Expression and Prognostic Significance of PDCD4 in Human Epithelial Ovarian Carcinoma

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Abstract. *Background: Programmed cell death 4 (PDCD4) is a newly discovered tumor suppressor. The aim of this study was to investigate the expression and prognostic significance of PDCD4 in epithelial ovarian cancer. Materials and Methods: PDCD4 expression in 20 normal human ovaries and 69 serous ovarian tumors was examined by RT-PCR and immunohistochemistry. The relationships between PDCD4 expression, clinicopathological data and patient survival were evaluated. Results: PDCD4 expression was found to be lost or significantly lower in serous cystadenocarcinomas compared with that in normal ovaries and serous cystadenomas ($p<0.05$). The loss or reduction of PDCD4 expression in serous cystadenocarcinomas was significantly associated with higher pathological grade ($p=0.0118$) and poorer disease-specific survival of patients ($p=0.0011$). Multivariant Cox regression analysis revealed that PDCD4 expression was an independent prognostic factor for serous cystadenocarcinoma. Conclusion: Lost or reduced PDCD4 expression is associated with the progression of serous cystadenocarcinomas and may serve as an important prognostic marker.*

Epithelial ovarian cancer is the third leading common gynecological cancer and primary cause of gynecological cancer death in women (1). Serous cystadenocarcinoma is the most common histological type of epithelial ovarian carcinoma (2, 3). Early symptoms of ovarian cancer patients are generally minor and easily overlooked. Two-thirds of patients have advanced metastatic lesions when diagnosed. Over the past three decades, there has been little improvement in the 5-year survival for patients with ovarian carcinomas (4). For this reason, there is a pressing need for the development of novel diagnostic and therapeutic methods to manage effectively advanced and recurrent ovarian carcinomas.

Programmed cell death 4 (PDCD4) is a newly identified tumor suppressor which directly interacts with the eukaryotic initiation factor (eIF) 4A complex to inhibit protein translation (5). Overexpression of PDCD4 inhibited tumor promoter-induced transformation in the mouse JB6 model system (6). Furthermore, PDCD4-deficient mice developed spontaneous tumors of lymphoid origin (7) and PDCD4 transgenic mice showed significant resistance to tumor induction (8). Recently, it was reported that PDCD4 protein was reduced to different degrees or lost in lung, liver, and colorectal cancer, and glioma (9-13). However, PDCD4 expression and its association with prognosis in gynecological cancer has not been evaluated.

To investigate the expression pattern and prognostic significance of PDCD4 in epithelial ovarian cancer, PDCD4 expression was examined in normal ovarian tissues and benign or malignant serous ovarian tumors, and the relationship between PDCD4 expression, clinicopathological features and patient survival was analyzed.

Materials and Methods

Tumor samples. Sixty-nine serous ovarian tumor samples (26 serous cystadenomas and 43 serous cystadenocarcinomas) were obtained from patients aged between 35 and 74 years (median=53 years) who underwent surgical operations at the 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

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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 32 cases of 43 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 at 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.

Semi-quantitative RT-PCR. From 20 normal ovaries of donors and 69 serous ovarian tumor patients, samples of 2 normal ovaries, 2 cystadenomas and 14 cystadenocarcinomas were snap frozen at the time of surgery in liquid nitrogen. Total RNAs were extracted from frozen samples using a modified TRIzol® one-step extraction method (Sangon Biotech Co., Ltd, Shanghai, P. R. China). Total RNAs (3 μg) were reverse transcribed to cDNA using the Reverse-Transcribe Kit (Promega Co., Madison, USA). cDNA (1 μl) was amplified by PCR using PDCD4-specific primers (sense 5' TCA GCG ACA GTG GGA GT 3' and antisense 5'AGC ACG GTA GCC TTA TC 3'). The PCR mixture was denatured at 94˚C for 2 min, followed by 35 cycles of 95˚C for 1 min 30 s, 66˚C for 1 min 30 s, and 72˚C for 1 min 30 s, with an extension cycle at 72˚C for 5 min. Amplified cDNAs were analyzed by 2% agarose gel electrophoresis. RT-PCR was performed at least three times for each sample.

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded tissue sections from 20 normal ovaries and 69 serous ovarian tumors were cut at 4-6 μm and transferred to slides. The tissues were deparaffinized in xylene and rehydrated through an alcohol gradient. The slides were washed, blocked for endogenous peroxidase activity, preincubated with goat serum and then incubated with a polyclonal rabbit anti-PDCD4 antibody (1: 800) (Cat. 3975; ProSci Incorporated, Poway, USA) for 1 h at room temperature in a humid chamber. The slides were washed, incubated with biotin-labeled secondary antibody and streptoavidin-peroxidase for 10 min each. The tissues were stained with DAB Peroxidase Substrate kit (Maixin Co., Fuzhou, P. R. China). The nuclei were counterstained with hematoxylin. Negative controls for the specificity of immunohistochemical reactions were performed by replacing the primary antibody with phosphate-bufferd saline (PBS). IHC was performed twice for each sample.

All slides were evaluated by two independent pathologists without knowledge of the patients. The nuclear and cytoplasmic PDCD4 staining was divided into four grades according to staining intensity: – (score 0), + (score 1), ++ (score 2), and +++ (score 3). The percentage of PDCD4-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 PDCD4 staining score, defined as follows: no expression (total score 0); weak expression (total score 1 and 2); moderate expression (total score 3 and 4); strong expression (total score 5 and 6). When there were discrepancies between two pathologists, the average score was determined.

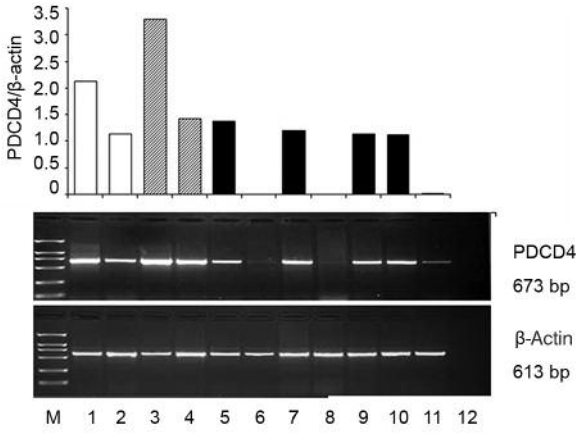

Figure 1. *PDCD4 mRNA expression in normal ovaries and serous ovarian tumors as detected by RT-PCR. M: DL2000 marker; 1, 2: normal ovaries; 3, 4: serous cystadenomas; 5-11: serous cystadenocarcinomas; 12: negative control without cDNA (H_2O). The bands of interest were further analyzed by densitor meter. Data were normalized to β-actin.*

Statistical analysis. The SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. χ^2 test was used to compare the difference of PDCD4 expression between serous cystadenocarcinomas and normal ovarian tissues or serous cystadenomas; χ^2 test was also used to analyze the association of PDCD4 protein expression with clinicopathological parameters. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between PDCD4 expression and patient survival. Cox regression was used to determine whether PDCD4 expression was an independent prognostic factor. A *p*-value less than 0.05 was considered statistically significant.

Results

PDCD4 mRNA expression in normal ovaries and serous ovarian tumors. To explore the expression of PDCD4 in ovarian carcinoma, we firstly detected PDCD4 mRNA expression in normal ovarian tissues and serous ovarian tumors by RT-PCR. As shown in Figure 1, moderate or high levels of PDCD4 mRNA expression were observed in serous cystadenomas as well as normal ovarian tissues. However, 57.1% (8/14) of serous cystadenocarcinomas samples exhibited a loss or reduction of PDCD4 mRNA expression.

PDCD4 protein expression in normal ovaries and serous ovarian tumors. To further determine the status and location of PDCD4 protein expression in serous ovarian tumors, we examined PDCD4 protein expression in normal ovarian tissues and serous ovarian tumors by IHC. As shown in Figure 2, PDCD4-specific staining was mainly found in the cytoplasm of normal ovarian surface epithelial cells, serous cystadenomas epithelial cells and tumor cells of serous cystadenocarcinomas, rarely in the nucleus. All

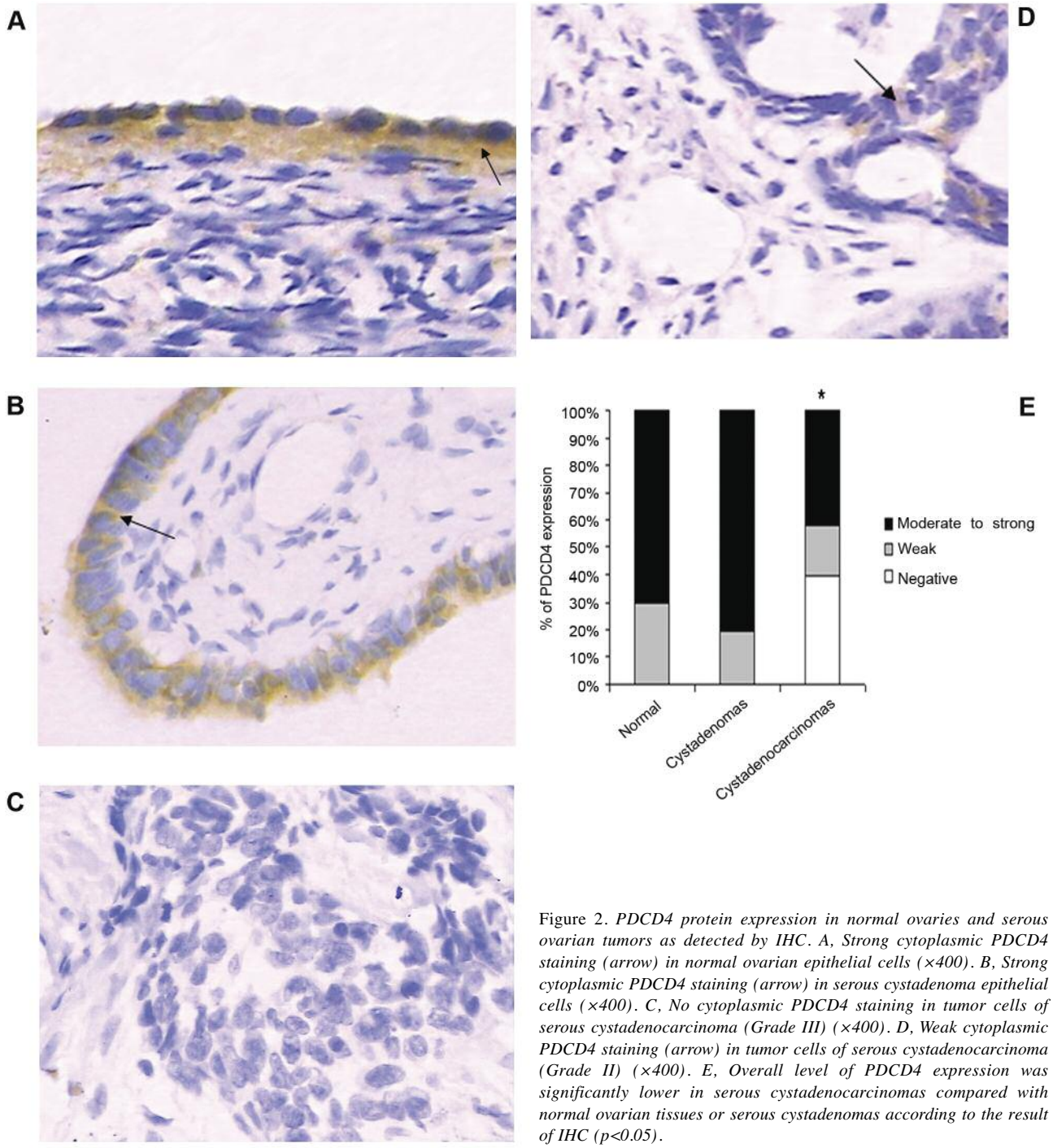

Figure 2. *PDCD4 protein expression in normal ovaries and serous ovarian tumors as detected by IHC. A, Strong cytoplasmic PDCD4 staining (arrow) in normal ovarian epithelial cells (×400). B, Strong cytoplasmic PDCD4 staining (arrow) in serous cystadenoma epithelial cells (×400). C, No cytoplasmic PDCD4 staining in tumor cells of serous cystadenocarcinoma (Grade III) (×400). D, Weak cytoplasmic PDCD4 staining (arrow) in tumor cells of serous cystadenocarcinoma (Grade II) (×400). E, Overall level of PDCD4 expression was significantly lower in serous cystadenocarcinomas compared with normal ovarian tissues or serous cystadenomas according to the result of IHC ($p<0.05$).*

normal ovaries and serous cystadenomas tested by IHC were positive for PDCD4 expression. Among them, 80% (21/26) of serous cystadenomas and 70% (14/20) of normal ovarian tissues showed moderate or strong PDCD4 protein expression (Figure 2A and B). In contrast, 39.5% (17/43) of serous cystadenocarcinomas had no detectable PDCD4 protein expression (Figure 2C), while 18.6% (8/43) exhibited weak PDCD4 expression (Figure 2D). The overall expression of PDCD4 in serous cystadenocarcinoma was significantly lower compared with normal ovarian tissues and serous cystadenomas ($p<0.05$) (Figure 2E).

Table I. *Relationship between PDCD4 expression and clinicopathological characteristics of serous cystadenocarcinoma.*

Clinicopathological features	PDCD4 expression			
Value	n	Negative, Weak	Moderate, Strong	*p*
Age (years)				
<60	26	15	11	
≥60	17	10	7	0.9414
Site of origin				
Left	7	5	2	
Right	13	10	3	
Double	23	10	13	0.1095
Metastasis				
Positive	34	19	15	
Negative	9	6	3	0.8390
Pathological grade				
I-II	19	7	12	
III	24	18	6	**0.0118**
FIGO stage				
I-II	15	10	5	
III-IV	28	15	13	0.4068

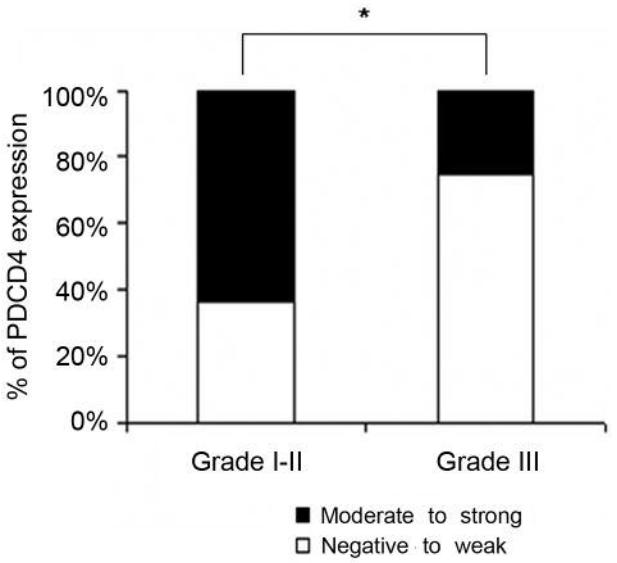

Figure 3. *The loss or reduction of PDCD4 expression correlated with higher grade serous cystadenocarcinoma (p=0.0118). Of high-grade (III) carcinomas, 75% (18/24) showed lost or reduced PDCD4 expression, whereas only 36% (7/19) of low-grade carcinomas (I-II) showed lost or reduced PDCD4 expression. *p<0.05.*

Loss or reduction of PDCD4 expression was significantly associated with high-grade serous cystadenocarcinoma. To investigate the clinical significance of lost or reduced PDCD4 expression in serous cystadenocarcinomas, we further analyzed the correlation between PDCD4 expression and clinicopathological features of serous cystadenocarcinomas (Table I). There was no significant correlation of lost or reduced PDCD4 expression with age, site of origin, metastasis or FIGO stage (p>0.05). However, the loss or reduction of PDCD4 expression was significantly associated with high- grade serous cystadenocarcinomas (p=0.0118). Of high-grade (III) carcinomas, 75% (18/24) had lost or reduced PDCD4 expression, whereas only 36% (7/19) of low-grade carcinomas (I-II) had lost or reduced PDCD4 expression (Figure 3).

PDCD4 expression was an independent prognostic factor for serous cystadenocarcinoma. To assess the association of PDCD4 expression with patient survival, the survival data from 32 patients with serous cystadenocarcinomas were generated by follow-up. According to the final PDCD4 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 PDCD4 expression tumors (n=18) and those with low PDCD4 expression tumors (n=14) was evaluated by Kaplan-Meier method and log-rank test. The result demonstrated that the level of PDCD4 expression significantly correlated with disease-specific survival of patients (p=0.0011) (Figure 4). The mean survival time for patients with high PDCD4 expression tumors was longer than

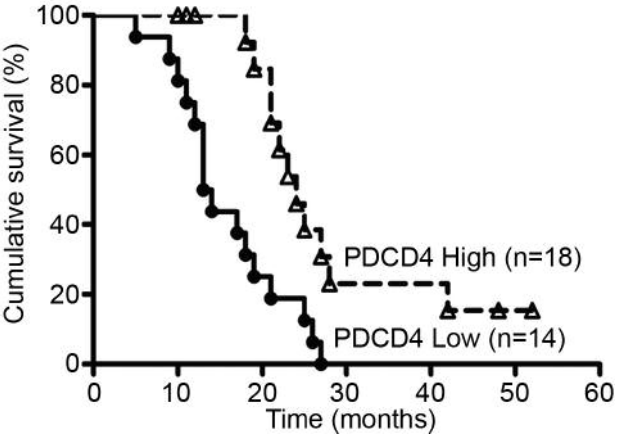

Figure 4. *The loss or reduction of PDCD4 expression was associated with poor prognosis in patients with serous cystadenocarcinoma (p=0.0011). Patients with a low level of PDCD4 expression had a significantly poorer disease-specific survival than those with a high level of PDCD4 expression.*

that for those with low PDCD4 expression tumors (23.3±12.3 months *vs.* 16.4±7.0 months, p<0.05).

To examine whether the PDCD4 expression was an independent prognostic factor for serous cystadenocarcinomas, we performed a multivariant Cox regression analysis, including PDCD4 expression, age, site of origin, metastasis, pathological grade and FIGO stage, for 32 patients with serous cystadenocarcinomas. Besides grade being an independent

Table II. *Multivariant Cox regression analysis of PDCD4 expression in 32 cases of serous systadenocarcinoma.*

Variable	Relative risk (95% CI)	*p*-Value
Age	3.028 (0.982-9.338)	0.054
FIGO stage	1.866 (0.484-7.190)	0.365
Site of origin	1.476 (0.481-4.528)	0.496
Metastasis	2.237 (0.499-10.029)	0.293
Pathological grade	2.527 (1.120-5.703)	**0.026**
PDCD4	0.298 (0.128-0.697)	**0.005**

CI: confidence interval; Note: Coding of variables: Age was coded as 0 (<60 years) and 1(≥60 years); FIGO stage was coded as 0 (Stage I-II) and 1 (Stage III-IV); Sites of origin were coded as 1 (left), 2 (right) and 3 (double); Metastasis was coded as 0 (positive) and 1 (negative); Pathological grade was coded as 0 (Grade I-II) and 1 (Grade III); PDCD4 expression was coded as 0 (loss or weak expression) and 1 (moderate or strong expression).

prognostic factor for disease-specific survival, the level of PDCD4 expression was able to significantly predict the patient outcome independent of other clinicopathological variables for disease-specific survival (relative risk, 0.298; 95% confidence interval, 0.128-0.697; p=0.005; Table II).

Discussion

Activation of oncogenes and inactivation of tumor suppressor genes contribute to ovarian oncogenesis (14, 15). Many tumor suppressor genes, such as *p53, p16, PTEN, BRCA* and *ARH1*, are mutated or down-regulated in human ovarian cancer (16-20). Restoration of their expression reduces the malignant phenotype of tumor cells (21-23). This indicates that tumor suppressor genes play important roles in ovarian cancer development.

PDCD4 was identified as a suppressor of transformation (6, 24, 25), tumorigenesis (8), tumor growth, progression and invasion (26-28). It has been reported that loss or reduction of PDCD4 expression was found in some malignant tumors, such as lung and colorectal cancer (12). However, PDCD4 expression level and the association with prognosis in ovarian cancer have not been fully investigated. To investigate the status and location of PDCD4 expression in epithelial ovarian cancer, we firstly compared the difference of PDCD4 expression level among normal ovarian tissues, serous cystadenomas and serous cystadenocarcinomas. Although a low level of PDCD4 expression was also found in a few normal ovarian tissues and serous cystadenomas, actual loss of PDCD4 expression was only found in serous cystadenocarcinomas. Statisical analysis showed the overall expression of PDCD4 in serous cystadenocarcinoma to be significantly lower compared with normal ovarian tissues and serous cystadenomas. In addition, the result of IHC from normal ovaries and serous ovarian tumors showed PDCD4 expression to be mainly in the cytoplasm and rarely in the nucleus. These results are inconsistent with those from colorectal cancer, which showed that the expression of PDCD4 in both adenoma and adenocarcinoma was reduced significantly compared with that in normal colonic mucosa (13). Moreover, PDCD4 expression was found in both the nucleus and cytoplasm. The ratio between nuclear and cytoplasmatic staining decreased significantly from normal tissues to adenomas and tumor tissues (13). This difference of results from various tumors implies that the role of PDCD4 in carcinogenesis may be tissue-specific.

In this study, we also revealed that loss or reduction of PDCD4 expression was significantly associated with high-grade serous ovarian carcinoma and poor prognosis of patients but not with age, site of origin, metastasis or FIGO stage. This finding is consistent with the report by Chen Y *et al*. showing that PDCD4 protein underexpression was linked to a high-grade of lung adenocarcinoma, while no association was found with sex, age, tumor stage, size or nodal status (9). However, unlike lung adenocarcinoma, the loss of PDCD4 expression in colorectal cancer was significantly correlated with the presense of distant metastasis, stage and lymphangiosis. No significant correlations were observed for sex or tumor locations (13). These results also indicate potential tissue-specific roles for the PDCD4 gene.

Additionally, our multivariant analysis showed PDCD4 expression to be an independent predictor of disease-specific survival. Consequently, loss or reduction of PDCD4 expression may also be a prognostic factor for serous cystadenocarcinoma. The mechanism of PDCD4 expression affecting the prognosis of patients is unclear at present. It has been reported that overexpression of PDCD4 could enhance the sensitivity to geldanamycin in renal cancer cells (29). Our preliminary result also showed overexpression of PDCD4 in the ovarian cancer SKOV3 cell line can enhance sensitivity to cisplatin (data not published), suggesting PDCD4 may prolong survival of patients through enhancing sensitivity to chemotherapy.

Conclusion

In the present study, we found that PDCD4 expression is clearly reduced or lost in serous cystadenocarcinoma compared with normal ovarian tissues and serous cystadenomas. Moreover, we identified loss or reduction of PDCD4 expression to be associated with progression of serous cystadenocarcinoma and this may serve as a novel prognostic marker for patients with this disease.

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