Programmed cell death 4 protein in esophageal cancer

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Abstract. Screening for genes down-regulated in esophageal cancers (Oncomine database) pinpointed programmed cell death 4 (*PDCD4*) as one of the most consistently involved. *PDCD4* is a new putative tumor suppressor gene implicated in cell transformation, tumorigenesis, and invasiveness. Based on such a biological rationale, the aim of the present study was to evaluate the prognostic value of PDCD4 in esophageal cancers. The immunohistochemical expression of PDCD4 protein was assessed in 111 consecutive esophageal cancers (63 adenocarcinomas and 48 squamous cell carcinomas) and paired non-cancerous samples. PDCD4 immunostaining was significantly lower in cancer samples than in non-cancerous mucosa (p<0.001). In all cases, the native esophageal epithelium consistently expressed nuclear PDCD4, which was significantly less expressed (37/111 cases) or completely lacking (31/111 cases) in the cancer samples. A significant inverse correlation emerged between nuclear *PDCD4* expression and tumor stage (p=0.002), pT (p<0.001), nodal metastasis (p=0.038), and with both vascular (p=0.005) and perineural invasion (p=0.004). Nuclear PDCD4 expression was associated with a longer disease-free (p=0.011) and overall (p=0.021) survival. PDCD4 expression predicts the patient outcome in esophageal cancers. Additional functional studies should look into the role of *PDCD4* in the multistep process of esophageal oncogenesis also inquiring on the clinical usefulness of the protein expression as prognostic marker in esophageal precancerous lesions.

Introduction

Esophageal carcinoma (EC) is a highly lethal malignancy and neither radical surgery nor multimodal therapeutic protocols

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significantly affect its early recurrence and mortality rate (1).

Little is known as yet about the molecular mechanisms behind esophageal oncogenesis. Expanding on such biological information might point to new prognostic markers and targeted therapies (2,3).

On screening for genes differently expressed in EC (Oncomine database) (4), the programmed cell death 4 (*PDCD4*) gene is one of those most frequently down-regulated in esophageal epithelial malignancies. The PDCD4 protein is involved in the apoptotic machinery (5) and, by interacting with translation initiation factors eIF4A and eIF4G, it suppresses cell transformation, tumorigenesis, and invasion (6,7). The molecules regulated by *PDCD4* include p21 (8), Cdk4, ornithine decarboxylase (9), carbonic anhydrase II (10), and JNK/c-Jun/AP-1 (11,12). It is worth adding that miR-21, a promoter of cell transformation, has recently been shown to target PDCD4 expression (13-17).

Recent studies have demonstrated that *PDCD4* is down-regulated in different human cancers, as well as in cancer cell lines (9,13,14,18-26). Moreover, loss of PDCD4 expression (as assessed by immunohistochemistry in the nucleus and/or cytoplasm) is associated with unfavorable cancer outcome (18,22). Taken together, the available information points to a role of *PDCD4* as a tumor suppressor gene. No studies have addressed PDCD4 expression in large series of human esophageal cancers, and the protein's expression has not been correlated with patient survival.

This study explored PDCD4 expression in a series of 111 esophageal cancers [63 esophageal adenocarcinomas (EAC) and 48 squamous cell carcinomas (ESCC)] and their paired non-neoplastic samples; the protein's expression was correlated both with the clinico-pathological features of the cancer and with clinical outcome.

Materials and methods

cDNA microarray analysis. The Oncomine database and gene microarray analysis tool, a repository for published cDNA microarray data (http://www.oncomine.org) (4,27), was queried (on 15 December 2008) for PDCD4 mRNA expression in esophageal non-neoplastic tissues and primary cancers. A statistical analysis of any differences in PDCD4

Table I. Clinico-pathological characteristics, clinical outcome and their association with PDCD4 nuclear expression in 111 patients treated for esophageal cancer.

	EAC	ESCC	Total	P-value
No. of cases	63	48	111	NS
Age	65.7±10.6 (range 39-86)	65.1±8.1 (range 45-81)	65.4±9.6 (range 39-86)	NS
Sex				0.011
Male	58	35	93	
Female	5	13	18	
Tumor stage				0.002
SI	14	9	23	
SIIA-IIB	15	23	38	
SIII	14	8	22	
SIV	20	8	28	
Tumor grade				NS
G1-2	33	39	72	
G3-4	30	9	39	
Vascular invasion				0.005
Yes	43	29	72	
No	20	19	39	
Perineural invasion				0.003
Yes	16	21	37	
No	47	27	74	
Tumor recurrence				0.011
No	44	33	77	
Yes	11	8	19	
Patient survival				0.021
Alive	44	29	73	
Deceased	11	12	23	

NS, not significant.

expression was accomplished using the Oncomine algorithms, which enable multiple comparisons between different studies (4,27). Only studies with results achieving a $p<0.05$ were considered.

Patients. One hundred and eleven esophageal cancer patients (48 ESCC and 63 EAC) who had undergone radical eso-phagectomy (R0) between 2002 and 2006 at the Clinica Chirurgica III-University of Padova were considered. Patients who had neo-adjuvant therapy or who died within 2 months of their operation were excluded. After surgery, 96 (41 ESCC and 55 EAC) of the 111 patients were followed up for at least 6 months or until they died (mean follow-up 22.3±14.4 months; median 18.0; range 6.0-59.8). Tumor recurred in 19 patients, and 17 of them died of disease (the median time from recurrence to death was 4.6 months); 6 other patients died of unrelated causes (Table I; Fig. 3).

All the patients considered in this study gave their written informed consent.

Pathological study. The gross surgical specimens were all examined according to a standardized protocol. Gross serial sections were obtained from the whole resected esophagus, enabling tissue samples to be collected from both cancer and native esophageal mucosa. The tissue samples were routinely processed for histological examination; serial sections (4-6 μm thick) were obtained from paraffin blocks and stained with hematoxylin and eosin (H&E) or used for immuno-phenotyping. The pTNM stage was assessed according to current criteria (28). The clinico-pathological characteristics of the series are described in Table I.

Immunohistochemical (IHC) study. In all cases, immuno-histochemical staining was performed automatically (Ventana
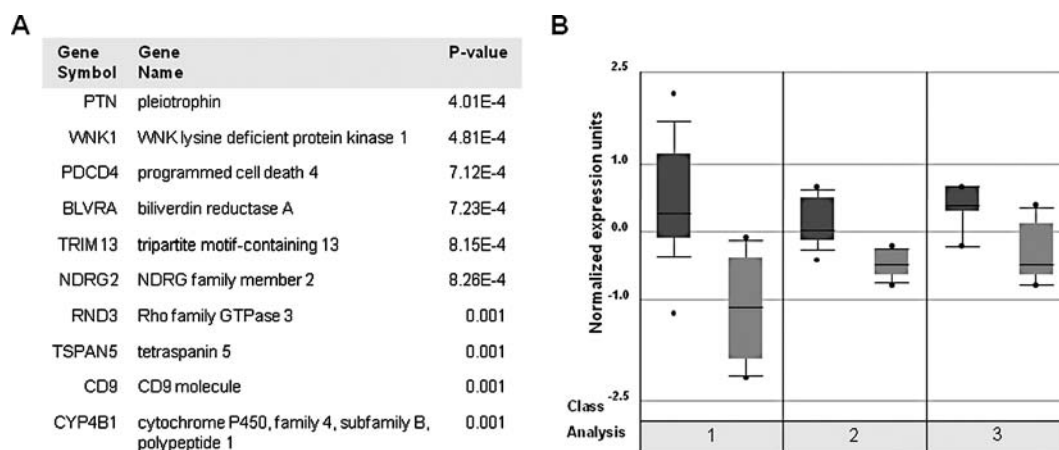
Figure 1. Oncomine analysis. Expression array analyses of multiple available esophageal cancer microarray data sets were collected and analyzed for down-regulated genes, and statistical significance was calculated using the Oncomine database and gene microarray data analysis tool. (A) *PDCD4* was identified as the third most down-regulated gene in cancer among the three available esophageal adenocarcinoma data sets (29-31). (B) *PDCD4* mRNA expression in publicly-available esophageal cancer microarray studies, showing *PDCD4* expression in normal tissues (dark grey) and esophageal adenocarcinomas (light grey). Class analysis 1, Wang_Esophagus (t-test=5.294; p=6.9E-5; 24 normal, 9 EAC) (30); class analysis 2, Hao_Esophagus (t-test=5.522; p=1.2E-4; 15 normal, 5 EAC) (31); class analysis 3, Kimchi_Esophagus (t-test=3.985; p=0.001; 8 normal, 8 EAC) (29).

Benchmark XT system, Touchstone, AZ) for PDCD4 (catalog #HPA001032; Atlas Antibodies, Stockholm, Sweden; 1:100) according to the manufacturer's instructions. Sections were lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently.

Criteria for assessing the immunohistochemical results. PDCD4 expression was jointly scored by two pathologists (M.F. and G.P.) with no knowledge of the clinical history of the patients. Ten high power fields (x400) were randomly selected, all of them representative of cancer tissue. Both nuclear and cytoplasmic PDCD4 immunostains were considered initially. Since only nuclear PDCD4 down-regulation has been found consistently associated with (unfavorable) cancer outcome (18), nuclear PDCD4 immunoreaction was considered for the purposes of this study and cases were dichotomized as PDCD4-positive (positive staining of at least 5% of the tumor cells' nuclei) vs. PDCD4-negative (no immunoreaction). In the cancer samples, the presence of positive stromal and inflammatory cells served as an internal control. All discrepancies in scoring were reviewed and a consensus was reached.

Statistical analysis. Univariate analysis was performed on each variable. The Mann-Whitney, Fisher's exact and Pearson's correlation tests were used to analyze the differences and correlations. For the purposes of this study, the outcomes considered were the disease-free interval (defined as the months elapsing between surgery and first tumor recurrence), and overall survival (defined as the time from surgery to the patient's death). Kaplan-Meier product limit curves were also calculated. Patients with no recurrent disease were censored at the time of their last follow-up or death (due to causes unrelated to their esophageal cancer). Disease-free and overall disease-free survival rates were compared across the levels of prognostic factors using the log-rank test. P-values <0.05 were considered significant. All the statistical assessments were performed with STATA software (Stata Corporation, College Station, TX, USA).

Results

PDCD4 is one of the most down-regulated genes in publicly-available EC mRNA microarray studies. In screening for genes specifically down-regulated in EC, a gene expression analysis was performed by checking different publicly-available EC microarray studies using the Oncomine database and gene microarray data analysis tools (4,27). The analysis took into account the mRNA expression levels for each of the studies; the significance of the gene expression across the available studies was also taken into account. In the three independent data sets of human esophageal adenocarcinomas considered, PDCD4 was consistently one of the most down-regulated genes: PDCD4 mRNA expression levels were significantly lower in primary EC tissues than in non-neoplastic controls (Pearson's correlation, p=7.12E-4) (29-31) (Fig. 1).

PDCD4 expression is down-regulated in EC. In all the normal esophageal tissue samples, the basal squamous epithelial layer (proliferative zone) featured strong PDCD4 nuclear expression (mostly coexisting with weak-moderate cytoplasm staining) (Fig. 2). PDCD4 nuclear expression was always a feature of non-epithelial cells (fibroblasts, lymphocytes, smooth muscle cells, and endothelia) in both normal and cancer samples (positive internal control) (8,18,32).

Among the cancer samples, only 37/111 showed any PDCD4 nuclear expression (Mann-Whitney, p<0.001), and no PDCD4 immunoreaction (at nuclear or cytoplasmic level) was seen in 31 cases (Fig. 2).

No significant differences were observed in the protein's expression according to the cancer histotype (ESCC vs. EAC).

Prognostic value of PDCD4 expression. An inverse correlation emerged between PDCD4 nuclear expression and female gender (Fisher, p=0.011), pT (Mann-Whitney, p<0.001), lymph node metastasis (Fisher, p=0.038), pathological tumor
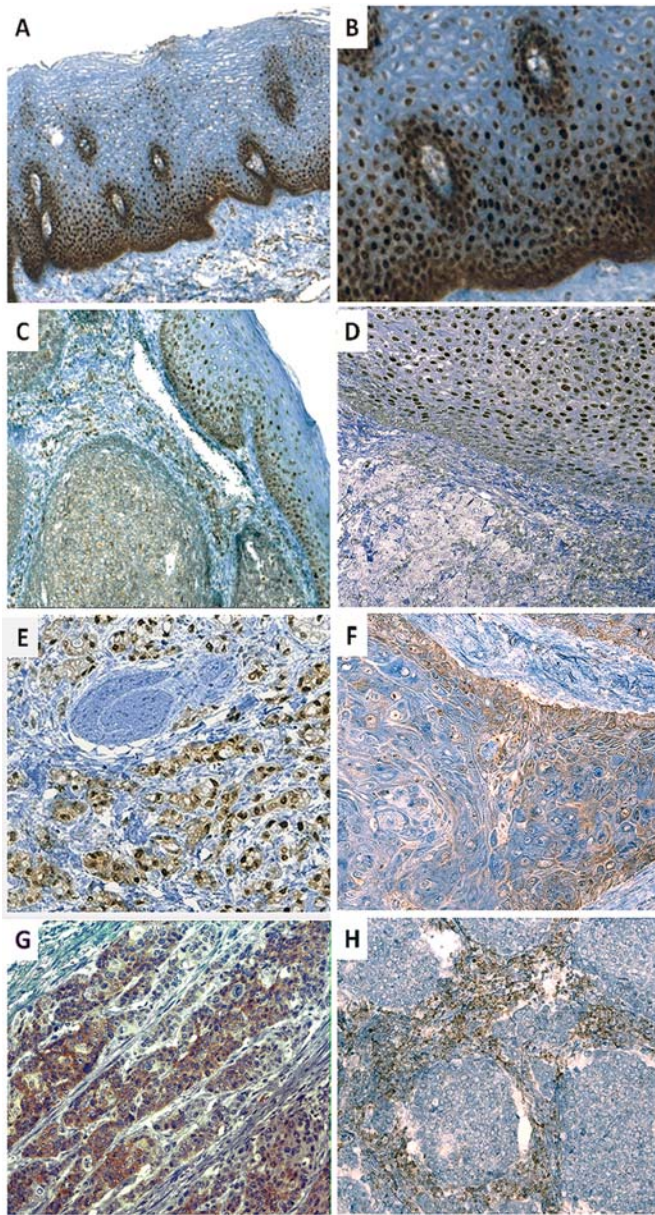
Figure 2. Representative *PDCD4* immunostaining of non-cancerous and cancer tissue samples. *PDCD4* immunostaining of native squamous esophageal epithelium (A and B) and squamous epithelium next to tumor cells (C and D). Normal esophageal basal cells feature strong nuclear and weak-moderate cytoplasmic staining; cancer tissue shows weak (C) or no (D) protein expression. Strong nuclear and weak-moderate cytoplasmic immunostaining of an EAC (E). Weak cytoplasmic immunostaining of an ESCC (F) and an EAC (G). A Pdcd4-negative ESCC surrounded by a nuclear-positive lymphocytic infiltrate (H). (Original magnification, 20 and x40).
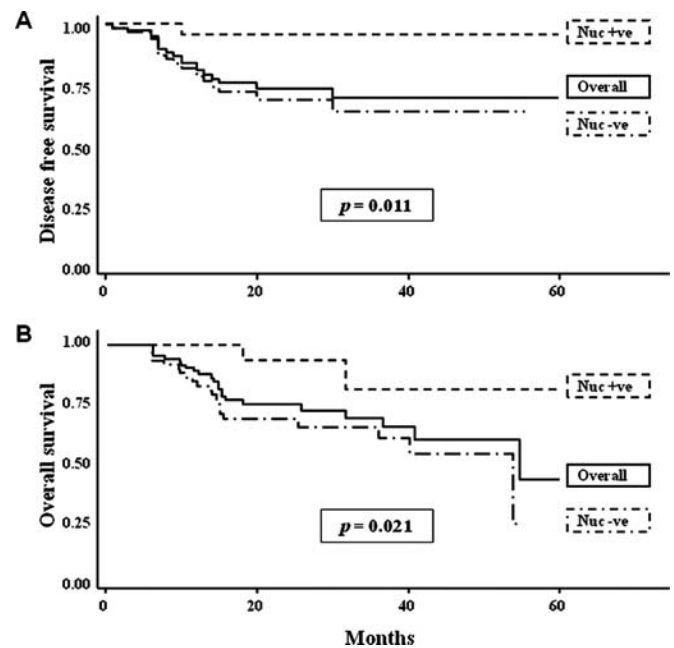

Figure 3. Disease-free (A) and overall survival (B) analyzed by Kaplan-Meier curves in 96 patients with radically treated esophageal carcinomas and stratified by *PDCD4* immunohistochemical nuclear positivity.

stage (Mann-Whitney, p=0.002), and both vascular (Fisher, p=0.005) and perineural cancer invasion (Fisher, p=0.003) (Table I). PDCD4 nuclear expression was associated to both longer disease-free and overall survival (log-rank test, p=0.011 and 0.021, respectively; Fig. 3).

Discussion

Lower PDCD4 expression (both total and nuclear) has been detected in several human malignancies (18,19,25,26) and PDCD4 has been shown to inhibit neoplastic transformation in both cancer cell lines (33,34) and *in vivo* models (i.e. skin carcinoma in transgenic mice) (9).

The available cDNA microarray databases consistently identify *PDCD4* as one of the genes most frequently down-regulated in EC. Hiyoshi and colleagues (17) recently found *PDCD4* gene expression (RT-PCR, Western blot analysis) regulated by miR-21 in a series of ESCC, but no previous study has extensively investigated PDCD4 expression in large series of ECs.

This study considered the immunohistochemical PDCD4 expression in a cohort of consecutive esophageal cancers (both ESCC and EAC). PDCD4 protein was completely lost or significantly reduced in almost 60% of the tumor samples considered. No differences were found in PDCD4 expression in the two main histotypes of esophageal cancer (EAC vs. ESCC). Notably, similar non-histotype-specific patterns were achieved on testing the protein's expression in different lung cancer histotypes (26). These findings would suggest that *PDCD4* is associated with a major tumor suppressor function that would be lost early in different carcinogenesis pathways.

The prognostic value of PDCD4 down-regulation was consistently supported by its association with the most common clinico-pathological variables associated to cancer aggressiveness (i.e., tumor p-stage, pT, presence of nodal metastasis, vascular and perineural invasion). Additionally, a significant correlation was demonstrated with both overall and disease-free survival. The achieved results support the hypotheses that PDCD4 acts as a tumor suppressor and that (irrespective of the cancer histotype) it discriminates between prognostically different tumors (18).

Inconsistent information is available about the different biological meaning of the protein subcellular expression (i.e. cytoplasmic vs. nuclear). In this study, according to previous experiences, only unequivocal nuclear expression

was considered. Various hypotheses have been advanced to explain the molecular mechanisms underlying nuclear or cytoplasmic PDCD4 expression. Under normal growth conditions, *in vitro* studies have demonstrated that PDCD4 is located mainly in the nucleus (35). So the significant loss of nuclear PDCD4 expression in cancer may theoretically be the result of epigenetic and/or genetic *PDCD4* gene de-regulation.

In conclusion, the loss of PDCD4 expression in the two main types of EC strongly supports the putative role of *PDCD4* in the multistep process of esophageal oncogenesis and is consistent with pivotal in silico studies which proved an elective involvement of *PDCD4* in esophageal carcino-genesis. Further efforts are needed to validate the prognostic impact of PDCD4 IHC expression. Restoring PDCD4 (and its downstream signaling pathways) may potentially pave the way to the development of novel therapeutic strategies.

Acknowledgements

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