Clinicopathological and prognostic significance of PDCD4 and microRNA-21 in human gastric cancer

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Abstract. Recent studies have demonstrated that the novel tumor suppressor protein programmed cell death 4 (PDCD4) is downregulated in several human solid cancer types and is suppressed by microRNA-21 (miR-21). The objectives of this study were: i) to establish the clinicopathological and prognostic significance of *PDCD4* mRNA, and ii) to elucidate any correlation between *PDCD4* mRNA and miR-21 in gastric cancer. The expression status of *PDCD4* mRNA was investigated by qRT-PCR and protein expression was analyzed by an immunohistochemical study. We analyzed *PDCD4* mRNA expression with respect to various clinicopathological factors in 105 gastric cancers. We also performed an association study comparing *PDCD4* mRNA and miR-21 in eight cell lines and 49 gastric cancers. Expression of *PDCD4* mRNA in cancer tissues was significantly lower than in non-cancer tissues ($P<0.05$). Patients with low *PDCD4* mRNA expression was significantly correlated with size, depth, lymph node metastasis, venous invasion, advanced stage, and poor clinical prognosis ($P<0.05$). Expression of miR-21 in cancer tissues was significantly higher than in non-cancer tissues ($P<0.05$). Elevated miR-21 expression was significantly correlated with size and depth ($P<0.05$). An inverse correlation between *PDCD4* mRNA and miR-21 was found in gastric cancer. This study revealed that low PDCD4 expression correlates with biological aggressiveness and poor prognosis in gastric cancer. Furthermore, our findings suggest that *PDCD4* mRNA is negatively regulated by miR-21 in gastric cancer and may serve as a target for effective therapies.

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Introduction

Programmed cell death 4 (PDCD4) was first identified as a gene that is up-regulated upon induction of apoptosis in murine cell lines (1). Since then, several studies have gained insight into PDCD4 function by identifying binding partners and regulatory elements involved in tumorigenesis. PDCD4 has been characterized as a novel tumor suppressor gene that inhibits tissue polypeptide antigen (TPA)-induced neoplastic transformation (2,3) and tumor promotion/progression in a transgenic mice (4). Supporting evidence was provided by Schmid and colleagues who demonstrated that activation of both PI3K and MEK/ERK signaling is required to maintain downregulation of PDCD4 by TPA in tumorigenesis (5). The molecular basis for PDCD4-mediated suppression has been linked to its high affinity MA-3 domains that sequester the eukaryotic translation initiation factors eIF4A and eIF4G to inhibit protein synthesis (6-9).

Furthermore, PDCD4 plays a key role in suppressing tumorigenesis by regulating several other genes involved in multiple processes including, apoptosis, cell cycle, and cell proliferation. Among these proteins regulated by PDCD4 are p21 (10), CDK4, ornithine decarboxylase (4), carbonic anhydrase II (11), JNK/c-Jun/AP-1 (12,13) and u-PAR (14). Additionally, PDCD4 has been shown to be regulated by a diverse set of molecules affecting several pathways including topoisomerase-inhibitors (15), COX-2-inhibitors (16), v-Myb (17), Akt (18,19) and mitogens (20).

PDCD4 is ubiquitously expressed in human tissues. Several studies observed loss of PDCD4 expression in various human solid neoplasias (19,21-24). These reduced PDCD4 levels have proven to be a poor prognostic factor in several human cancers (19,21,24,25). To our knowledge, however, the clinicopathological and prognostic values of PDCD4 expression have not been thoroughly investigated in human gastric cancer.

MicroRNAs (miRNAs) are a class of mature non-coding small RNA-21-25 nucleotides that have been implicated in diverse cellular processes including development, differentiation, proliferation, migration, and apoptosis (26,27). They target protein-coding mRNAs at the post-transcriptional level by direct cleavage of the mRNA or by inhibition of protein

synthesis (28,29). Recent evidence indicates that some miRNAs can function as oncogenes or tumor suppressors (29).

Overexpression of oncogenic miR-21 has been reported in several types of human malignant solid tumors (30-35). Furthermore, recent studies demonstrate that miR-21 promotes carcinogenesis through inhibition of apoptosis, proliferation, invasion, migration, and metastasis (32-34,36-38). Recently, several studies revealed that PDCD4 is negatively regulated by miR-21 in pancreatic tumors (31), colon cancer (36), breast cancer (37,39) and in glioblastoma cell lines (40). To our knowledge, there are no reports on a possible correlation between PDCD4 and miR-21 in gastric cancer.

In this study, we investigated the expression of PDCD4 in 105 tumor samples to determine its clinicopathological and prognostic value. We also investigated possible associations between *PDCD4* mRNA and miR-21 expression levels in patient samples and established gastric cancer cell lines.

Materials and methods

Tissue samples and cell lines. One hundred and five gastric tumor samples and matched controls in the experimental panel were obtained from the Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan. All samples were derived from patients who had not received adjuvant treatment including radiotherapy or chemotherapy prior to surgery in order to eliminate potential treatment-induced changes to gene expression profiles. Immediately following surgical resection, tissues were frozen in liquid nitrogen and kept at -90˚C until RNA extraction. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board, and this study was conducted under the supervision of the ethics board of Kyushu University. The follow-up periods ranged from 0.1 to 11.2 years with a mean of 2.5 years. Human gastric carcinoma cell lines MKN1, MKN7, MKN45, MKN74, NUGC3, NUGC4, AZ521, and KATOIII were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) and maintained according to recommended protocols.

RNA preparation for reverse transcription-PCR. Total RNA was isolated by the modified acid guanidinium-phenol chloroform procedure (41).

Quantitative real-time reverse transcription-PCR. Complementary DNA (cDNA) was synthesized from 8 μg of total RNA using random hexamer primers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as described previously (42). The primers for *PDCD4* amplification (NM_014456) were as follows: sense 5'-GTATGATGTGG AGGAGGTGGAT-3' and antisense 5'-CCCTCCAATGCTA AGGATACTG-3'. The primers for *GAPDH* were as follows: sense primer 5'-TTGGTATCGTGGAAGGACTCA-3' and antisense primer 5'-TGTCATCATATTTGGCAGGTT-3' (43).

PCR amplifications for quantification of *PDCD4* and *GAPDH* mRNA were done in a LightCycler system (Roche Applied Science, Indianapolis, IN, USA) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). In brief, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of LC DNA Master SYBR Green I mix, 50 ng of primers, and 2.4 μl of 25 nmol/l $MgCl_2$. The amplification conditions for 35 cycles consisted of denaturation at 95˚C for 10 sec, annealing at 65˚C for 10 sec, and extension at 72˚C for 10 sec. The products were then subjected to a temperature gradient from 68˚C to 95˚C at 0.1˚C/sec, with continuous fluorescence monitoring to produce melting curves of the products. The expression levels were normalized to *GAPDH* mRNA expression (44).

Quantitative real-time reverse transcriptase-PCR for miRNA. Total RNA was extracted from cell lines and tissue samples of gastric cancer using TRIzol (Invitrogen) as per the manufacturer's protocol. The miR-21 and RNU6B (as an internal control) -specific cDNA were synthesized from total RNA using gene-specific primers according to the *Taq*Man MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). Reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/l stem-loop RT primer, 1X RT buffer, 0.25 mmol/l each of deoxynucleotide triphosphate (dNTP), 3.33 U/μl MultiScribe reverse transcriptase, and 0.25 U/μl RNase Inhibitor. The 7.5-μl reaction volumes were incubated in Bio-Rad i-Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well plate for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C, and then held at 4˚C.

Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. The 10-μl PCR included 0.67 μl of RT products, 1X *Taq*Man Universal PCR master mix, and 1 μl of primers and probe mix of the *Taq*Man MicroRNA Assays. The reactions were incubated in 96-well optical plates at 95˚C for 10 min and then followed by 45 cycles of 95˚C for 15 sec and 60˚C for 10 min. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B, and relative to a calibrator sample.

In situ hybridization for PDCD4 protein detection. Immunohistochemical studies of PDCD4 protein were done on surgical specimens from 5 selected gastric cancer patients using the avidin-biotin-peroxidase method (LSAB2 kit; Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues. After deparaffinization and blocking, the antigen-antibody reaction was carried out overnight at 4˚C. The LSAB2 kit was applied to detect the signal of the PDCD4 antigen-antibody reaction. The rabbit polyclonal antibody against human PDCD4 (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) was used at 1:500.

Statistical analysis. Biostatistical analyses were performed with JMP 5.0.1a for Windows software (SAS Institute, Cary, NC, USA). Possible differences between groups were analyzed using Student's t-test and Chi-square (χ^2) test. The association between expression levels of *PDCD4* mRNA and miR-21 was analyzed by Spearman correlation coefficient. Survival curves were obtained by the Kaplan-Meier method (45), comparison between curves was made by log-rank test. A probability level of 0.05 was chosen for statistical significance.

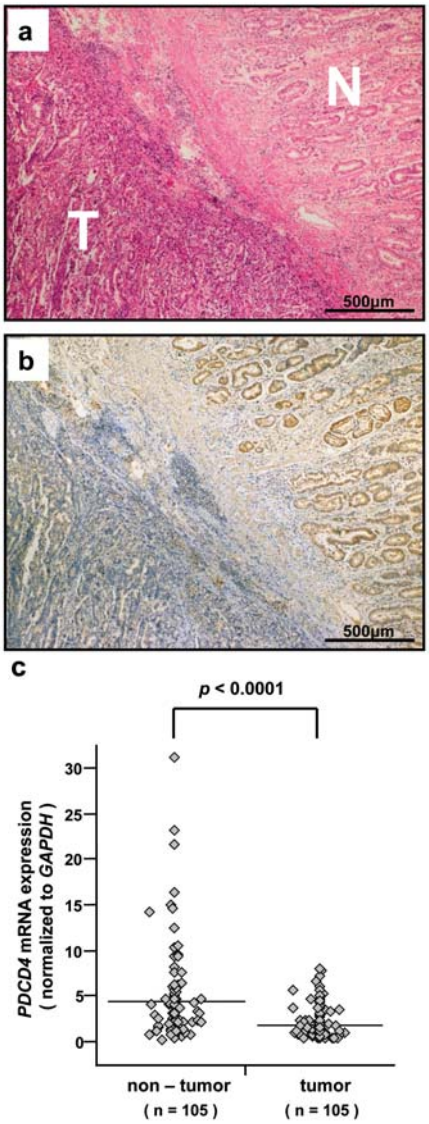

Figure 1. Detection of PDCD4 protein expression in representative examples of gastric cancer by *in situ* hybridization (a and b). Original magnification x40, H&E staining (a), original magnification x40, PDCD4 staining (b), quantitative real-time RT-PCR analysis of *PDCD4* mRNA in tumor and non-tumor samples of 105 gastric cancer cases (c). Horizontal lines indicate mean value of each sample.

Results

PDCD4 mRNA and protein expression is lower in primary gastric cancer tissues. We performed *in situ* hybridization on representative formalin-fixed, paraffin-embedded tissues in order to visualize expression of PDCD4 protein in gastric cancer and adjacent normal tissues (Fig. 1). H&E staining highlights apparent differences between gastric tumor and normal epithelium in primary tissue samples (Fig. 1a). More importantly, we probed tissue samples with an anti-PDCD4 antibody to show that PDCD4 protein levels are markedly lower in gastric tumor than adjacent normal tissue (Fig. 1b), where highest PDCD4 concentrations are primarily localized in the nucleus and the cytoplasm.

In order to corroborate this observation, we examined 105 primary patient samples for *PDCD4* mRNA levels by quantitative real-time reverse transcriptase-PCR (qRT-PCR) to assess differential expression within tissues. After normalization to *GAPDH* gene expression levels, the majority of patient tissues, 77 of 105 (77.3%), showed a lower expression level of *PDCD4* mRNA in tumor than in non-tumor tissues. Additionally, the mean (± SD) expression level of *PDCD4* mRNA was significantly lower in tumor tissues (1.82±1.77) than in non-tumor tissues (4.22±5.03) (P<0.0001, Fig. 1c).

Low PDCD4 mRNA expression group correlates with clinicopathological variables and poor prognosis. In order to assess correlations between *PDCD4* mRNA values and standard clinicopathological variables listed in Table I, the 105 clinical

Table I. Clinicopathological data and *PDCD4* mRNA expression in 105 gastric cancers.

Clinicopathologic variables	*PDCD4* mRNA expression T<N (n=77)	T>N (n=28)	P-value
Size (cm)			
≥5	29	18	0.02[a]
<5	48	10	
Histological type			
Well	13	7	0.09
Moderately	22	10	
Poorly	33	7	
Signet	4	4	
Mucinus	5	0	
Depth			
m, sm, mp	20	17	<0.01[a]
ss, se, si	57	11	
Lymph node metastasis			
Absent	24	16	0.02[a]
Present	53	12	
Lymphatic invasion			
Absent	20	11	0.19
Present	57	17	
Venous invasion			
Absent	52	25	0.02[a]
Present	25	3	
Liver metastasis			
Absent	73	28	0.11
Present	4	0	
Peritoneal dissemination			
Absent	62	26	0.11
Present	15	2	
Stage			
I, II	35	20	0.02[a]
III, IV	42	8	

[a]P<0.05.

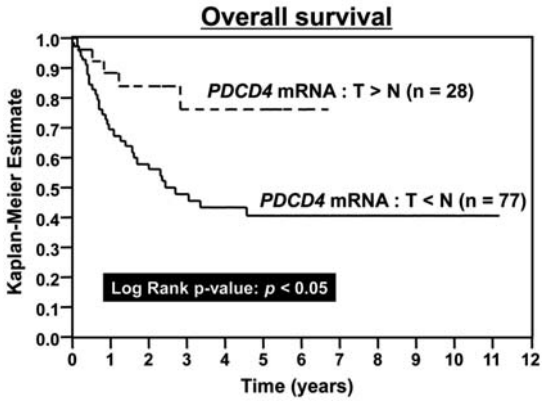

Figure 2. Overall survival rates of clinical cases tested are presented using Kaplan-Meier estimates. The 105 clinical cases were divided into two groups: low *PDCD4* group (T<N, n=77) in which the expression levels of *PDCD4* mRNA were lower in tumor than in non-tumor tissues and high *PDCD4* group (T>N, n=28) in which the expression levels of *PDCD4* mRNA were higher in tumor than in non-tumor tissues.

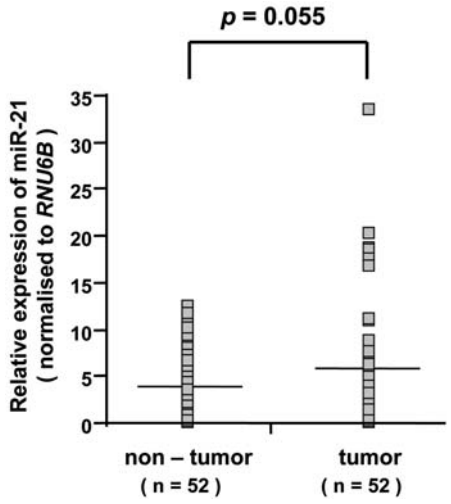

Figure 3. Quantitative real-time reverse transcriptase-PCR analysis of miR-21 expression in clinical samples of gastric cancer patients. Fifty-two tissue gastric tumor and non-tumor samples were selected. MiR-21 expression levels were normalized to RNU6B as an internal standard. Horizontal lines indicate mean value of each sample.

cases were divided into two groups: the low *PDCD4* group (T<N, n=77) in which the expression levels of *PDCD4* mRNA were lower in tumor than in non-tumor tissues and the high *PDCD4* group (T>N, n=28) in which the expression levels of *PDCD4* mRNA were higher in tumor than in non-tumor tissues. Table I shows the correlation between expression of *PDCD4* mRNA and the clinicopathological data of the 105 gastric cancer patients. The low *PDCD4* group was significantly associated with tumor size (<5 cm), depth (ss, se, si), lymph node metastasis (present), venous invasion (present), and advanced stage (III, IV) (P=0.02, <0.01, 0.02, 0.02 and 0.02, respectively). On the other hand, no significant differences were observed regarding histological type, lymphatic invasion, liver metastasis or peritoneal dissemination. Moreover, by log-rank test, patients in the low *PDCD4* group showed significantly poorer prognosis than those in the high *PDCD4* group (P<0.05, Fig. 2).

Table II. Clinicopathological data and miR-21 expression in 49 gastric cancers.

Clinicopathologic variables	miR-21 expression High (n=24)	Low (n=25)	P-value
Size (cm)			
≥3	1	9	<0.01[a]
<3	23	16	
Histological type			
Well	4	3	0.26
Moderately	11	5	
Poorly	7	13	
Signet	1	3	
Mucinus	1	1	
Depth			
m, sm, mp	3	12	<0.01[a]
ss, se, si	21	13	
Lymph node metastasis			
Absent	6	12	0.09
Present	18	13	
Lymphatic invasion			
Absent	3	8	0.09
Present	21	17	
Venous invasion			
Absent	17	17	0.82
Present	7	8	
Liver metastasis			
Absent	22	25	0.08
Present	2	0	
Peritoneal dissemination			
Absent	20	21	0.95
Present	4	4	
Stage			
I, II	12	14	0.67
III, IV	12	11	

[a]P<0.05.

Expression of miR-21 in clinical samples and clinicopathological characteristics. We performed qRT-PCR on 52 selected specimens to evaluate miR-21 expression in clinical samples of gastric cancer patients, and found that 34 of 52 (65.4%) showed a higher expression level of miR-21 in tumor than in non-tumor tissues. As shown in Fig. 3, after normalization to RNU6B expression levels, the mean (± SD) expression level of miR-21 was higher in tumor tissues (5.92±6.41) than in non-tumor tissues (3.97±3.41) (P=0.055). Based upon these elevated miR-21 expression levels and the knowledge that *PDCD4* mRNA correlated to tumor size, etc., we selected 49 clinical cases to evaluate miR-21/clinico-

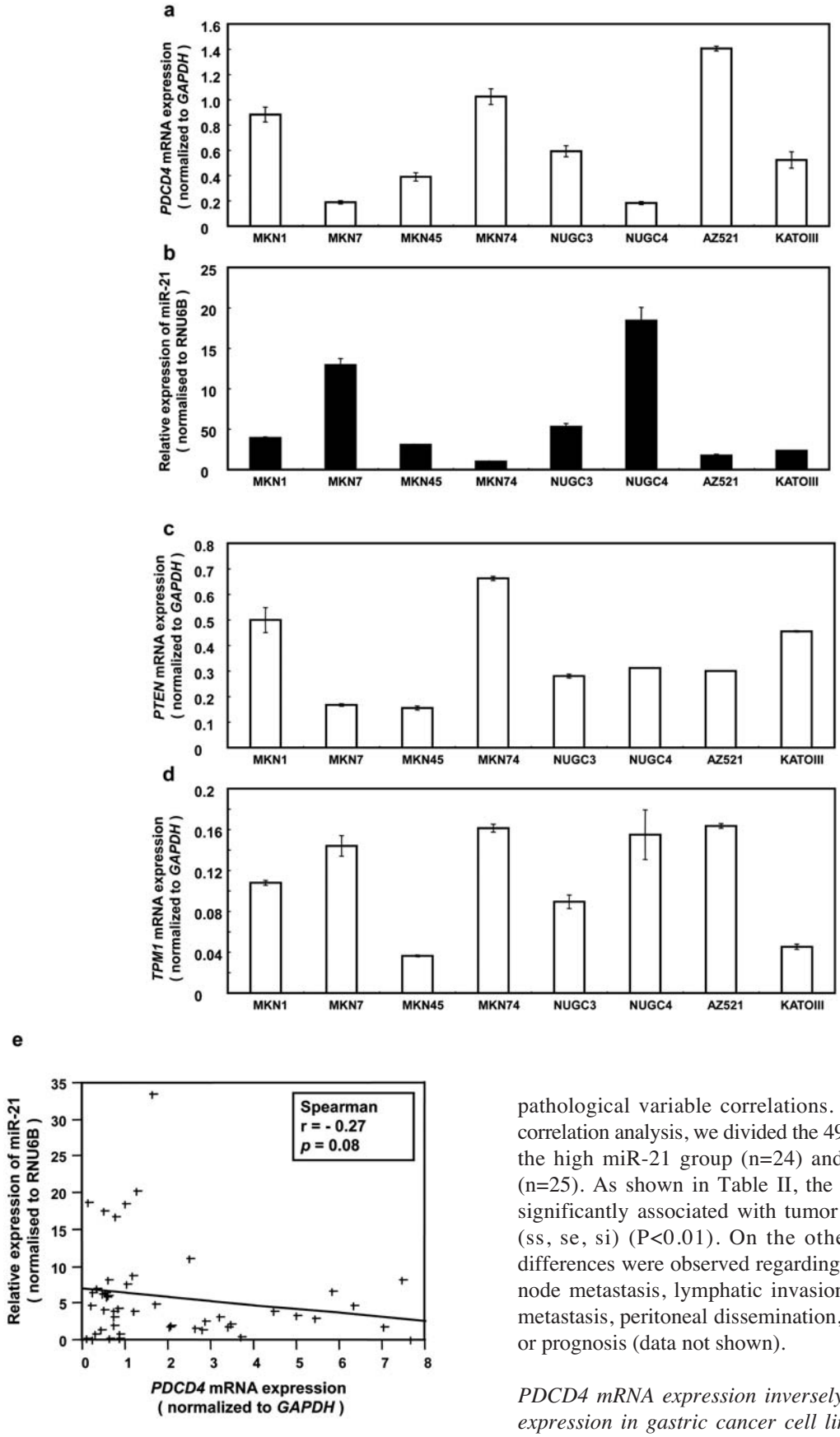

Figure 4. Quantitative real-time reverse transcriptase-PCR analysis and correlation data between *PDCD4* mRNA and miR-21 in a panel of eight gastric cancer cell lines. *PDCD4* mRNA and miR-21 levels were normalized to *GAPDH* and RNU6B, respectively, as internal standards (a and b). Expression of *PTEN* mRNA and *TPM1* mRNA was normalized to *GAPDH* in the same afore-mentioned gastric cancer cell lines (c and d). Relationship between *PDCD4* mRNA and miR-21 in gastric cancer patients is plotted in (e).

pathological variable correlations. In order to perform the correlation analysis, we divided the 49 patients into two groups: the high miR-21 group (n=24) and the low miR-21 group (n=25). As shown in Table II, the high miR-21 group was significantly associated with tumor size (<3 cm) and depth (ss, se, si) (P<0.01). On the other hand, no significant differences were observed regarding histological type, lymph node metastasis, lymphatic invasion, venous invasion, liver metastasis, peritoneal dissemination, advanced stage (III, IV) or prognosis (data not shown).

PDCD4 mRNA expression inversely correlates with miR-21 expression in gastric cancer cell lines and gastric cancers. To evaluate the correlation between *PDCD4* mRNA and miR-21, we analyzed *PDCD4* mRNA and miR-21 in gastric cancer cell lines (MKN1, MKN7, MKN45, MKN74, NUGC3, NUGC4, AZ521, and KATOIII). As shown in Fig. 4a and b, we found an inverse correlation between PDCD4 mRNA and miR-21 in gastric cancer cell lines (P<0.05). On the other

hand, no significant correlations between *PTEN* mRNA or *TPM1* mRNA and miR-21 were observed (Fig. 4c and d), though previous studies reported that identified targets for miR-21 include PTEN (32,33) and TPM1 (46).

We then surveyed miR-21 expression in 49 out of 105 gastric cancers in which expression levels of *PDCD4* mRNA were analyzed. As shown in Fig. 4e, we found an inverse correlation between *PDCD4* mRNA and miR-21 in 49 clinical gastric cancer patients (Spearman's correlation coefficient = -0.26, P=0.08).

Discussion

In this study, we demonstrated through a panel of primary specimens and cell culture models that PDCD4 mRNA/protein expression is lower in gastric cancer tissues compared to corresponding normal tissues (Fig. 1b and c). Previous studies have reported that reduced PDCD4 expression was found in various human solid tumors including primary lung cancer (21), primary pancreatic cancer (22), glioma (23), colon cancer (19) and ovarian cancer (24). However, to our knowledge, there are no reports concerning the clinicopathological significance of reduced PDCD4 expression in gastric cancer. Thus, we studied *PDCD4* mRNA expression with respect to various clinicopathological factors in 105 gastric cancer patients.

PDCD4 has been reported as a suppressor of transformation (2,3), tumorigenesis and progression (4), invasion and metalloproteinase activation (13) and an inducer of apoptosis (1,16). Leupold *et al* demonstrated that PDCD4 inhibits invasion and intravasation by inhibiting the invasion- and progression-related molecule u-PAR (14). These observations supported our findings that loss of *PDCD4* mRNA expression in gastric cancer tissues was significantly associated with parameters of pathological aggressiveness such as tumor size, depth, lymph node metastasis, venous invasion, and advanced stage (Table I). To our knowledge, this is the first study to link diminished PDCD4 expression to gastric carcinogenesis.

We also found that the underexpression of *PDCD4* mRNA in gastric cancer patients was significantly associated with poor prognosis and low overall survival (Fig. 2). Reduced PDCD4 is a negative prognostic factor in primary lung cancer (21), colon cancer (19), ovarian cancer (24) and glioma (25), suggesting that PDCD4 might be a critical indicator for the prediction of survival in patients with gastric cancer.

Overexpression of miR-21 has been reported in several human malignant solid tumors (30-35), supporting our results that expression of miR-21 in gastric cancer tissues was higher than in non-cancer tissues. MiR-21 is one of the most prominent miRNAs implicated in the genesis and progression of human cancer. Increased expression of miR-21 has been related to various processes involved in carcinogenesis, including inhibition of apoptosis (30), promotion of cell proliferation (31), stimulation of tumor growth (34) and chemoresistance (32). Recently, Zhu *et al* demonstrated that suppression of miR-21 reduced invasion in a breast cancer cell line (38), consistent with our results that overexpression of miR-21 was significantly associated with parameters of pathological aggressiveness such as tumor size and depth (Table II).

There have been a number of reports of PDCD4 as a target of miR-21 (31,36,37,39,40). We thus investigated whether *PDCD4* mRNA was negatively regulated by miR-21 in gastric cancer. As shown in Fig. 4, we found an inverse relationship between PDCD4 mRNA and miR-21 in eight gastric cancer cell lines and 49 clinical gastric cancer samples.

MiRNAs have been reported to exert their biologic effects by targeting specific mRNAs in two different ways: i) direct cleavage of the target mRNA using interference machinery (mRNA cleavage), and ii) inhibition of protein synthesis (translational repression) (29). An inverse correlation between *PDCD4* mRNA and miR-21 was reported in pancreatic tumors (31) and the MCF-7 breast cancer cell line (39), supporting our results that *PDCD4* mRNA was negatively regulated by miR-21 in gastric cancer. In addition, a correlation between *PDCD4* mRNA and miR-21 was found in colon cancer cell lines and tissues (36). However, no significant correlation between *PDCD4* mRNA and miR-21 was reported in glioblastoma cell lines (40). The molecular mechanism for the relationship between PDCD4 and miR-21 is not yet clear. However, it may indicate different and potentially tissue-specific roles. To the best of our knowledge, this is the first report showing a correlation between *PDCD4* mRNA and miR-21 in gastric cancer.

In conclusion, the present study shows that PDCD4 is a new gastric cancer-related gene and loss of PDCD4 is associated with biological aggressiveness and poor prognosis in gastric cancer. Furthermore, our findings indicate that increased miR-21 is associated with biological aggressiveness and miR-21 may regulate *PDCD4* mRNA negatively in human gastric cancer. The results of our study add further support to the expectation that elevating PDCD4 expression, inhibitory strategies against miR-21, or strategies interfering with the PDCD4/miR-21 interaction, offer promising possibilities for treatment of gastric cancer.

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