Clinical and prognostic significance of lost or decreased PDCD5 expression in human epithelial ovarian carcinomas

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Abstract. Programmed cell death 5 (PDCD5) is a novel apoptosis-promoting protein. Although the decreased expression of PDCD5 has been recently found in a few types of human tumors, the status and significance of PDCD5 in ovarian cancer has not been evaluated. In the present study, we detected PDCD5 expression in 20 normal human ovaries and 26 serous cystadenomas and 41 serous cystadeno-carcinomas by RT-PCR, Western blotting and immunohisto-chemistry, and analyzed the relationship between PDCD5 expression and clinicopathological data or patient survival. PDCD5 was expressed in all normal ovaries and serous cystadenomas, 80% (16/20) of normal ovarian tissues and 76.9% (20/26) of serous cystadenomas with moderate or strong PDCD5 protein expression. In contrast, 22% (9/41) of serous cystadenocarcinomas had no detectable PDCD5 protein expression and 46.3% (19/41) exhibited weak PDCD5 expression. The overall expression of PDCD5 in serous cystadenocarcinoma was significantly lower compared with normal ovarian tissues or serous cyst-adenomas (p<0.01). Furthermore, lost or decreased PDCD5 expression in serous cystadenocarcinomas was associated significantly with FIGO stage (p<0.05) and poorer disease-specific survival of patients (p<0.05). In conclusion, our data suggest that lost or reduced PDCD5 expression may contribute to the pathogenesis of human serous cyst-adenocarcinomas.

Introduction

Ovarian cancer is the leading cause of death from gyneco-logical malignancy worldwide and serous cystadenocarcinomas

is the most common histologic type of epithelial ovarian carcinomas. Since early symptoms of patients with serous cystadenocarcinomas are frequently nonspecific, and there is a lack of reliable early detection methods, the majority of ovarian cancer patients were diagnosed at late stage, and the prognosis is very poor with a 5-year survival rate of ~45% (1). Therefore, it is necessary to develop drugs to target specific molecular pathways and improve understanding of the molecular pathways involved in ovarian carcinogenesis.

Programmed cell death 5 (PDCD5), also designated as TF-1 cell apoptosis-related gene-19 (TFAR19), is a novel gene cloned from TF-1 cells undergoing apoptosis (2), and it is up-regulated in the process of cell apoptosis and translocation from the cytoplasm to the nucleus (3). PDCD5 is a strong candidate of apoptosis-regulated protein and its overexpression promotes apoptosis triggered by certain stimuli, while blocking PDCD5 action suppresses apoptosis (2,4). In addition, adeno-virus carrying PDCD5 gene exerts potent antitumor effect on human leukemic cells *in vitro* and *in vivo* (5). PDCD5 can interact with Tip60 (a histone acetyltransferase), and promote DNA damage-induced apoptosis. The phosphorylation of PDCD5 by CK2, a multifunctional kinase, is an important process for its apoptotic potential (6,7). Recent studies have demonstrated that PDCD5 is down-regulated in some human tumor tissues such as gastric cancer (8), hepatocellular carcinoma (9), breast cancer (10), acute and chronic myeloid leukemia (11) and glioma (12). However, PDCD5 expression and its association with prognosis in gynecological cancer have not been evaluated.

In this study, we detected the status of PDCD5 expression in three kinds of human serous cystadenocarcinoma cell lines, normal ovarian tissues and serous ovarian tumors using RT-PCR, Western blotting and immuohistochemistry. Furthermore, we analyzed the association of PDCD5 expression with clinicopathological features and survival of patients.

Materials and methods

Tumor samples. Sixty-seven serous ovarian tumor samples (26 serous cystadenomas and 41 serous cystadenocarcinomas) were obtained from patients aged between 35 and 74 years (median, 53 years) who underwent surgical operations at the

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Department of Gynecology, Qilu Hospital and the Second Hospital, Shandong University from 2001 to 2007. None of the patients studied had received adjuvant immunosuppressive treatments such as radiotherapy or chemotherapy prior to surgery in order to eliminate their effects on gene expression. Tumor samples were graded based on Gynecologic Oncology Group criteria and staged in accordance with the International Federation of Gynecology and Obstetrics (FIGO) system. Survival data were from 30 cases of 41 patients with serous cystadenocarcinomas. The disease-specific survival time was defined as the time from primary surgery to death of the patient from ovarian cancer or to the end of the follow-up. Twenty normal ovarian tissues were obtained from the normal ovaries of donors during surgery for other gynecological diseases in Qilu Hospital, Shandong University. The final protocol for the use of patient samples in our study was approved by the local Institutional Review Board and informed consent was obtained from all patients and controls.

Cell lines. Human serous cystadenocarcinoma cell lines SKOV3 was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), CAOV3 and NIH-OVCAR3 were purchased from China Center for Type Culture Collection (Wuhan, China). SKOV3 were cultured and maintained in Rosewell Park Memorial Institute (RPMI)-1640 and containing 10% fetal calf serum (FMG Bio, Shanghai, China). CAOV3 was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. NIH-OVCAR3 was cultured in RPMI-1640 medium, supplemented with 20% fetal bovine serum and 0.01 mg/ml bovine insulin. All media contain 1% penicillin/streptomycin.

Semi-quantitative RT-PCR. Total RNA was extracted using a modified TRIzol® one-step extraction method (Invitrogen Corp., Carlsbad, CA, USA). RNA concentrations were determined based on the absorbance at 260 nm. Total RNAs (3 μg) were reverse transcribed to cDNA using the Reverse-Transcribe kit (Promega Co., Madison, WI, USA). PCR was performed using PDCD5 specific primers (sense 5'-CCA TGG CGG ACG AGG AGC TTG-3', and anti-sense 5'-TCA ATA ATC GTC ATC TTC ATC-3') for 30 cycles at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec followed by an extension cycle at 72˚C for 7 min. Amplified cDNAs were analyzed by 2% agarose gel electrophoresis. Human ß-actin primer was as a positive control. RT-PCR was performed at least 3 times for each sample.

SDS-PAGE and Western blotting. The proteins were extracted from tissue samples and ovarian cell lines using a modified TRIzol one-step extraction method. The concentration of the protein was determined by Bradford analysis. The protein extract was dissolved in a loading buffer (1 mM Tris·Cl, 3% SDS, 60% glycerol and 75 mM DTT) and each sample was analyzed by SDS-PAGE on a 15% gel. The PVDF membrane was incubated with mouse anti-human PDCD5 (1:1000) or rabbit anti-human ß-actin (1:2000) at 4˚C overnight. Immunoreactive bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (ECL, Amersham Biosciences, UK).
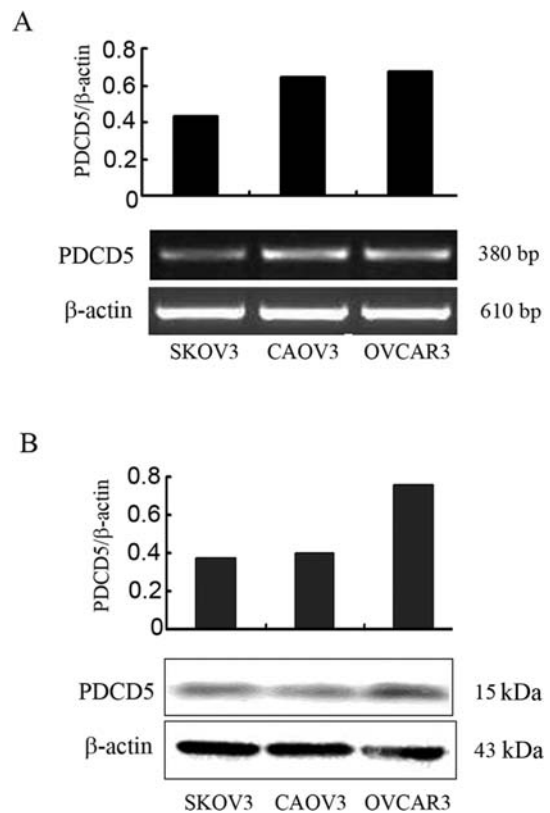

Figure 1. Expression of PDCD5 mRNA and protein in human serous cystadenocarcinoma cell lines. The expression of PDCD5 mRNA and protein in human serous cystadenocarcinoma cell lines, SKOV3, CAOV3 and OVCAR3, were detected by RT-PCR (A) and Western blotting (B). The bands of interest were further analyzed by densitometer. Data were normalized to ß-actin. The upper panel shows the normalized PDCD5 expression level.

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded tissue sections from 20 normal ovaries and 67 serous ovarian tumors were cut at 4-6 μm and transferred to slides. The tissues were deparaffinized in xylene and rehydrated through an gradient alcohols. The slides were washed, blocked for endogenous peroxidase activity, preincubated with goat serum and then incubated with a polyclonal rabbit anti-PDCD5 antibody (1: 250) for 1 h at room temperature in a humid chamber. Secondary staining with HRP-conjugated anti-rabbit IgG was performed using a MaxVision™ kit and a DAB Peroxidase substrate kit (Maixin Co., Fuzhou, China). The nuclei were counterstained with hematoxylin. Negative control for the specificity of immunohistochemical reactions was performed by replacing the primary antibody with IgG of non-immunized rabbit. IHC was performed twice for each sample. All slides were evaluated by two independent pathologists without knowledge of the patients. The PDCD5 staining was divided into four grades according to staining intensity: - (score 0), + (score 1), ++ (score 2), and +++ (score 3). The percentage of PDCD5-positive cells was also classified into four categories: - (<1%, score 0), + (1-33%, score 1), ++ (34-66%, score 2), and +++ (67-100%, score 3). The sum of intensity and percentage scores was used as the final PDCD5 staining score, defined as follows: no expression (total score: 0); weak expression (total score: 1-2); moderate expression (total score: 3-4); strong expression (total score: 5-6).
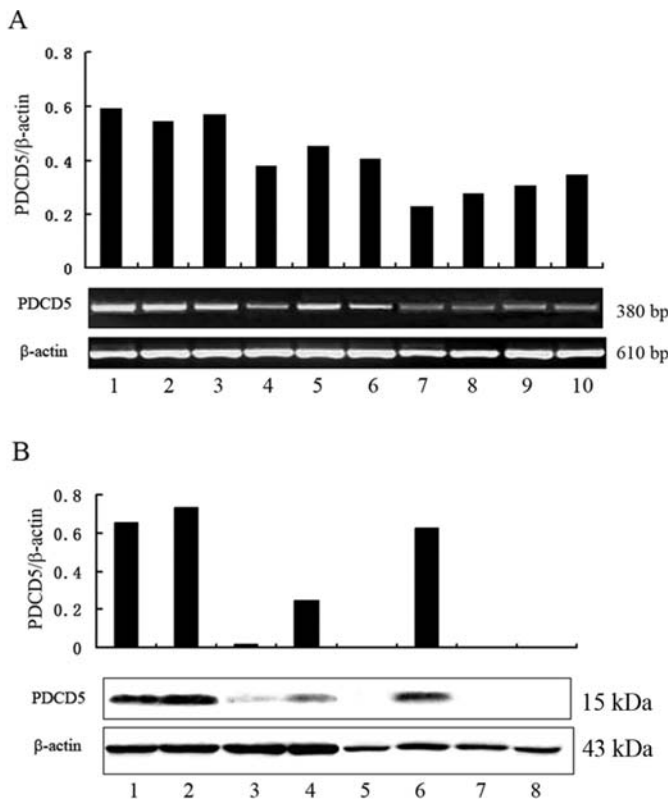
Figure 2. Expression of PDCD5 mRNA and protein expression in human normal ovaries and serous ovarian tumors. (A) The expression levels of PDCD5 mRNA in human ovarian tissues detected by RT-PCR. Lanes 1, 2, normal ovaries; 3, 4, serous cystadenomas; 5-10, serous cystadenocarcinomas; the bands of interest were further analyzed by densitormeter. Data were normalized to ß-actin. (B) The expression levels of PDCD5 protein in human ovarian tissues detected by Western blotting. Lane 1, normal ovaries; 2, serous cystadenomas; 3-8, serous cystadenocarcinomas; the bands of interest were further analyzed by densitormetry. Data were normalized to ß-actin.

When there were discrepancies between two pathologists, the average score was determined.

Statistical analysis. The Chi-square and Fisher's exact test was used to compare the expression of PDCD5 with clinico-pathological parameters. Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. P<0.05 was considered statistically significant. The calculations were performed using the SPSS statistical software.

Results

Expression of PDCD5 mRNA and protein in human ovarian cancer cell lines. To explore the potential roles of PDCD5 in ovarian cancer, we detected the status of PDCD5 expression in three ovarian cancer cell lines which derived from serous cystadenocarcinomas by RT-PCR and Western blotting. As shown in Fig. 1, the OVCAR3 cell line expressed high levels of PDCD5 mRNA and protein, whereas the SKOV3 cell line showed low levels of PDCD5 expression. Although CAOV3 cells showed relatively high PDCD5 mRNA expression, their protein expression was relatively low. These results suggested that the expression of PDCD5 may be decreased in human serous cystadenocarcinomas.

Expression of PDCD5 mRNA and protein in human normal ovarian tissues and ovarian carcinomas. To explore the expression of PDCD5 in ovarian carcinoma, we detected PDCD5 mRNA expression in normal ovarian tissues and serous ovarian tumors by RT-PCR. As shown in Fig. 2A, high levels or moderate of PDCD5 mRNA expression were observed in serous cystadenomas as well as normal ovarian tissues. However, 64.3% (9/14) of serous cystadenocarcinoma samples exhibited loss or reduction of PDCD4 mRNA expression. Then, the expression of PDCD5 protein in ovarian carcinoma was examined by Western blotting and immuno-histochemistry (IHC). As shown in Fig. 2B, PDCD5 protein expression was obviously reduced or even lost in serous cystadenocarcinomas compared with normal ovarian tissues and serous cystadenomas. The results from IHC showed that PDCD5-specific staining was mainly found in the cytoplasm of normal ovarian surface epithelial cells, serous cystadenomas epithelial cells and tumor cells of serous cystadeno-carcinomas, rarely in the nucleus. All normal ovaries and serous cystadenomas tested by IHC were positive for PDCD5 expression. Among them, 80% (16/20) of normal ovarian tissues and 76.9% (20/26) of serous cystadenomas showed moderate or strong PDCD5 protein expression (Fig. 3A and B). In contrast, 46.3% (19/41) of serous cystadenocarcinomas exhibited weak PDCD5 expression and 22% (9/41) had no detectable PDCD5 protein expression (Fig. 3C and D). The overall expression of PDCD5 in serous cystadenocarcinoma was significantly lower compared with normal ovarian tissues or serous cystadenomas (p<0.01) (Fig. 3E).

Correlation of the expression level of PDCD5 with the FIGO stage of serous cystadenocarcinoma. To determine the clinical significance of lost or reduced PDCD5 expression in serous cystadenocarcinoma, we examined the correlation of PDCD5 expression with the clinicopathological parameters of serous cystadenocarcinoma by Chi-square and Fisher's exact test. The results showed that there was no significant correlation among PDCD5 expression and age, site of origin, metastasis and pathological grade (Table I). However, the expression of PDCD5 correlated significantly with clinical FIGO stage. The percentage of cases with PDCD5 high expression is lower in FIGO stage III and IV patients (19.2%, 5 of 26) than that in FIGO stage I and II patients (53.3%, 8 of 15) (p<0.05).

Loss or reduction of PDCD5 expression was significantly associated with survival of patients with serous cystadeno-carcinoma. To assess the association of PDCD5 expression with patient survival, the survival data from 30 patients with serous cystadenocarcinomas were generated by follow-up. According to the final PDCD5 staining score in the results of IHC, these patients were divided into a high expression group (score: 3-6) and a low expression group (score: 0-2). The difference in survival time between patients with high PDCD5 expression tumors (n=16) and those with low PDCD5 expression tumors (n=14) was evaluated by Kaplan-Meier method and log-rank test. The result demonstrated that patients with a low level of PDCD5 expression had a significantly poorer disease-specific survival than those with a high level of PDCD5 expression (p<0.05). This indicates that the level
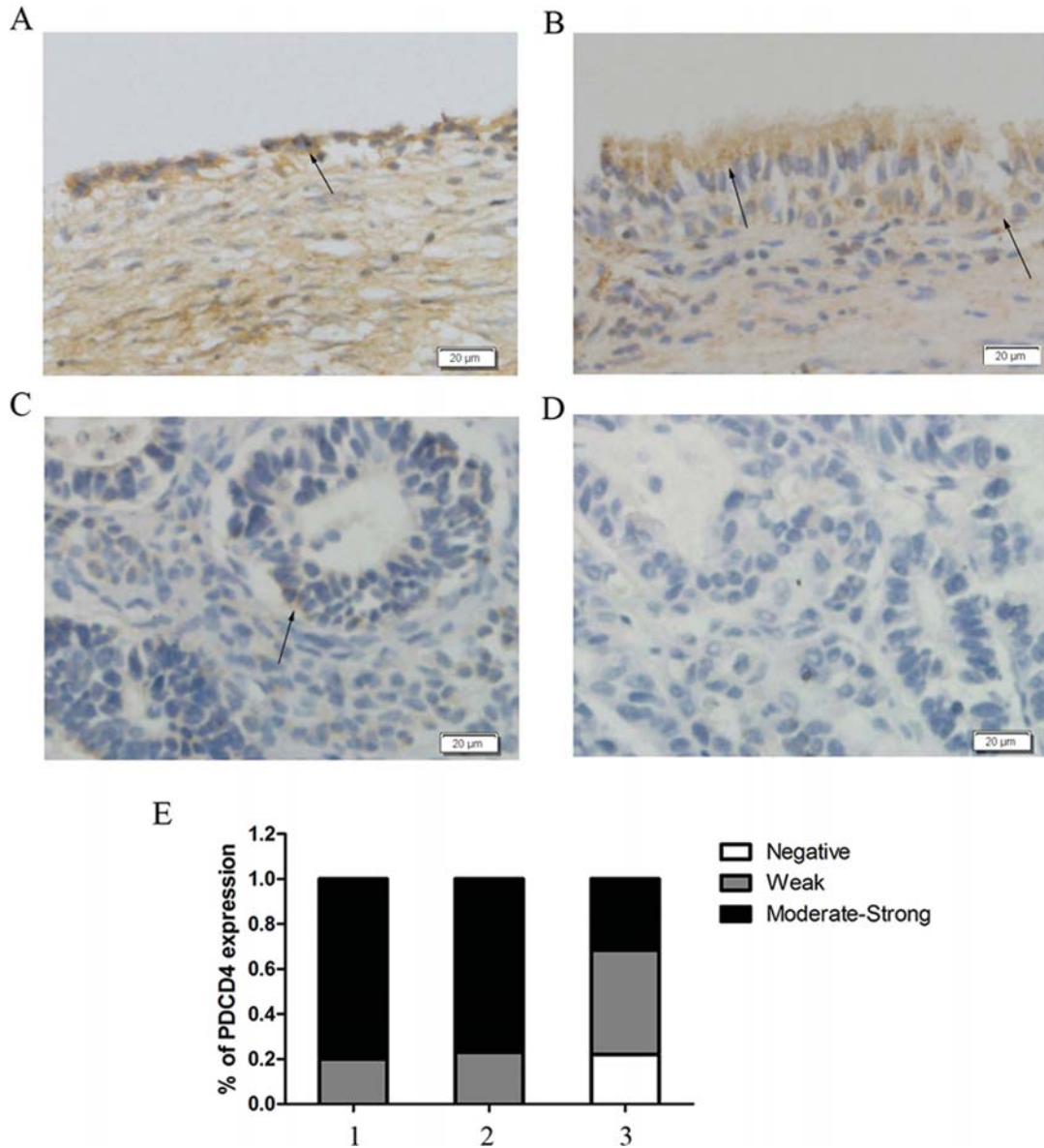
Figure 3. PDCD5 protein expression in normal ovaries and serous ovarian tumors detected by immunohistochemistry. (A) Strong cytoplasmic PDCD5 staining (black arrow) in normal ovarian epithelial cells. (B) Moderate cytoplasmic PDCD5 staining (black arrows) in serous cystadenoma epithelial cells. (C) Weak cytoplasmic PDCD5 staining (black arrow) in tumor cells of serous cystadenocarcinoma. (D) No cytoplasmic PDCD5 staining in tumor cells of serous cystadenocarcinoma (magnification, x400). (E) Overall level of PDCD5 expression was significantly lower in serous cystadenocarcinomas compared with normal ovarian tissues or serous cystadenomas according to the result of IHC ($p < 0.05$).

of PDCD5 expression significantly correlated with prognosis of patients with serous cystadenocarcinoma (Fig. 4).

Discussion

In the present study, we demonstrated, for the first time, that PDCD5 expression decreased significantly in serous cystadenocarcinomas compared with normal ovaries or serous cystadenomas tissues, and correlated with the FIGO stage of serous cystadenocarcinomas. More importantly, longitudinal studies of a cohort of patients revealed that PDCD5 expression had statistically significant impact on the prognosis of patients with serous cystadenocarcinoma.

Epithelial ovarian carcinoma is one of the most common gynecologic malignancies in women. Many tumor suppressor genes and apoptosis-related genes, such as p53, Bcl-2, PTEN,

BRCA and ARH1, play important roles in the development of epithelial ovarian carcinoma. Mutations in the p53 tumor suppressor gene are found in ~50% of advanced stage carcinomas and the apoptotic inhibitor, Bcl-2, is overexpressed in ~40-60% of epithelial ovarian carcinomas (13,14). Restoration of p53, PTEN and ARH1 expression reduces the malignant phenotype of tumor cells (15-17). PDCD5 is a strong candidate of apoptosis-regulated protein. Recent studies have demonstrated that PDCD5 is down-regulated in some human tumor tissues such as gastric cancer, hepatocellular carcinoma, breast cancer, glioma and acute and chronic myeloid leukemia (8-12). In the current study, we demonstrated that the expression of PDCD5 mRNA and protein decreased or even lost in serous cystadenocarcinoma (Figs. 2 and 3). Statistical analysis showed the overall expression level of PDCD5 in serous cystadenocarcinoma markedly reduced

Table I. Relationship between PDCD5 expression and clinicopathological characteristics of serous cystadenocarcinoma.

Clinical and pathological features	n	PDCD5 expression		p-value
		Negative-Weak	Moderate-Strong	
Age				
<6	25	18	7	0.7319
≥6	16	10	6	
Site of origin				
Left	7	6	1	0.4347
Right	11	8	3	
Double	23	14	9	
Metastasis				
Positive	32	22	9	0.6979
Negative	9	6	4	
Pathological grade				
I-II	21	15	6	0.7442
III	20	13	7	
FIGO stage				
I-II	15	7	8	0.0376
III-IV	26	21	5	

There was no significant correlation of lost or reduced PDCD5 expression with age, site of origin, metastasis or pathological grade (p>0.05). However, the loss or reduction of PDCD4 expression was significantly associated with FIGO stage of patients with serous cystadenocarcinomas (p=0.0376). The p-value was calculated by Chi-square and Fisher's exact test.
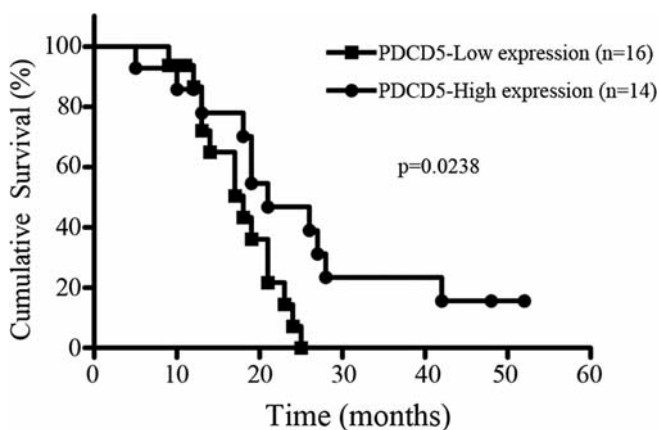

Figure 4. The loss or reduction of PDCD5 expression was associated with poor prognosis in patients with serous cystadenocarcinoma (p<0.05). Patients with a low level of PDCD5 expression had a significantly poorer disease-specific survival than those with a high level of PDCD5 expression.

compared with normal ovarian tissues or with serous cystadenomas (Fig. 4). These data suggested that PDCD5 may contribute to the development of serous cystadenocarcinoma. However, more studies are needed to clarify this issue.

Until now, little is known about the clinical significance of PDCD5 down-regulation in tumors. Previous studies showed that the decreased expression of PDCD5 in astrocytoma, renal clear cell carcinomas significantly correlated

with the high-grade tumor (12,18), and the reduced expression of PDCD5 correlates with short survival periods of patients with gastric tumor tissues (8,19). However, the association of PDCD5 expression in ovarian cancer with prognosis is still unclear. In this study, we revealed that loss or reduction of PDCD5 expression was significantly associated with FIGO stage of serous ovarian carcinoma and with disease-specific survival of patients with serous cystadenocarcinoma but not with pathological grade. The data suggest that loss or reduction of PDCD5 expression may also be a prognostic factor for serous cystadenocarcinoma. It has been reported that adenovirus-mediated PDCD5 gene transfer sensitizes K562 cells to apoptosis induced by idarubicin *in vitro* and *in vivo*, and blocking PDCD5 action by anti-PDCD5 antibody is able to suppress apoptosis induced by etoposide and knocking down its expression by PDCD5-specific siRNA attenuates cell apoptosis induced by Bax overexpression (4,20). Our preliminary result also showed overexpression of PDCD5 in the glioma cells can enhance cisplatin induced apoptosis (unpublished data), suggesting PDCD5 may prolong survival time of patients with cancers by enhancing apoptosis of tumor cells. However, the mechanism of PDCD5 expression affecting the prognosis of patients remains to be further investigated.

In conclusion, we found that PDCD5 expression is clearly reduced or lost in serous cystadenocarcinoma compared with normal ovaries and serous cystadenomas tissues. Moreover, we identified loss or reduction of PDCD5 expression to be associated with progression of serous cystadenocarcinoma.

Acknowledgements

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