

Original Article

Programmed cell death 2 functions as a tumor suppressor in osteosarcoma

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Received May 31, 2015; Accepted July 21, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: *Objectives:* To investigate the role of programmed cell death 2 (PDCD2) in osteosarcoma (OS), along with correlations between PDCD2 and CD4⁺/CD8⁺. *Methods:* Sprague-Dawley (SD) rats were randomly assigned to control group and OS group. The OS group rats were subjected to induce models of OS by transplantation with UMR106 cells. Peripheral blood was collected to test the percentages of the CD4⁺ and CD8⁺ cell subsets using flow cytometry (FCM). Western blotting was performed to determine the PDCD2 protein level. The correlations between PDCD2 and CD4⁺/CD8⁺ were analyzed by Pearson correlation coefficient. Besides, specific small interfering RNAs (siRNA) against PDCD2 and nonspecific (NS)-siRNA were transfected into UMR106 cells. Cell viability and invasive ability were determined after transfection. *Results:* CD4⁺ cells percentages were significantly decreased in the OS group, while CD8⁺ cells were significantly increased ($P < 0.05$). The PDCD2 protein levels were markedly lower than that in the control group ($P < 0.05$). Additionally, PDCD2 was positively correlated with CD4⁺ ($R^2 = 0.66$, $P < 0.05$), but was negatively correlated with CD8⁺ ($R^2 = -0.94$, $P < 0.05$). Moreover, the cell viability and invasion ability were significantly higher than that in the control group and the NS siRNA group after transfection with PDCD2 siRNA ($P < 0.05$). *Conclusion:* These results suggest that PDCD2 is involved in the pathogenesis of OS, and PDCD2 may play an important role in tumor suppression. These mechanisms might be related to immune response induced by CD4⁺ and CD8⁺ T cells.

Keywords: Osteosarcoma, programmed cell death 2, CD4⁺, CD8⁺

Introduction

Osteosarcoma (OS) is a relatively rare cancer, which is responsible for 5% of childhood tumors and 8.9% of tumor-related deaths in teenagers [1]. But it is the most frequent pediatric primary malignant bone tumor around the world [2], with males in predominance (male/female = 1.6:1) [3, 4]. Additionally, OS develops rapidly, progresses aggressively and usually has a high occurrence of lung metastasis [5]. Although advances in the tailored therapies and improvement in the 5-year disease-free survival rate have taken place [6-8], amputation is still the principal treatment in most cases [5], resulting in physical disability and psychological distress for both the patients and their families. Therefore, there is an urgent need to unveil the pathogenesis of OS to improve treatment.

Recently, dysregulation of immune system has been reported to be associated with the development of OS [9-11]. A previous study found that programmed cell death (PDCD)1 was significantly increased on both OS patients' peripheral CD4⁺ and CD8⁺ T cells, suggesting that PDCD1 is associated with the pathogenesis of OS and the progression of disease [12]. However, the role of PDCD2 in OS has not been explored. PDCD2, a highly conserved nuclear protein, was identified by Kawakami *et al.* [13]. It has been paid attention since aberrant PDCD2 expression is involved in cell apoptosis [14]. Besides, previous studies reported that PDCD2 plays an important role during embryonic development and stem cell differentiation [15, 16]. In addition, abnormal expression of PDCD2 is associated with many tumors, such as leukemia and gastric cancer [17, 18], and may be regarded as an attractive and a novel

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potential targeting anticancer treatment [17]. Little information is available regarding the correlations between PDCD2 and CD4⁺/CD8⁺ in OS.

Therefore, in the present study, we explored the role of PDCD2 in OS, and the relationship between PDCD2 expression and CD4⁺/CD8⁺ T cell percentages. We firstly established the OS model using transplantation with UMR106 cell line. Next, the percentages of CD4⁺/CD8⁺ were evaluated, and the PDCD2 protein level was confirmed in both OS and health controls. Further, the cell viability and invasive ability were assessed after silencing of expression of PDCD2. Our results may provide an essential research for searching a new target therapy of OS.

Materials and methods

Cell line, rats and tumors

The UMR106 OS cell line, provided by the American Type Culture Collection (ATCC; Manassas, VA, USA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The medium and the drugs were all purchased from Invitrogen, Carlsbad, CA, USA. The cells were maintained in a 5% CO₂ incubator at 37°C.

Fifty-six male Sprague-Dawley (SD) rats (2-3 weeks old, weighing 40-50 g) were used in our experiment. The animal care and use was in consistent with the Guide for Care and Use of Laboratory Animals published by the China National Institutes of Health. All animals were randomly divided into two groups (n = 28): OS group and control group. The animal models of OS were established according to previous studies [19, 20]. Briefly, after washing twice with 0.01 mmol/L phosphate buffer solution (PBS, pH = 7.4), the UMR106 OS cells were re-suspended in PBS at a concentration of 2 × 10⁷/ml. Under sterile conditions, the cell suspension (0.5 ml) and PBS (0.5 ml) were slowly injected into the subcutaneous tissue on the back of OS group rats and control group rats, respectively. At 8-12 days after transplantation, the tumors diameter reached approximately 1.0 centimeters.

Flow cytometry (FCM)

Venous whole blood samples were collected from each rat in the both two groups at 30 days after transplantation, and stored at -80°C until use. Fluorescently labeled anti-CD4⁺ and anti-CD8⁺ monoclonal antibodies were added to the blood samples. After 15 min incubation at room temperature, 1 ml hemolysin was added to the mixture. Then the mixture was kept away from the light at room temperature for another 15 min. After 5 minutes centrifugation at 5000 r/min, the supernatant was discarded, and the cells were washed twice with PBS. The percentages of the CD4⁺ and CD8⁺ cell subsets in the peripheral blood were determined using FCM (Becton Dickinson, USA).

Western blotting

Rats were sacrificed to acquire the tissues. Tumor tissues from the OS group and distal femoral or proximal tibial tissues from the control group were harvested and stored at -80°C until used. For Western blotting analysis, the tissues were washed with PBS. Protein density was determined using BCA assay kit (TaKaRa BIO INC, Japan) according to the manufacturer's instruction. Protein samples (15 µL) from each group were separated by 10-12% standard electrophoresis sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PDVF) membranes (Nihon Millipore Kogyo, Tokyo, Japan). Defatted milk powder (5%) was used to block nonspecific protein-binding sites on the PVDF membranes for 2 h at room temperature. After washing with Tris-buffered saline Tween (TBST), the membranes were incubated overnight at 4°C with anti-PDCD2 antibody (1:500; Proteintech) or anti-β-actin antibody (1:1000, Novus Biologicals, Littleton, CO). Then the membranes were incubated with appropriate peroxidase-conjugated secondary antibodies at room temperature. After another washing with TBST, enhanced chemiluminescence and densitometric analysis was performed. Finally, Gel-Pro analyzer software (version 4, Toyobo, Osaka, Japan) was used to assess the band.

Transfection

After post-culturing in 10% FBS/DMEM for 24 h, the UMR106 cells were seeded in a 24-well plate. The small interfering RNAs (siRNA)

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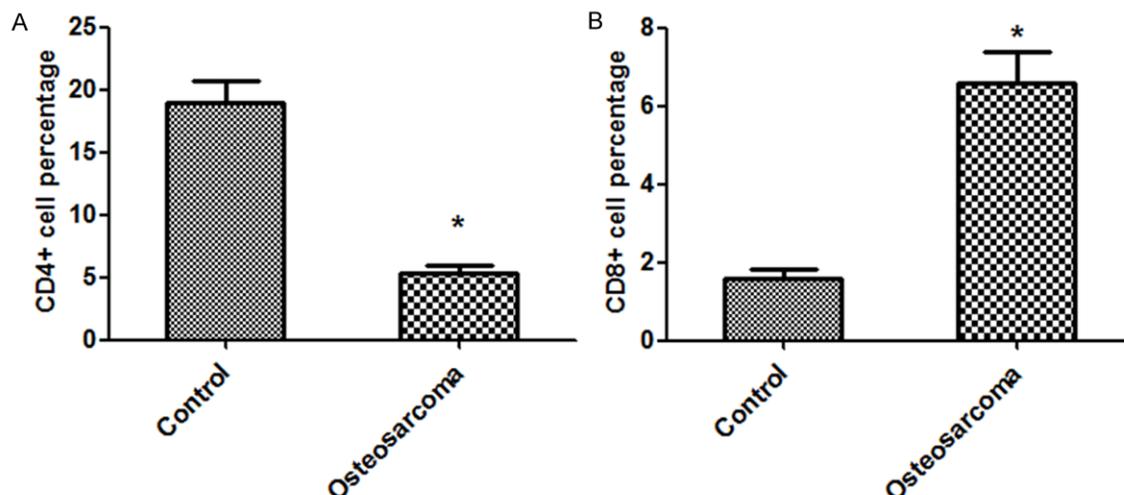


Figure 1. Expression of CD4⁺ and CD8⁺ T cells after transplantation with UMR106 cells. A. Expression of CD4⁺ cells after transplantation with UMR106 cells; B. Expression of CD8⁺ cells after transplantation with UMR106 cells; **P* < 0.05 compared with the control group.

against PDCD2 and nonspecific (NS)-siRNA (Qiagen, Venlo, Netherlands) were transfected into UMR106 cells with Lipofectamine 2000 (Invitrogen, USA) based on the manufacturer's protocols. Experiments were carried out 48 h later for further analysis.

Cell proliferation assay

The UMR-106 cells were collected for measurement of cell viability using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the manufacturer's guidelines. Briefly, the UMR 106 cells were washed with PBS and placed in 96-well plate at a final concentration of 1×10^5 cells/well. After transfection at 0 h and 24 h, 10 μ l MTT (0.5 mg/mL) was added to each well, and the plates were incubated at 37°C for another 2 h. The absorbance at 570 nm was measured using a microplate reader (Molecular Devices Corp., Sunnyvale CA). Experiments were performed 3-5 times.

Matrigel invasion assay

The Matrigel invasion assay was conducted to evaluate the effects of PDCD2 on the invasive properties of the UMR106 cells. A 12-well plate with Transwell inserts insisting of 12.0- μ m pore (Becton-Dickinson, Franklin Lakes, NJ, USA) were coated with 200 μ l Matrigel matrix basement membrane (BD Biosciences, San Jose,

CA, USA). The cells were harvested after transfection with siRNAs for 48 h later, and then the cells were suspended in serum-free DMEM media at a density of 1×10^5 cells/ml. A total of 100 μ l cell suspensions from each sample were placed to each well in triplicate. After incubation 48 h at 37°C in a humidified incubator containing 5% CO₂, the membranes were stained with 0.5% crystal violet in methanol and the remaining cells were calculated using a light microscope (Olympus, Tokyo, Japan) at $\times 100$ magnification. Each experiment was carried out in duplicate. Eight high powered fields were randomly collected and counted for each membrane.

Statistical analysis

One-sample K-S test was firstly performed to confirm the normal distribution. Chi-square test or rank-sum test was used to analyze the enumeration data. The collected data, expressed as mean \pm standard deviation (SD), were analyzed by statistical package for the social sciences (SPSS) (version 19.0; SPSS Inc., Chicago, IL). Student t-test and analysis of variance (ANOVA) were used to analyze the measurement data for two groups and more than three groups, respectively. Further post-hoc Tukey test was performed to evaluate the comparisons between groups. Pearson correlation coefficient was used to determine the relationship between PDCD2 and CD4⁺/CD8⁺. A statistical significance was defined when *P* < 0.05.

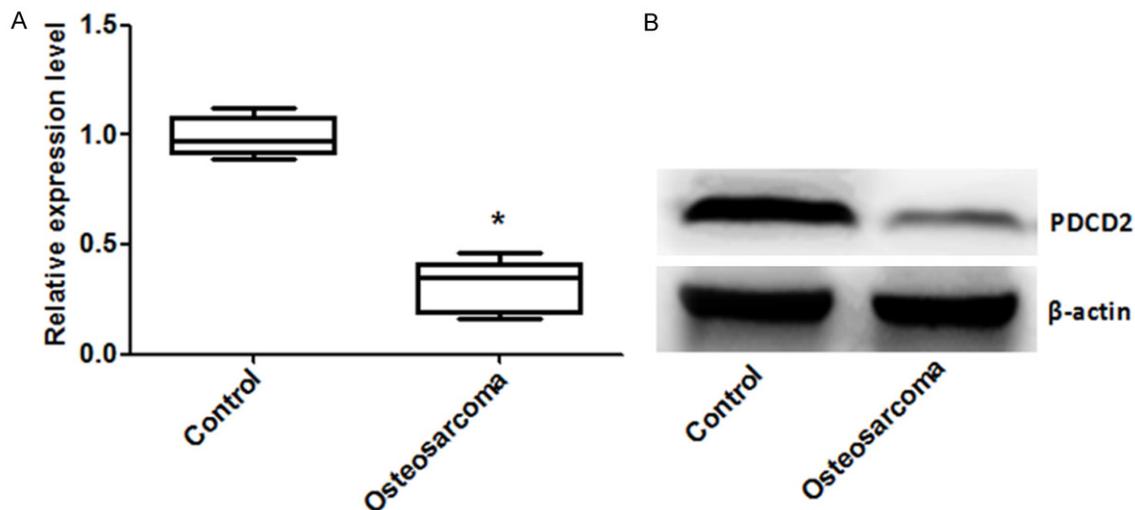


Figure 2. Expression of PDCD2 after transplantation with UMR106 cells. A. Relative protein expression level of PDCD2; B. Western blotting picture of PDCD2; PDCD, programmed cell death; * $P < 0.05$ compared with the control group.

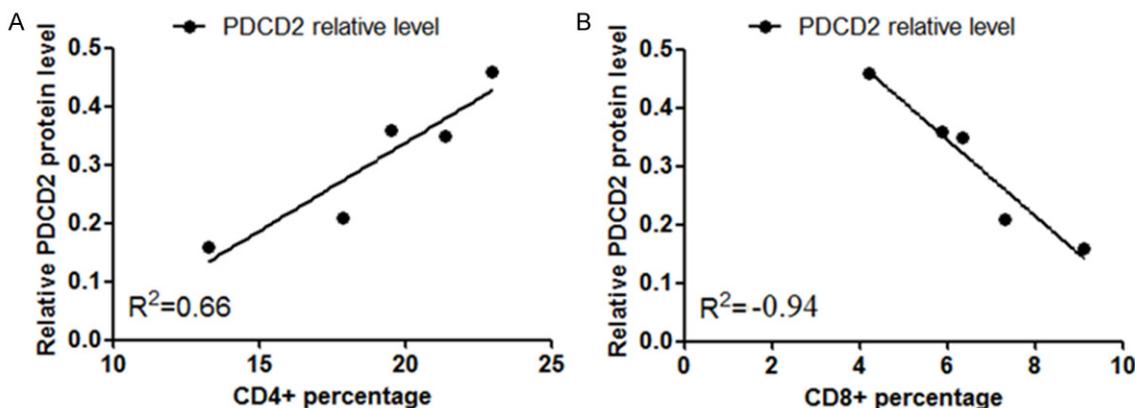


Figure 3. Correlations between PDCD2 and CD4⁺/CD8⁺. A. The correlation between PDCD2 and CD4⁺; B. The correlation between PDCD2 and CD8⁺; PDCD, programmed cell death.

Results

Expression of CD4⁺ and CD8⁺ T cells after transplantation with UMR106 cells

To understand the expression of CD4⁺ and CD8⁺ T cells in OS, we firstly induced the model of OS by transplantation with UMR106 cells. The FCM results showed that the percentage of CD4⁺ cells percentage was significantly decreased in the OS group than that in the control group ($P < 0.05$), but CD8⁺ T cells percentage was significantly increased in the OS group ($P < 0.05$) (Figure 1A and 1B).

Expression of PDCD2 after transplantation with UMR106 cells

To confirm the expression of PDCD2 in UMR106 cells, we performed Western blotting to assess the protein expression of PDCD2. As shown in Figure 2A and 2B, the protein expression level of PDCD2 was lower than that in the control group, and there was a significance between the two groups ($P < 0.05$).

Correlations between PDCD2 and CD4⁺/CD8⁺

To evaluate the relationship between the expression of PDCD2 and CD4⁺/CD8⁺, Pearson

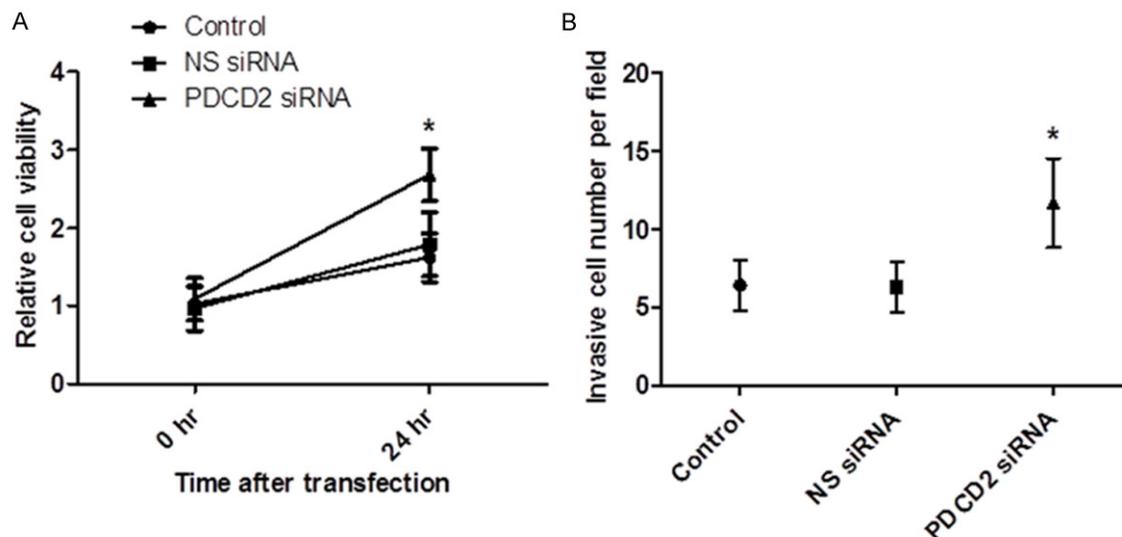


Figure 4. Silencing of PDCD2 to UMR106 cells increases cell viability and invasion ability. A. Silencing of PDCD2 to UMR106 cells increases cell viability; B. Silencing of PDCD2 to UMR106 cells increases invasion ability; PDCD, programmed cell death; siRNA, small interfering RNAs; NS, nonspecific; * $P < 0.05$ compared with the control group.

correlation coefficient was carried out. As shown in **Figure 3A** and **3B**, we found that the expression of PDCD2 was correlated strongly positive with $CD4^+$ ($R^2 = 0.66$, $P < 0.05$), but was correlated strongly negative with $CD8^+$ ($R^2 = -0.94$, $P < 0.05$).

Silencing of PDCD2 to UMR106 cells increases cell viability and invasion ability

To examine the effect of silencing of PDCD2 on the cell viability and invasion ability, we performed the target sequence for PDCD2-specific siRNA into UMR106 cells. The MTT results showed that the cell viability was significantly higher than that in the control group and the NS siRNA group by transfection with PDCD2 siRNA ($P < 0.05$, **Figure 4A**). In addition, the Matrigel invasion assay showed that the average invasive cell numbers pre field in the control group and the NS siRNA group were 5-6, while in the PDCD2 siRNA group it was 12.3. The cell invasion ability was significantly increased in the PDCD2 siRNA group than that in the other two groups ($P < 0.05$, **Figure 4B**).

Discussion

PDCD2 protein is responsible for embryonic development and tissue remodeling by induction of apoptosis, and alteration of PDCD2 expression is associated with development of human cancers. In the present study, we

explored the role of PDCD2 in OS and the correlations between PDCD2 and $CD4^+/CD8^+$. We found that the PDCD2 protein levels were significantly reduced in the OS group than that in the control group. In addition, the cell viability and invasion ability were significantly increased after silencing of the expression of PDCD2. Moreover, PDCD2 was correlated with both the $CD4^+$ and $CD8^+$ cells percentages. These results demonstrate that PDCD2 is involved in the pathogenesis of OS, and PDCD2 may function as an important factor in tumor suppression.

PDCD2 gene, the human homologue of the rat Rp8 gene, is reported to be located on chromosome 6q27, which encodes a nuclear protein found in many tissues [17]. It has been identified as a target of the transcriptional repressor BCL6 that is required for lymph node germinal center development. The function of PDCD2 is mainly explored in hematological tumors in previous studies. A study conducted by Baron *et al.* [21] indicated that the apoptosis in human erythroleukemia cells induced by expression of PDCD2 through activation of caspases. Another study showed that knockdown of PDCD2 expression in both leukemia Jurkat cells and lung cancer A549 cells could impair cell proliferation and progression to S phase of the cell cycle [17]. Recently, the role of PDCD2 in other human cancers has gained more attention. Zhang *et al.* reported that loss of PDCD2

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expression is involved in the development and progression of gastric cancer, and that the cell growth is arrested at the early S phase of the cell cycle [18]. Fan *et al.* showed that the expression of PDCD2 decreased in multidrug-resistant colon cancer cells [22]. However, the role of PDCD2 in OS has been rarely reported. Therefore, we speculated that PDCD2 may be associated with OS.

To confirm the hypothesis, we evaluated the protein expression level of PDCD2 in OS model. The OS model was successfully established by transplantation with UMR106 cells. The Western blotting showed that the PDCD2 protein expression was significantly decreased in the OS model group than that in the control group. The results indicated that PDCD2 might be involved in the pathogenesis of OS. To further assess the function of PDCD2 in OS, we silenced the expression of PDCD2 in UMR106 cell line. The cell viability and invasion ability were determined. The results demonstrated that silencing of PDCD2 expression resulted in increase of cancer cell viability and invasion ability, indicating that PDCD2 might be function as a potential tumor suppression factor. The above results were in line with previous studies [18, 21, 22].

It has been well accepted that CD4⁺ T cells are essential for antitumor immune responses [23-26]. CD4⁺ T cells remove tumors by activation and recruitment of some effector cells, such as macrophages and eosinophils [23, 27]. However, CD8⁺ T cells can directly and destroy tumor masses *in vivo* by infiltrating the cancer cell nest [28]. Besides, CD4⁺ T cells are benefit for prime CD8⁺ T cells responses [25]. Moreover, CD4⁺ T cells play an important role in maintaining effector functions of CD8⁺ T cells by secreting some cytokines (e.g. interleukin 2) that is required for growth and proliferation of CD8⁺ T cells [29]. Furthermore, the cooperative role of CD4⁺ T cells and CD8⁺ cytotoxic T lymphocytes (CTLs) has been reported to in tumor suppression *in vivo* [30]. Zheng *et al.* found that the OS patients presented significantly upregulated percentages of PDCD1 on both peripheral CD4⁺ and CD8⁺ T cells. Therefore, we speculated that PDCD2 expression might also be associated with peripheral CD4⁺ and CD8⁺ T cells. We found that OS group presented lower CD4⁺ cells percentages and higher CD8⁺ T cells. The results indicated that the immune function of

OS patients might be inhibited, leading to reduced ability to remove tumor cells. Additionally, the correlations between the PDCD2 expression and CD4⁺ and CD8⁺ T cells percentages were investigated. Our results showed that PDCD2 was strongly positive with CD4⁺ cells percentages, but negatively correlated with CD8⁺ T cells percentages, suggesting that the tumor suppression of PDCD2 might be involved with immune response.

In conclusion, our results suggest that PDCD2 is involved in the pathogenesis of OS, and PDCD2 may play an important role in tumor suppression. These mechanisms might be related to immune response induced by CD4⁺ and CD8⁺ T cells.

Disclosure of conflict of interest

None.

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