Regulatory role of programmed cell death 4 in precancerous gastric lesions and early gastric cancer

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Abstract

Background Programmed cell death 4 (PDCD4) as a newly identified tumor suppressor is involved in inhibiting tumorigenesis and tumor progression. Epithelial mesenchymal Transition (EMT) is also associated with tumorigenesis of gastric cancer. However, few studies have elucidated the role of PDCD4 in regulation of EMT during precancerous gastric lesions (PLGC) and early gastric cancer. **Methods** In this study, the expression of PDCD4 and EMT-associated proteins in PLGC and early gastric cancer tissues were detected by immunohistochemistry. The relationship between the expression of PDCD4 and EMT-associated proteins and clinical and pathological parameters were analyzed. The PDCD4 high-expressed SGC-7901 cell line was also used to evaluate the function of PDCD4 in vitro and in vivo. **Results** PDCD4 and EMT associated proteins expression were significantly altered in PLGC and early gastric cancer tissues. After transfection with the PDCD4 gene, the expression of E-cadherin was significantly increased, the expression of N-cadherin and Vimentin were also remarkably inhibited. PDCD4 high-expressed also inhibited tumor growth and pathological features in nude mice xenograft model. **Conclusion** This study elucidates a regulatory role of PDCD4 in PLGC and early gastric cancer and provide insights into EMT-associated target which may provide novel therapeutic recourse.

Background

Gastric cancer is often described via a sequence of events, a stepwise progression from non-active gastritis (NAG), chronic active gastritis (CAG), precursor lesions of gastric cancer (PLGC) and gastric cancer [1]. Although many researchers have studied the molecular mechanisms of carcinogenesis and tumor progression of gastric cancer, the pathogenesis has remained largely unknown [2–3]. Thus, it is very important to investigate the mechanisms of gastric cancer initiation and progression and find an effective preventive method for gastric cancer [4].

Programmed cell death protein 4 (PDCD4) is a novel tumor suppressor protein involved in programmed cell death [5]. Its association with cancer progression and survival in patients with gastric cancer [6]. In recent years, scientific attention has turned to the mechanistic and functional of PDCD4 in the tumorigenesis of early gastric cancer, much of which remains unknown [7].

Epithelial-mesenchymal transition (EMT) describes a developmental switch from a polarized epithelial phenotype to a highly motile mesenchymal phenotype, is associated with the cell invasion and motility in gastric cancer [8–9]. It plays an important role in gastric cancer initiation and progression [10].

The present study examined the expression of PDCD4 and EMT-associated proteins in gastric tissues of multiple pathologic conditions, and to explore the possible biological function of PDCD4 in gastric cancer cells. These findings shed important light on the molecular mechanisms of PDCD4 in regulation of EMT during PLGC and early gastric cancer and find novel diagnostic and therapeutic targets for early gastric cancer.

Methods

Patients and tissue specimens

Gastric tissues were collected from patients from the First Affiliated Hospital of Anhui Medical University from July 2017 to June 2018. A total of 150 subjects were enrolled in this study, including 30 healthy subjects who underwent routine endoscopic screening for gastric cancer, 90 patients with atrophic gastritis associated with various degrees of dysplasia (mild, moderate and severe, $n = 30$ in each group) and 30 patients with early gastric cancer. All specimens were histopathologically confirmed. This study was approved by the Human Research Ethics Committee of Anhui Medical University. Written informed consents were obtained from all participants.

Immunohistochemistry

Paraffin sections were baked overnight and routinely processed. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide for 10 minutes. Then, the antigen were then blocked for 20 minutes with Bovine serum albumin (BSA, 5%) and the slides were incubated with antibody at 4°C overnight. Finally, the tissues were incubated with SABC working solution and visualized with 3,3-diaminobenzidine (DAB). The images were acquired under the microscope (Nikon, Japan) at 200× magnification.

Cell culture and transfection

The human gastric cancer cell line SGC-7901 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies) at 37°C in a humidified atmosphere with 5% CO₂. pcDNA3.1/PDCD4 plasmid was kindly provided by Dr. Qian-ben Wang (University of Duke, USA). Lipid-mediated transfection was used to obtain PDCD4 high-expressed SGC-7901 cell line according to the manufacturer's protocol.

Western blot

After the protein was processed, the protein liquid was separated by SDS-PAGE and transferred into nitrocellulose membranes (Millipore, Bedford, MA). The blot was blocked in blocking buffer (5% not-fat dry milk and 1% Tween-20 in PBS) for 2 hours at room temperature, and then incubated with appropriate primary antibodies in blocking buffer overnight at 4°C. The membranes were washed and incubated with secondary antibodies and were analyzed with an enhanced chemiluminescent reagent. The bands from western blotting were quantified by Quantity One analysis software (Bio-Rad).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.04% Triton X-100 in PBS, blocked in DMEM with 10% FBS. Primary antibodies were incubated at 4°C overnight and after washed 3 times with PBS, the secondary antibodies were incubated at 37°C for an hour. The nuclei were dyed with 4, 6-diamidino-2-phenylindole (DAPI) (Bioworld Technology, St Louis, USA). The cells were observed under a fluorescence microscope at ×400 magnification and photographed.

Real-time PCR (RT-PCR)

RT-PCR was performed on an Applied Biosystems 7500 Real-time PCR System using SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China) following the manufacturer's protocols. Primers were obtained from Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China) and their sequences were: PDCD4, forward primer 5'- CAG TTG GTG GGC CAG TTT ATT G -3', reverse primer 5'- AGA AGC ACG GTA GCC TTA TCC A -3'; E-cadherin, forward primer 5'-ATG AGG TCG GTG CCC GTA TT-3', reverse primer 5'-CGT TGG TCT TGG GGT CTG TGA-3'; N-cadherin, forward primer 5'-TCA GTG GCG GAG ATC CTA C-3', reverse primer 5'-GTG CTG AAT TCC CTT GGC TA-3'; Vimentin, forward primer 5'-AAG CAG GAG TCA AAC GAG TA-3', reverse primer 5'-GTT GGC AGA GGC AGA GAA AT-3'; GAPDH, forward primer 5'-CTC AAC TAC ATG GTC TAC ATG TTC CA-3', reverse primer 5'-CTT CCC ATT CTC AGC CTT GAC T-3'. The level of GAPDH mRNA transcript was used to normalize all reported gene expression levels, and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Animal studies

BALB/c nude mice were obtained from the Experimental Animal Center (License No. scxk (WAN) 2011-002) of Anhui Medical University (Anhui, China). Twenty nude mice were randomly divided into four groups. Stable PDCD4 high-expressed SGC-7901 cells were screened and confirmed. Mice were observed daily for tumor occurrence and growth for 30 days. The tumor dimensions and body weight of each mouse were measured every 3 days after treatment. On completion of the study, the mice were killed by cervical dislocation, and their tumors were excised and weighed.

Statistical analysis

All data were presented as Mean ± SD and performed using SPSS 20.0 statistical software. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test. *P* values below 0.05 were considered statistically significant.

Results

Expression and relationship of PDCD4 and EMT-associated proteins in PLGC and early gastric cancer

To study the expression and relationship of PDCD4 and EMT-associated proteins in PLGC and early gastric cancer, 150 clinical tissue samples from patients were harvested. Biopsies were divided into normal tissue, mild dysplasia, moderate dysplasia, severe dysplasia, early gastric cancer by hematoxylin and eosin staining (Fig. 1). The expression of PDCD4 and E-cadherin appeared to gradually decrease as the gastric lesions evolved from very mild forms to more severe forms, while the expression of N-cadherin and Vimentin were opposite to that of change (Fig. 1 and Table1). As well, decreased PDCD4 expression was associated with changed EMT-associated proteins (Table 2). Therefore, PDCD4 may play an important role in PLGC and early gastric cancer via EMT pathway.

Transfection with PDCD4 into SGC-7901 cells

To investigate the function of PDCD4 in tumorigenesis and progression of gastric cancer, SGC-7901 cells were stably transfected with pcDNA3.1/PDCD4. Western blot was used to analyze the transfection results (Fig. 2A). Immunofluorescence and RT-PCR also revealed the protein and mRNA expression of PDCD4 in stable PDCD4 high-expressed SGC-7901 cells (Fig. 2B-C). These results suggested that a stable PDCD4 high-expressed SGC-7901 cell line was successfully established, and can be used for the further biological study.

Effect of PDCD4 on EMT-associated proteins in transfected SGC-7901 cells

In order to further study the effects of PDCD4 on EMT-associated proteins in transfected SGC-7901 cells, the expression of E-cadherin, N-cadherin, Vimentin, with both RT-PCR and immunofluorescence assay were detected. The results showed that transfection of SGC-7901 cells with the PDCD4 expression vector resulted in increased levels of E-cadherin expression while it reduced Vimentin and N-cadherin expression (Fig. 3). This data confirm that high expression of PDCD4 has a certain inhibition effect on EMT process, thus providing a basis for the gene target of gastric cancer.

Effect of PDCD4 on tumor growth of gastric cancer in xenograft model

Nude mice were selected for normal group, control group, pcDNA3.1 and pcDNA3.1/PDCD4-expressing xenograft for the assessment of the role of PDCD4 on tumor growth. After thirty days treatment, the results showed that the tumor appearance in the PDCD4 high-expressed group was visibly smaller than control group. Following removal and measurement of all tumors, it was clear that tumor weights were

significantly lower in the PDCD4 high-expressed group than those in control group (Fig. 4). These findings indicate that over-expression of PDCD4 could inhibit the gastric cancer cell line in nude mice tumorigenic ability.

Effect of PDCD4 on EMT process of gastric cancer in xenograft model

The results of immunohistochemical staining showed that PDCD4 positive signals was brown in colour and located in the cytoplasm and nucleus while E-cadherin was mainly located on the cytoplasm-facing side of the cell membrane and partially in the cytoplasm. N-cadherin and Vimentin, appeared to be exclusively located in the cytoplasm. Interestingly, PDCD4 high-expressed group observably diminished the expression of N-cadherin and Vimentin protein in the tumors, compared to control groups. In contrast, PDCD4 high-expressed group enhanced the expression of E-cadherin (Fig. 5). The present observations indicate that results from the *in vivo* study were in good agreement with the *in vitro* results.

Discussion

Gastric cancer is one of the most common types of cancer worldwide, particularly in East Asian populations [11]. Researchers have devoted much to study the mechanisms of carcinogenesis and tumor progression, but the exact information of tumor initiation is remained largely unknown [12].

PDCD4 is a novel tumor suppressor gene and a promising target for anti-cancer therapies[13]. PDCD4 is frequently down-regulated in various human cancers including gastric cancer [14]. However, the role of PDCD4 in patients with PLGC and early gastric cancer have not been fully elucidated. In the present study, we observed a much reduced level of PDCD4 expression in the majority of gastric tissues with severe dysplasia and early gastric cancer, as opposed to the normal gastric mucosa and tissues with mild dysplasia. Therefore, we speculate that PDCD4 might be a potentially valuable molecular target in diagnosis and therapy for early gastric cancer [15].

A key process in promoting tumorigenesis is the EMT, which is a process by which epithelial cells lose their epithelial attributes and acquire a mesenchymal cell phenotype [16]. At the molecular level, EMT is characterized by loss of epithelial cell markers, including the cell adhesion protein, E-cadherin, and by the acquisition of mesenchymal markers, such as N-cadherin and Vimentin [17]. In the past several years, EMT had emerged as one of the most interesting topics in the field of PLGC, and had caused widespread concern [18–19]. The current study indicates that N-cadherin/Vimentin expression in severe dysplasia and early gastric cancer tissues were higher than normal gastric mucosa tissues. However, E-cadherin and PDCD4 decreased in the process of PLGC and early gastric cancer. Interestingly, it exists on a negative correlation to the expression of N-cadherin/Vimentin. These findings indicate that PDCD4 participates in tumorigenesis through the regulation of EMT process [20].

To investigate the hypothesized regulatory role of PDCD4 on the EMT associated proteins, a stable PDCD4 high-expressed SGC-7901 cell line was established. Then, we detected the expression of EMT-associated proteins. The results show that expression of E-cadherin is increase, while expression of N-cadherin and Vimentin is decrease. Taken together, our data demonstrated that high expression of PDCD4 may inhibit gastric tumorigenesis through EMT process.

The *in vitro* data prompted us to test the anti-tumorigenic role of PDCD4 *in vivo* [21]. Therefore, we established a successful animal model of PDCD4 high-expressed for the first time in a xenograft model in nude mice. The research shows that PDCD4 high-expressed suppressed tumor growth and pathological features, confirming the tumorigenic role of PDCD4. In addition, we also expand our current understanding of the regulatory role of PDCD4 on EMT in gastric cancer, positioning EMT pathway as downstream mediators of PDCD4 function.

Conclusion

The present study discovered that PDCD4 may be a potential therapeutic target and biomarker in gastric cancer patients in the forthcoming future. To our knowledge, our finding is significant because it might be beneficial in the tumorigenesis mechanism of early gastric cancer, and might provide insights in the development of gastric cancer therapeutic strategies.

Abbreviations

CAG: Chronic Active Gastritis, DAB: 3,3-Diaminobenzidine; DAPI: 4, 6-Diamidino-2-Phenylindole; EMT: Epithelial Mesenchymal Transition; NAG: Non-Active Gastritis; PDCD4: Programmed Cell Death 4; PLGC: Precancerous Gastric Lesions

Declarations

Ethics approval and consent to participate

The research protocol was reviewed and approved by the Human Research Ethics Committee of Anhui Medical University, and written informed consent was obtained from each patient included in the study. The animal work was approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Anhui Medical University.

Consent for publish

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

YDZ was the principle investigator of the research, performed the experiments, analysed data and wrote the paper; LL, QP, HB, WJS and WJD performed the experiments; PL and MZ provided technical support and all the chemical. All authors read and approved the final manuscript.

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Tables

Table 1 Correlation between clinicopathologic feature and expression of PDCD4 and EMT markers in PLGC and early gastric cancer

characteristic	Total	PDCD4			E-cadherin			N-cadherin			Vimentin		
		pos	neg	<i>p</i>	pos	neg	<i>p</i>	pos	neg	<i>p</i>	pos	neg	<i>p</i>
Ages (y)													
≥60	44	29	15	>0.05	18	26	>0.05	31	13	>0.05	29	15	>0.05
<60	106	84	22		28	78		32	74		31	75	
Gender													
Male	73	37	36	>0.05	32	41	>0.05	30	43	>0.05	34	39	>0.05
Female	77	39	38		38	39		36	41		38	39	
Grade													
Normal	30	26	4	<0.01	4	26	<0.01	24	6	<0.01	25	5	<0.01
Mild	30	21	9		9	21		21	9		20	10	
Moderate	30	16	14		12	18		15	15		16	14	
Serve	30	11	19		16	14		11	19		12	18	
Cancer	30	7	23		22	8		6	24		8	22	

pos positive ,neg negative

Table 2 Correlation between PDCD4 and EMT markers expression in PLGC and early gastric cancer

		PDCD4			χ^2	<i>P</i> value
		positive	negative	total		
E-cadherin	positive	42	4	46	9.11	<0.01
	negative	71	33	103		
	total	113	37	150		
N-cadherin	positive	34	29	63	26.68	<0.01
	negative	79	8	87		
	total	113	37	150		
Vimentin	positive	29	31	60	39.23	<0.01
	negative	84	6	90		
	total	113	37	150		

Figures

Figure 1

Expression of PDCD4 and EMT associated proteins in various gastric tissues (Normal tissue, Mild dysplasia, Moderate dysplasia, Severe dysplasia, Early gastric cancer). Representative graphs of hematoxylin-eosin (H&E) staining and of immunohistochemical. Images taken at ×200 magnification.

Figure 2

Confirmation of stable PDCD4 high-expressed SGC-7901 cells. Plasmids of pcDNA3.1 and pcDNA3.1/PDCD4 were respectively transfected into SGC-7901 cells for 48 h, and then cells were harvested for isolating total cellular RNA or protein. (a) The expression of PDCD4 was evaluated by western blot assay. GAPDH was served as an internal control of protein level. The relative density was normalized to GAPDH, which was determined by densitometric analysis. (b) The expression of PDCD4 was evaluated by Immunofluorescence assay. The positive cells in each group were indicated by arrows. Images taken at $\times 400$ magnification. (c) The mRNA expression of PDCD4 was measured using RT-PCR.

The data was analyzed using the $2^{-\Delta\Delta Ct}$ method. Data are presented as means $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ vs. control group.

Figure 3

PDCD4 regulated EMT associated proteins in transfected SGC-7901 cells. The corresponding plasmids were transfected into SGC-7901 cells. Then cells were harvested for immunofluorescence and RT-PCR analysis. (a-c) The expression of EMT markers were evaluated by Immunofluorescence assay. The positive cells in each group were indicated by arrows. Images taken at $\times 400$ magnification. (d) The mRNA

expression of EMT associated proteins were measured using RT-PCR. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method. Data are presented as means $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ vs. control group.

Figure 4

Tumor weight and volume in pcDNA3.1-PDCD4-xenografted mice. (a) Thirty days after SGC-7901 xenograft, all mice were die of cervical dislocation, tumors were removed and characterized. Representative photograph in xenografted mice. (b) The tumor volume curve of mice xenografted with

PDCD4 high-expressed group compared to controls over time. (c) Quantitative tumor weight assessment between the experimental and control group revealed significant differences. Data are presented as means $\pm$ SD. *p < 0.05 and **p < 0.01 vs. control group.

Figure 5

Immunohistochemical evaluation of PDCD4 and EMT associated proteins in xenografted mice. Images taken at $\times 200$ magnification.

Supplementary Files

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