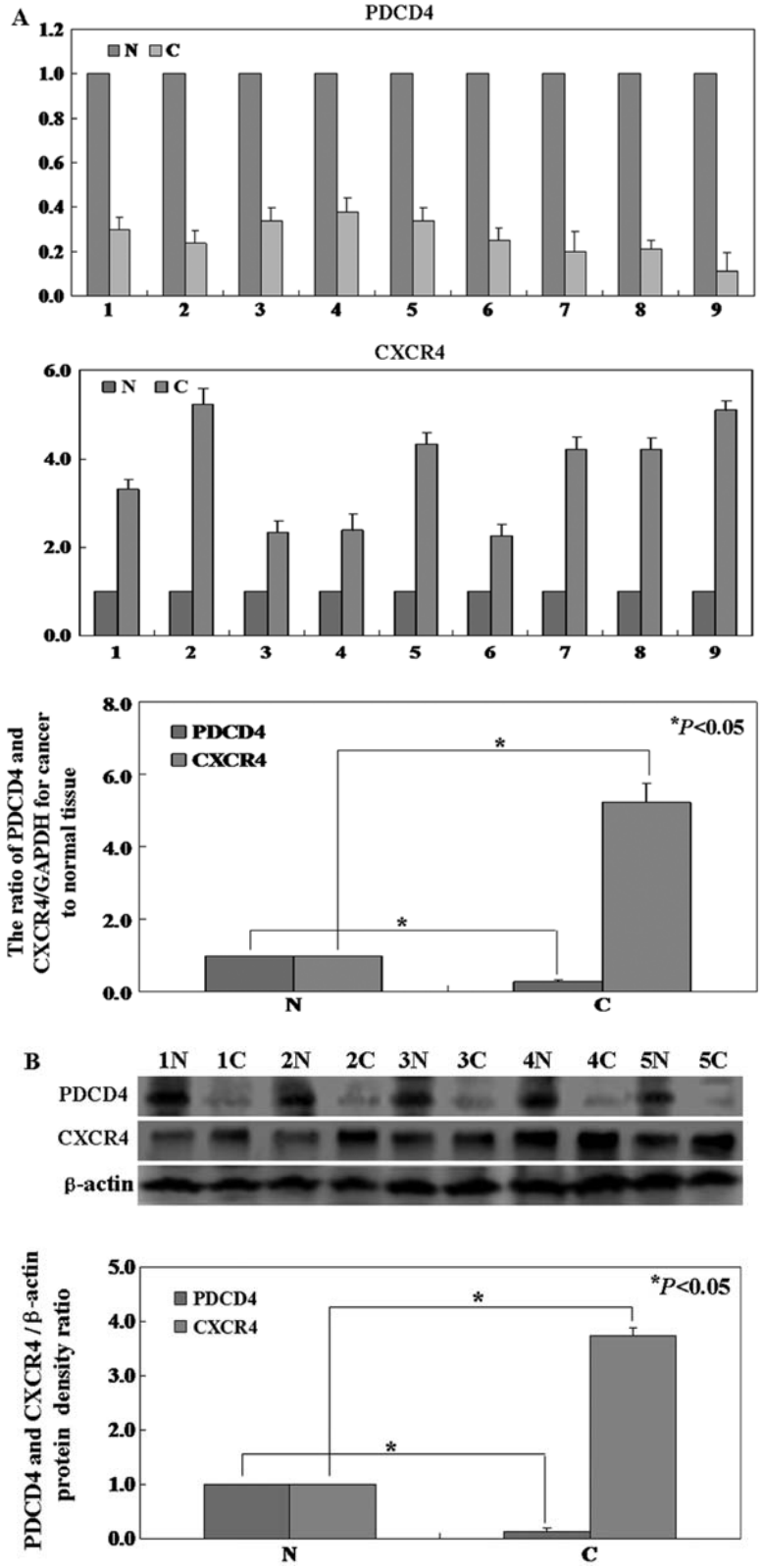

Figure 2. (A) The level of *PDCD4* and *CXCR4* mRNA measured in specimens using real-time PCR. The level of *PDCD4* mRNA was lower in cancer tissues than matched normal tissues (P<0.05). However, the level of *CXCR4* mRNA was higher in cancer tissues than matched normal tissues (P<0.05). The *GADPH* was used as an internal control. (B) Representative results of two paired of gastric cancer and corresponding normal tissue by western blotting with PDCD4 and CXCR4 antibodies. PDCD4 protein expression was lower in cancer tissues than matched normal tissues (P<0.05). CXCR4 protein showed a higher level in cancer tissues than matched normal tissues (P<0.05). β-actin was used as an internal control. N, normal; C, cancer.

Evaluation of the mRNA and protein levels of different PDCD4 fragments in B16 cells. B16 cells were transfected with PDCD4 fragments that included: intact (aa 1-469), truncated 1 (aa 164-469), truncated 2 (aa 164-275) and truncated 3 (aa 327-440). Levels of PDCD4 fragments were detected using immunofluorescence and RT-PCR assays. In RT-PCR

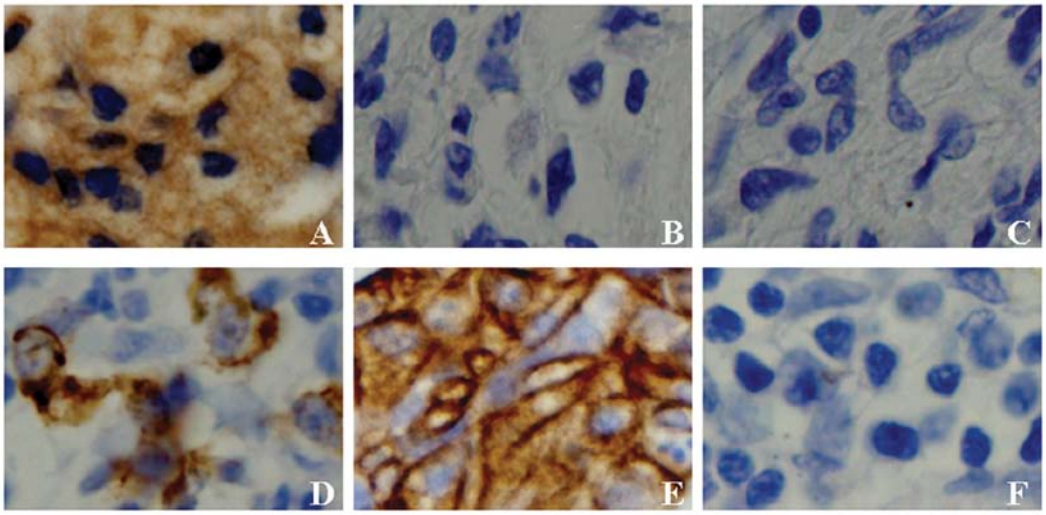

Figure 3. Immunohistochemical staining for PDCD4 and CXCR4 protein in specimens. PDCD4 was stained yellow with granules and localized to the nucleus and cytoplasm. CXCR4 was localized to the cytomembrane. The nuclei were counterstained with hematoxylin. (A and D) Paired normal tissue. (B and E) Gastric cancer tissue. (C and F) Negative control.

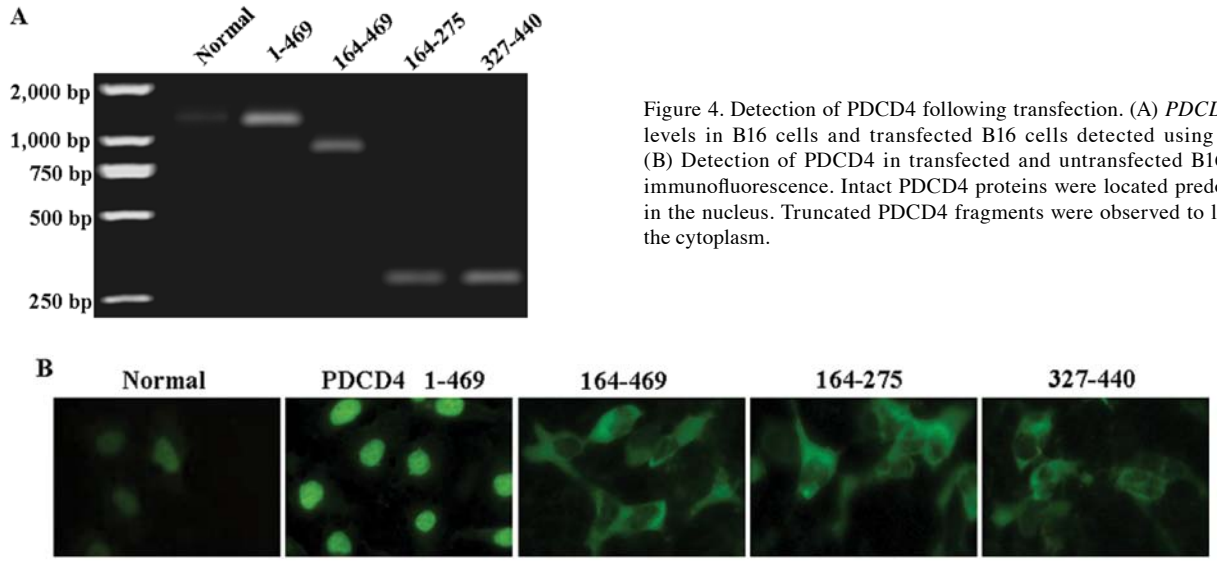

Figure 4. Detection of PDCD4 following transfection. (A) *PDCD4* mRNA levels in B16 cells and transfected B16 cells detected using RT-PCR. (B) Detection of PDCD4 in transfected and untransfected B16 cells by immunofluorescence. Intact PDCD4 proteins were located predominantly in the nucleus. Truncated PDCD4 fragments were observed to localize in the cytoplasm.

assay, levels of *PDCD4* mRNA were lower in B16 cells than in treated cells (Fig. 4A). In the immunofluorescence assay, levels of PDCD4 proteins were lower in untransfected B16 cells compared with transfected B16 cells (Fig. 4B). The results showed that intact PDCD4 proteins were able to shuttle between nucleus and cytoplasm. Under normal growth conditions, intact PDCD4 proteins were located predominantly in the nucleus. However, truncated PDCD4 fragments were observed to localize in the cytoplasm (Fig. 4B).

Upregulation of PDCD4 expression in B16 cell line results in decreased proliferation, increased apoptosis and diminished migration. We then investigated the functions of PDCD4 fragments in B16 cell line. The MTT assay showed that the proliferation ratio of intact PDCD4-expressing B16 cells was decreased compared with untransfected cells ($P<0.05$, Fig. 5A). Truncated 1 (aa 164-469) and truncated 3 (aa 327-440) had similar effects with intact PDCD4 on B16 cells. However, truncated 2 (aa 164-275) showed no effects on B16 cells. We determined that the percentage of apoptosis by using Annexin V and PI double-staining in untreated B16 cells was 0.34%, significantly lower compared to intact PDCD4 (2.71%), truncated 1 (2.68%) and truncated 3 (3.25%) cells ($P<0.05$, Fig. 5B). Truncated 2 cells (0.41%) showed no significant changes compared with untreated ones ($P>0.05$, Fig. 5B). To quantitatively assess the cell migration rate, transwell migration assay was applied. The cells in intact PDCD4, truncated 1 and truncated 3 groups showed 54, 56 and 53% less migration, respectively, than the untreated ones ($P<0.05$, Fig. 6A). We found that no significantly less of the truncated 2 cells migrated to the lower membrane compared to control cells ($P>0.05$, Fig. 6A). In addition, we evaluated expression of MMPs and observed the levels of MMP-2 in intact PDCD4, truncated 1 and truncated 3 cell lines were lower than that in untreated and truncated 2 cell lines (Fig. 6B).

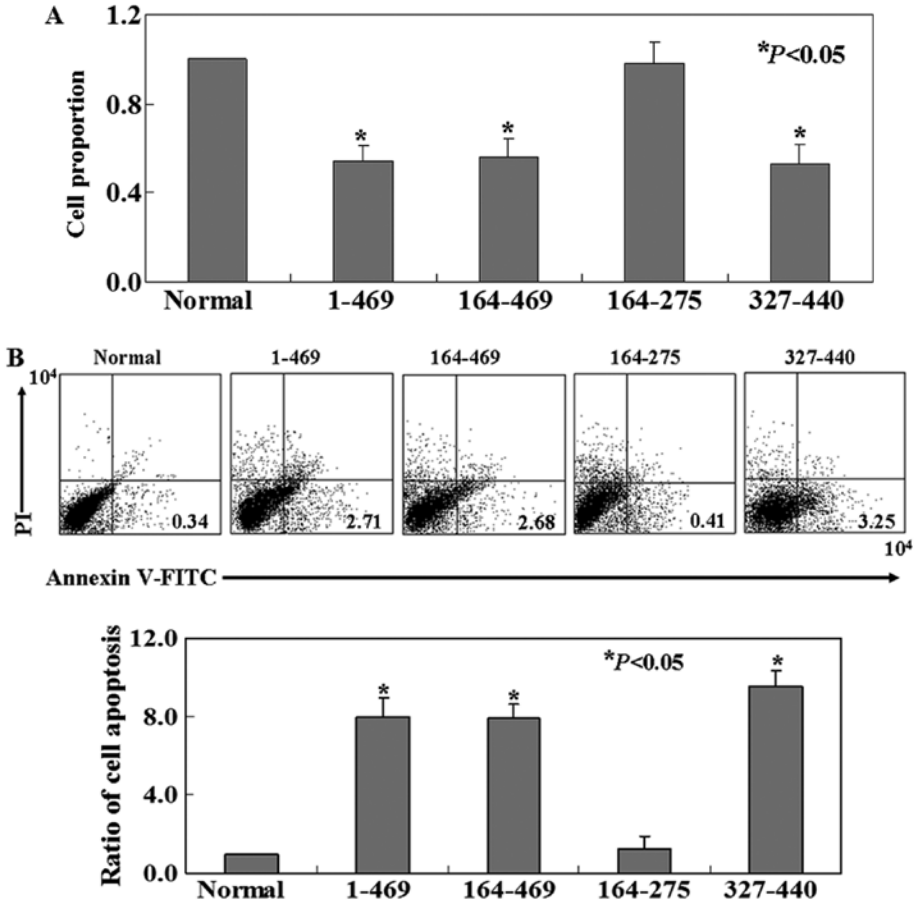

Figure 5. Expression of intact PDCD4 protein and truncated PDCD4 fragments regulates growth in B16 cells. (A) MTT assays were performed to determine the ratio of growth inhibition associated with each of the PDCD4 fragments transfected into B16 cells compared with untransfected cells. (B) Flow cytometric analysis of B16 cell apoptosis induced by PDCD4 fragments. The percentage of cell apoptosis was determined by flow cytometry with Annexin-V/FITC and PI double staining.

Cell surface expression of CXCR4 on human melanoma specimens and B16 cell lines. In order to determine the level of CXCR4 protein and mRNA in human melanoma specimens, western blotting, IHC and real-time PCR were performed respectively. The results showed both CXCR4 protein and mRNA were decreased in cancer tissues compared with normal tissues (Fig. 2). The immunohistochemical staining results showed CXCR4 proteins were located in cytomembrane (Fig. 3D and E). B16 cells with PDCD4 different fragment treatment expressed different relative levels of the cell surface receptor CXCR4. We observed that untreated B16 (55.8%) and truncated 2 (67.2%) cell lines stained highly positive for CXCR4 (Fig. 6C). In contrast, CXCR4 was expressed in intact PDCD4 (4.4%), truncated 1 (4.2%) and truncated 3 (6.6%) cell lines at lower levels (Fig. 6C). As shown in Fig. 6C, this is not in striking difference to CXCR7 expression, which was barely expressed on the cell lines.

Discussion

PDCD4 has been characterised as a new tumor suppressor gene, which is downregulated in several human malignancies (14-18). Consistent with our results, we also found the levels of PDCD4 protein and mRNA were lower in cancer tissues than that in normal tissues. Yang *et al* (22) found that microRNA-21 (miR-21) regulates the metastatic behavior of B16 melanoma cells by promoting cell proliferation, survival and migration/invasion. MicroRNA (miR) target prediction databases suggest that PDCD4 is regulated by miR-21 (25,26). The expression of PDCD4 is increased during apoptosis (27,28) and decreased during human and mouse carcinogenesis (29,30). Overexpression of PDCD4 inhibits tumorigenesis and tumor progression in a transgenic-mouse model and inhibits tumor cell invasion *in vitro* (27-31). We also confirmed the antitumor activities of PDCD4 in B16 cells. These results provided the first direct evidence for an essential role of the PDCD4 in melanoma.

Furthermore, the main purpose of our study was to identify the roles of PDCD4 protein distinct domains. We designed three deletion mutants, PDCD4Δ164-469, PDCD4Δ164-275 and PDCD4Δ327-440. Residues 1-140 of human PDCD4 protein were an RNA-binding domain, residues 164-275 were nMA3 and residues 327-440 were cMA3 (9). The localization of PDCD4 has been reported to vary in a cell type-dependent manner (27). One explanation for this variability is that PDCD4 cycles between the nucleus and cytoplasm (32). However, a nuclear localization signal has not been formally identified. Interesting, we found only intact PDCD4 protein was able to shuttle between the nucleus and the cytoplasm. The results indicated that the nuclear localization signal of PDCD4 may

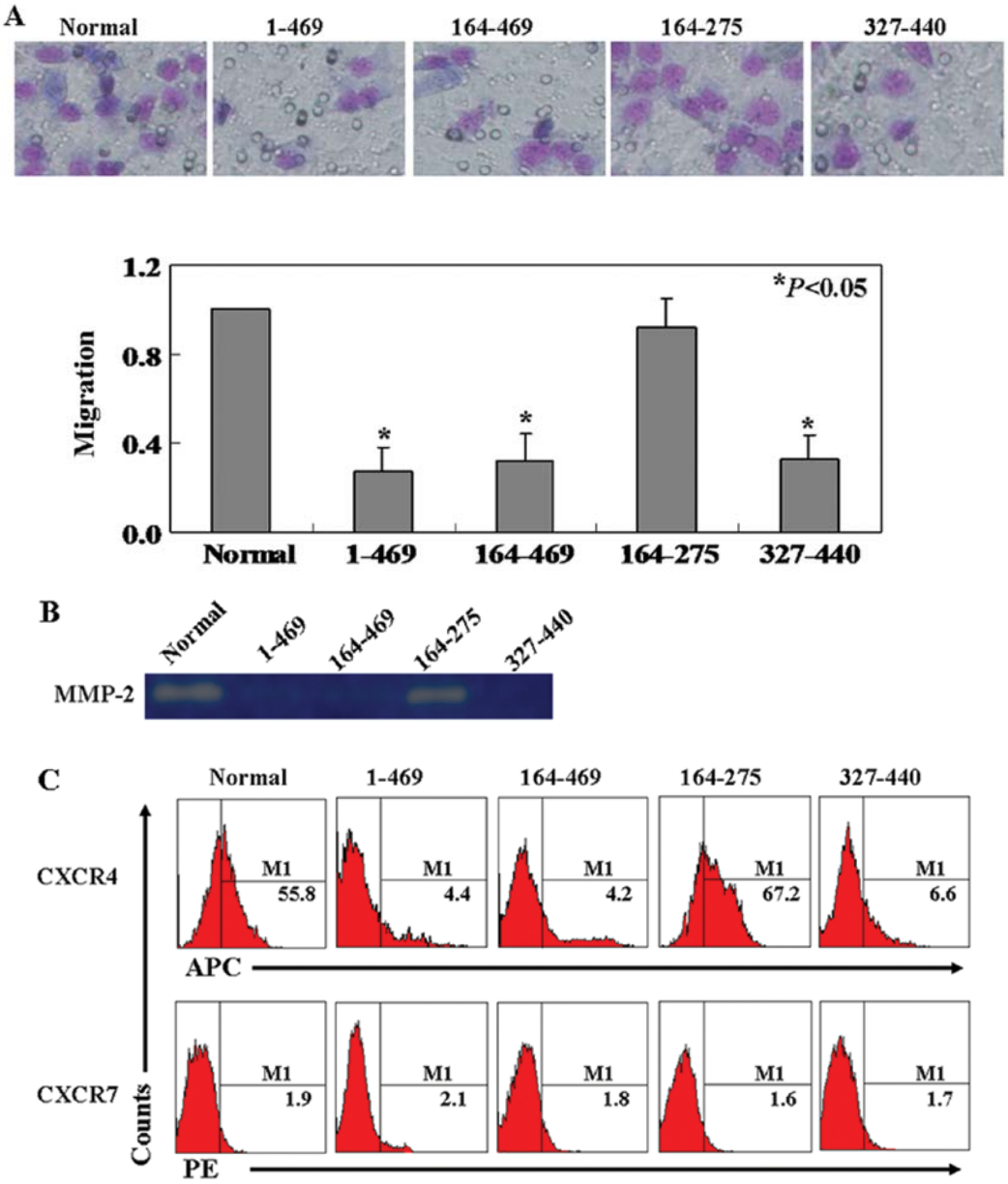

Figure 6. Distinct effects of different PDCD4 fragments on metastatic behavior of B16 cells. (A) Transwell assays were performed. Cells that migrated to the bottom side of the membrane were stained and counted. (B) Effect of PDCD4 fragments on gelatinolytic activities of MMP-2. Gelatinolytic activity of secreted MMP-2 of B16 cells was analyzed by zymography. (C) Expression of CXCR4 and CXCR7 on B16 cells. Flow cytometry was performed for detection of CXCR4 and CXCR7.

be included in residues 1-140. The only direct molecular function identified for PDCD4 is bound to and inhibits the function of the mammalian mRNA initiation factor eIF4A (11,33). As noted in the introduction, mutational analysis and NMR binding analysis have shown that PDCD4 uses both MA3 domains to interact with eIF4A (10,11). However, another study also demonstrated that the cMA3 domain alone is sufficient for the inhibition of RNA helicase and translation (13). Consistent with previous studies, our results showed that PDCD4Δ164-469 and PDCD4Δ327-440 had similar effects with intact PDCD4 on B16 cells. However, PDCD4Δ164-275 showed no biological activity.

Melanoma is an extremely aggressive disease with high metastatic potential (34). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the major MMPs secreted by activated inflammatory macrophages (35,36). MMP-2 is released as a latent enzyme and must be activated to degrade the matrix (37). In our study, intact PDCD4, PDCD4Δ164-469 and PDCD4Δ327-440 were able to reduce the gelatinolytic activity of MMP-2. Previous studies have proven that CXCR4 is a major chemokine receptor expressed in many types of cancer cells (38,39). CXCR4/SDF-1 axis plays a major role in migration and adhesion of various tumor cells, including colon cancer (40), breast cancer (41,42) and melanoma (43,44). Consistent with previous studies, we found that CXCR4 was highly expressed in melanoma tissue compared to normal tissue by real-time PCR, western blotting and IHC. Interesting, we confirmed that CXCR4 was decreased in B16 cells after treatment with intact PDCD4, PDCD4Δ164-469 or PDCD4Δ327-440. The biological significance of CXCR7 receptor expression on

B16 cells was not found. Our data presented from this study provide evidence that PDCD4 could inhibit mobility of B16 cells by regulating CXCR4 expression. However, the mechanism remains unclear.

Above all, our study demonstrated that: i) the levels of PDCD4 mRNA and protein were deficient in melanoma, however, CXCR4 showed a higher level in cancer tissues than normal tissues; ii) the tumor suppressor activity of PDCD4 and its fragments *in vitro*. Especially, PDCD4 inhibited mobility of B16 cells by suppressing MMP-2 activity and CXCR4 expression. This is the first report to demonstrate that different fragments of PDCD4 protein played distinct roles in B16 cells. This report may provide novel information for melanoma treatment.

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