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Original Article

Down-regulation of programmed cell death 4 (PDCD4) associates with the progression of cervical cancer

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Abstract: Programmed cell death 4 (PDCD4) is a tumor suppressor, and it inhibits tumor progression and metastasis. Even though down-regulation of PDCD4 has been implicated with many different types of human cancers, its roles in the progression of cervical cancer remain unexplored. Here, we have systematically examined the expression pattern of PDCD4 in cervical tissues. 15 cervical normal tissues, 14 cervical intraepithelial neoplasia (CIN) or carcinoma in situ (CIS) of cervix tissues and 78 invasive cervical cancer (ICC) tissues were analyzed by immunohistochemistry. Compared with the cervical normal tissues, the expression of PDCD4 was significantly reduced in all cancerous tissues. The extent of PDCD4 reduction can be correlated with the cervical cancer progression and severity. Furthermore, the decreasing of PDCD4 expression correlated with lymph node metastasis, histological type and p53 status. Thus, our study demonstrated that the reduction of PDCD4 was closely associated with the progression of cervical cancer and it might serve as a potential diagnosis marker.

Keywords: Programmed cell death 4, tumor suppressor gene, cervical cancer, immunohistochemistry

Introduction

Cervical cancer is the second most common malignancy in women worldwide, and more than 260,000 women die of cervical cancer each year [1]. Compared with the past 30 years, its incidence and mortality rates have fallen obviously because of the effective screening, early diagnosis and treatment for pre-cancer and early cancer [1]. However, the 5-year survival rate of patients with recurrent cervical carcinoma was only 10% [2] and the recurrence was mainly a result of lymph nodes metastasis and high distant failure rates [3]. Despite screening programs and early treatment, the high incidence and recurrence remain threats to cervical cancer patients worldwide. Characterization and investigation of novel cervical cancer biomarkers correlate with early diagnosis, treatment and prognosis are urgently required.

Programmed cell death 4 (PDCD4), was first discovered as a gene up-regulated during apoptosis [4]. Subsequent work showed that PDCD4 was a tumor suppressor. Compared with normal tissues, PDCD4 was commonly reduced or even lost in carcinomas, such as human lung cancer [5], colorectal cancer [6], ovarian cancer [7], nasopharyngeal carcinoma [8], hepatocellular carcinoma [9], glioma [10], and gastric cancer [11], and such a loss had been demonstrated to be correlate with poor prognosis. PDCD4 inhibited tumor promoter-induced neoplastic transformation in the murine JB6 cell model system [12]. Previous work demonstrated that PDCD4 directly interacted with and inhibited the RNA helicase activity of eukaryotic translation initiation factor eIF4A [13, 14], thus subsequently inhibit translation of p53 [15], procaspase-3 [16] in tumor cell lines. The p53 protein is a key regulator of cell survival and death, and caspase-3 is well known as the final executioner of apoptosis. PDCD4 inhibited the transcription of mitogen-activated protein kinase 1 (MAP4K1) to suppress colon carcinoma cell invasion [17]. Studies also found that PDCD4 directly interacted with transcription factor Twist1 to consequently down-regulate

proliferation related Twist1 target gene YB-1 [18]. Moreover, PDCD4 inhibited transcriptional activity of NF-κB and then suppressed the expression of invasion related NF-κB target genes in glioblastoma cells [19].

Despite the above findings, the physiological roles of PDCD4 in human cervical cancer have not been thoroughly investigated. In this study, we investigated the expressions of PDCD4 in normal, CIN/CIS and invasive cervical cancer tissues and correlated the expressions with clinical pathological parameters to address the potential function of PDCD4 in invasive cervical cancer.

Materials and methods

Patients and samples

A total of 107 samples were gathered from consenting patients at the time of conization of cervix for cervical biopsy at Guangdong Women and Children's Hospital and Health Institute. The cervical tissues including 15 normal, 14 CIN/CIS and 78 invasive cervical cancer samples, were used for PDCD4 expression analysis by immunohistochemistry (IHC). Normal cervical tissue samples were obtained from patients with uterine fibroids. The mean age (± standard deviation) of patients with normal cervix, CIN/CIS and invasive cervical cancer were 51 years (±10.1), 34 years (±8.0), and 45 years (±8.9), respectively. The tumor stages of invasive cervical cancer were classified according to International Federation of Gynaecology and Obstetrics (FIGO). All study procedures were approved by Guangdong Women and Children's Hospital and Health Institute and its ethics committees.

Immunohistochemistry

Immunohistochemistry was used to reveal the location and expression level of PDCD4 of all the specimens (107 cases). The tissues were deparaffinized in xylene and rehydrated through alcohol gradient. Antigen retrieval was performed by microwave treatment in 0.01 M citrate buffer (pH=6.0) for 16 minutes. To prevent unspecific reactions, a preincubation was performed with non-immune goat serum for 30 minutes at room temperature. Sections were incubated with PDCD4 antibody (Santa Cruz) at a dilution of 1:100 overnight at 4°C followed by secondary antibody (Dako) at room temperature for 1 hour. The bound antibody was detected by DAB (Dako) as the chromogen and counter stained with hematoxylin to locate nucleus (blue).

Evaluation of staining

All slides were coded and evaluated by 2 independent pathologists without knowledge of the patients. The immunoreactivity was estimated and graded by calculating the percentage of positively stained cells (0-100%) and scoring the staining intensities. Staining intensity was graded using a scale of 0-3 as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Total histological score was equal to the score of staining intensity (0, 1, 2, and 3) multiply by the percentage of positively stained cells (0-100). And we classified PDCD4 expression into two groups with score 100 as a cut-off value (low expression ≤100, high expression >100).

Statistical analysis

All data analyses were performed by using SPSS software (version 20; SPSS Inc., Chicago, Ill) and significance was assumed if $P<0.05$. Fisher's exact test was used to compare PDCD4 expression differences among normal, CIN/CIS and invasive cervical cancer groups. Fisher's exact test and Continuity correction test were used to compare differences between two groups. Pearson chi-square, Fisher's exact test, Continuity correction test and Mann-Whitney u test were used to test the correlation between PDCD4 protein expression and clinical parameters.

Results

PDCD4 expression is down-regulated in cervical cancer tissues

Fifteen normal cervical and 14 CIN/CIS and 78 invasive cervical cancer paraffin tissue samples were available for immunohistochemical study. We evaluated the expression level of PDCD4 by the total histological score, which was equal to the score of staining intensity multiply by the percentage of positively stained cells. The average histological score of normal tissues was 129, 11 out of 15 (73.3%) showed high PDCD4 levels (**Figure 1A**, **1B**), only 4 cases (26.7%) showed low levels (**Table 1**). In contrast, in the 78 invasive cervical cancer tissue samples, low expression of PDCD4 (**Figure 1E**, **1F**) was observed in 59 (75.6%) samples, and 19 (24.4%) samples had a complete loss of PDCD4 staining (**Figure 1G**, **1H**). On the other

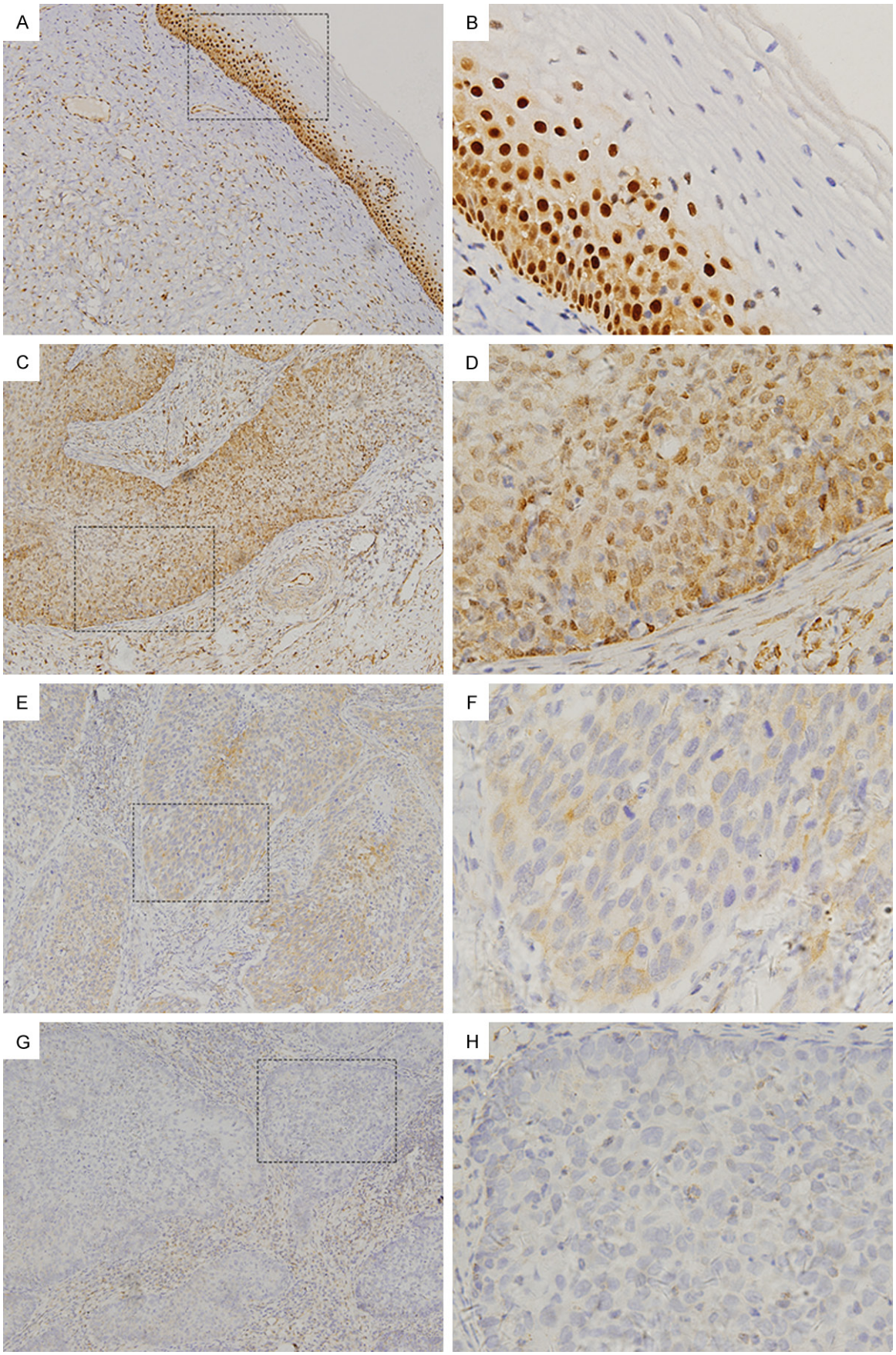
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Figure 1. Representative examples of immunohistochemical staining of PDCD4 in cervical tissues. (A, B) High expression of PDCD4 in normal cervical epithelial basal and stratum spinosum cells. (C, D) Low expression of PDCD4 in CIN/CIS tissue cells. (E, F) Low expression and complete loss (G, H) of PDCD4 in invasive cervical cancer tissues. (A, C, E, G) magnification, ×100; (B, D, F, H) magnification, ×400.

Table 1. Association between groups of cervical disease and PDCD4 expression

Groups	Case	Average score	PDCD4 expression		*p*-value
			High	Low	
Normal	15	129	11	4	0.000[a,*]
CIN/CIS	14	74	3	11	
ICC	78	15	0	78	
Normal vs. CIN/CIS					0.009[a,*]
Normal vs. ICC					0.000[b,*]
CIN/CIS vs. ICC					0.003[a,*]

CIN, Cervical intraepithelial neoplasia, CIS, Carcinoma in situ, ICC, Invasive cervical cancer. [a]Fisher's exact test, [b]Continuity correction test. [*]Statistically significant.

hand, we observed an intermediate situation in the CIN/CIS samples: the average histological score of CIN/CIS tissue samples was 74, high expression of PDCD4 was observed in 3 out of 14 (21.4%) samples, 11 out of 14 (78.6%) samples exhibited low expression (**Figure 1C**, **1D**; **Table 1**). Moreover, all the normal samples displayed strong nuclear staining and much weaker cytoplasmic staining (**Figure 1A**, **1B**), and the positive cells were localized in the basal layer and stratum spinosum. PDCD4 expressions in both invasive cervical cancer and CIN/CIS tissues were significantly lower than that in normal tissues (P<0.001 and P=0.009 respectively). And PDCD4 level of invasive cervical cancer was significantly lower than that of CIN/CIS (P=0.003) (**Table 1**). In normal cervical tissue samples, we calculated the percentage of positively stained cells in the basal layer and stratum spinosum. At the same time, we calculated the percentage of positively stained cells in dysplasia epithelium of CIN and in cancer cell mass of cervical cancer tissue samples. Some fibroblasts, lymphocytes, and endothelia had positive staining for PDCD4 in the stroma of normal, CIN, and cancer tissues, were not considered for IHC scoring.

Correlation of PDCD4 expressions with clinical parameters of cervical cancer

For invasive cervical cancers, clinical data were collected from patients' records. Since no high expression of PDCD4 in cancer tissues, we categorized invasive cervical cancer samples into PDCD4 positive group and negative group according to the presence of positive staining. In order to further understand roles of PDCD4 in the progression of cervical cancer, we assessed the association of PDCD4 expressions with clinical parameters of cervical cancer patients. The reduction of PDCD4 in cancers was found to be significantly associated with lymph node metastasis (P=0.046), histological type (P=0.042) and p53 (P=0.001), however, no significant correlation could be found between PDCD4 expression and other clinical parameters including age, tumor size, tumor stage, differentiation grade, depth of invasion, status of epidermal growth factor receptor (EGFR), Ki-67 and p16 (**Table 2**).

Discussion

The significant decreasing trend of PDCD4 in the sequence of normal, CIN/CIS and invasive cervical cancer tissue samples was observed. Our result was consistent with the findings in human lung cancer and hepatocellular carcinoma where loss of PDCD4 expression was found in cancer compared with normal tissues [5, 9]. We also found that the expression level of PDCD4 in cervical cancer was significantly associated with lymph node metastasis, histological type and p53 status.

PDCD4 has been identified as a suppressor of transformation [12], tumorigenesis, progression [20], proliferation [21], invasion [22], and as an inducer of apoptosis [21]. These tumor suppressor functions of PDCD4, were supported considerably by the current work, which demonstrated an overall decrease in PDCD4 protein levels from normal, to CIN, and further to invasive cervical cancer tissues and the correlation between PDCD4 and clinical parameters. In cervical cancers, the expression level of PDCD4 was found to be significantly associated with lymph node metastasis, histological type and p53 status, which suggested that PDCD4 might be a negative regulatory factor for the

Table 2. Clinicopathological characteristics of patients

Characteristics	n (%)	PDCD4 expression		*p*-value
		-	+	
Age	Mean 45.1 (range 27-69)			
		46.3±10.0	44.8±8.5	0.412[d]
Tumor size (cm)	Mean 3.4 (range 0.7-6.0)			
		3.2±1.1	3.5±1.1	0.418[d]
Differentiation grade				
Well	7	1 (7.7)	6 (11.1)	0.364[a]
Moderate	48	8 (61.5)	40 (74.1)	
Poor	12	4 (30.8)	8 (14.8)	
Tumor stage				
I	49	12 (63.2)	37 (62.7)	0.972[c]
II	29	7 (36.8)	22 (37.3)	
Depth of invasion				
≤1/2	17	5 (26.3)	12 (21.1)	0.847[b]
>1/2	59	14 (73.7)	45 (78.9)	
Lymph node metastasis				
Yes	41	14 (77.8)	27 (50.9)	0.046[c,*]
No	30	4 (22.2)	26 (49.1)	
Squamous carcinoma				
Yes	61	10 (52.6)	51 (87.9)	0.003[b,*]
No	16	9 (47.4)	7 (12.1)	
Ki-67 status				
Negative	2	0 (0)	2 (5.1)	1.000[a]
Positive	52	15 (100)	37 (94.9)	
p16 status				
Negative	1	0 (0)	1 (3.2)	1.000[a]
Positive	41	11 (100)	30 (96.8)	
p53 status				
Negative	27	13 (86.7)	14 (36.8)	0.001[c,*]
Positive	26	2 (13.3)	24 (63.2)	
EGFR				
Negative	1	0 (0)	1 (3.4)	1.000[a]
Positive	35	7 (100)	28 (96.6)	

[a]Fisher's exact test, [b]Continuity correction test, [c]Pearson chi-square test, [d]Mann-Whitney u test. [*]Statistically significant. EGFR, Epidermal growth factor receptor.

metastasis and progression of cervical cancer. Lymph node metastasis is associated with recurrence and poor prognosis of cervical cancer [23]. The negative association between PDCD4 and lymph node metastasis in our study suggested that PDCD4 might play a role in suppressing the development of cervical cancer. We also found that the positive rate (83.6%) of PDCD4 in squamous carcinomas was significantly higher than that (43.8%) in adenocarcinomas. It has been reported that patients with cervical adenocarcinoma showed lower response rate to the therapy, higher recurrence rate [24] and lower disease-specific survival [25]. The observation suggested that PDCD4 might be one of the protective factors in squamous cell carcinoma of the cervix.

Previous studies demonstrated that p53 is an important tumor suppressor gene, and the mutation and loss of p53 play an important role in carcinogenesis. The tumor suppressor gene p16 inhibits the cyclin dependent kinase 4 (CDK4) and cyclin dependent kinase 6 (CDK6) and further inhibits cell cycle G1 progression. The overexpression of p16 and nuclear proliferation marker Ki-67 indicates a high grade malignancy. Epidermal growth factor receptor (EGFR) is a cell surface receptor, which regulate cell proliferation, angiogenesis, and tumor metastasis [26]. It has been reported that EGFR is a potential therapeutic target of several tumors [27]. Studies have demonstrated that over-expression of p53, p16, Ki67, and EGFR was positively correlated with the progression, metastasis and recurrence of cervical cancer [28-31]. Therefore, we also collected the expression status of p53, p16, Ki67, and EGFR from patients' records. Reports have demonstrated that p53 gene has a low mutation rate, but a high level of positive expression in cervical cancer tissues and its positive rate ranges from 32 to 67.8% [31]. In our study, the positive rate of p53 was 49.1%, and the PDCD4 positive rate (92%) in p53 positive patients was much higher than that (52%) in patients without p53 (P=0.001). The observation indicated that there was a positive relationship between the two tumor suppressor genes, and it was conflicted with previous work, which demonstrated that PDCD4 suppresses the translation of p53 mRNA in an eIF4A-dependent manner [32]. The regulatory mechanisms of p53 are complicated and the relationship between PDCD4 and p53 need to be further investigated. In addition, although no correlation could be found between PDCD4 expression and differentiation grade, we observed

that well and moderate differentiated cervical cancer displayed higher PDCD4 positive rates (85.7%, 83.3%, respectively) than that (66.7%) of poor differentiated cervical cancer. Considering there were only 7 well, 48 moderate and 12 poor differentiated cervical cancer samples, by enlarging the sample size, we may achieve even more meaningful results.

PDCD4 is a nuclear-cytoplasmic shuttling protein, and it localizes predominantly in nucleus in normal cell lines, and the translocation from the nucleus to the cytoplasm was observed after serum starvation [33]. PDCD4 accumulated in the nucleus at the G0 phase of asynchronous cultures of human normal fibroblasts but was mainly localized in the cytoplasm in tumor cell lines such as HT1376 (bladder carcinoma), WiDr (colon adenocarcinoma), U373 (glioma), PC-3 (prostate adenocarcinoma) [34]. In colon tumor tissues PDCD4 staining was observed in both the cytoplasm and nucleus of the epithelium, but only observed in the nucleus of the lamina propria [22]. In this study, we demonstrated that PDCD4 localized predominantly in nucleus (**Figure 1A**, **1B**) of the normal cervical epithelium. With the progression of cervical lesions, nuclear PDCD4 was significantly reduced and even completely missing in some cervical cancer samples. We speculated that the accumulation of PDCD4 in the nucleus may play an important role in inhibiting the development of cervical cancer. The physiological roles and mechanisms of PDCD4 in human cervical cancer need to be thoroughly investigated.

Taken together, the significant reduction of PDCD4 in the process of cervical epithelial lesions, which from normal, to CIN, and further to invasive cervical cancer, supporting the notion that IHC for PDCD4 as a promising additional diagnostic tool to help discriminate benign from malignant cervical tissues. The correlation between PDCD4 and clinical parameters suggested that supporting the physiologic expression and/or function of PDCD4 may be promising strategies for the prevention of the development of invasive cervical cancer.

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Disclosure of conflict of interest

None.

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