Expression patterns of the tumor suppressor PDCD4 and correlation with β-catenin expression in gastric cancers

TAKASHI KAKIMOTO, RYOSUKE SHIRAISHI, RYUICHI IWAKIRI, KAZUMA FUJIMOTO, HIROKAZU TAKASHASHI, HIROSHI HAMAJIMA, TOSHIHIKO MIZUTA, HIROYUKI IDEGUCHI, SHUJI TODA, YOSHIHIKO KITAJIMA, IWATA OZAKI, and SACHIKO MATSUHASHI

Departments of Internal Medicine, Pathology, Surgery, and Health Administration Center, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan

Received June 28, 2011; Accepted August 16, 2011

DOI: 10.3892/or.2011.1450

Abstract. The expression patterns of PDCD4, a tumor suppressor, and β-catenin were immunohistologically investigated in gastric carcinoma tissues. In normal gastric tissues, PDCD4 was strongly expressed in the cell nuclei, but weakly expressed in the cytoplasm. In gastric adenocarcinoma tissues, nuclear PDCD4 expression was decreased, while cytoplasmic PDCD4 expression was unchanged or somewhat increased. In gastric signet ring cell carcinoma tissues, PDCD4 expression patterns were different from the expression patterns of the adenocarcinoma tissues, and PDCD4 was localized in the nuclei of the carcinoma cells as a belt in the middle of the epithelial layer. The nuclear localization of PDCD4 in the adenocarcinoma tissues was correlated with the membrane localization of β-catenin, the activation of which stimulates invasion of colon cancer cells. PDCD4 expression was correlated with β-catenin expression in gastric carcinoma cell lines, but not with E-cadherin, as the binding partner in the cell membrane.

Introduction

The human programmed cell death 4 (PDCD4) gene (H731) was first isolated as an antigen gene involved in the cell cycle (1,2) and mapped at 10q24 (3). Homologs in mice (MA-3/TIS) (4,5), chickens (6,7) and rats (DUG) (8) have also been reported. The human programmed cell death 4 (PDCD4) gene (H731) was first isolated as an antigen gene involved in the cell cycle (1,2) and mapped at 10q24 (3). Homologs in mice (MA-3/TIS) (4,5), chickens (6,7) and rats (DUG) (8) have also been reported.

In response to growth factors, PDCD4 protein was rapidly phosphorylated on S\(^{67}\) by the protein kinase S6K1, which is activated through the mitogen-activated Akt-mammalian target of rapamycin (mTOR) signaling pathway in cells (14). The phosphorylation of S\(^{67}\) promoted the phosphorylation of S\(^{71}\) and S\(^{66}\) in the canonical SCF\(^{TRCP}\) ubiquitin ligase-binding motif D\(^{SGRGDS}\) of PDCD4, resulting in degradation of PDCD4 via the ubiquitin-proteasome system (14). Loss of PDCD4 may stimulate protein synthesis, and the subsequent cell growth and proliferation led to carcinogenesis of the cells (14).

Mouse Pdc4 was shown to suppress the neoplastic transformation of JB6 mouse epidermal cells exposed to the promoter TPA (28). It was demonstrated that Pdc4 inhibited activator protein 1 (AP-1) transactivation in mouse epidermal JB6 cells (29,30), and that Pdc4 interfered with the phosphorylation of c-Jun by Jun N-terminal kinase (JNK) (31). Ectopic expression of Pdc4 in metastatic colon carcinoma cells suppressed invasion and inhibited the transcription of mitogen-activated...
protein kinase kinase kinase 1 (MAP4K1)/hematopoietic progenitor kinase 1, which is an upstream kinase of JNK activation (32). Suppression of MAP4K1 inhibited c-Jun phosphorylation and the consequent AP-1 transactivities led to cell proliferation and invasion. The regulation of AP-1 activities by Pdcd4 in vivo was demonstrated by generating transgenic mice that overexpress Pdcd4 in the epidermis (33). The epidermis of the transgenic mice is resistant to TPA-induced carcinogenesis (33), and PDCD4-knockdown mice show a shorter lifespan than normal siblings, mainly because of B-cell lymphoma development (34). PDCD4 knockdown stimulated the invasion of colon tumor HT29 cells and inhibited E-cadherin expression, resulting in accumulation of active β-catenin in the nuclei and stimulation of β-catenin/T cell factor (Tcf)-dependent transcription of genes such as u-PAR and c-Myc, which activate invasion as well as AP-1-dependent transcription (35,36). PDCD4 inhibited breast cancer cell migration and invasion by suppressing the expression of lysyl oxidase (LOX) mRNA (37). In gastric cell cancer, it has been reported that PDCD4 suppression is correlated with clinicopathological parameters and the clinical prognosis (38). However, the precise function of PDCD4 in gastric cells is unclear. In this study, we immunohistochemically investigated the PDCD4 expression patterns in human stomach cancers and the correlation with β-catenin, which functions in invasion of colon cancer.

Materials and methods

Patients. From January 2003 to December 2004, 21 consecutive patients with gastric carcinoma who agreed to participate in this study were enrolled. All the patients underwent a surgical gastrectomy at Arita Kyoritsu Hospital. The study was conducted with the approval of the Ethics Committee of Arita Kyoritsu Hospital. Written informed consent was obtained from all subjects before entry into the study. The tumor stage was classified according to the TNM staging system.

Tissues and cell lines. Tumor tissue specimens were obtained from stock samples surgically obtained from the patients. Each specimen was fixed in 10% formalin, paraffin-embedded, sectioned and stained with hematoxylin-eosin for histological evaluation. Normal stomach tissues obtained during routine surgical procedures at Saga University Hospital were used as controls for biochemical analyses. The procedures were performed with informed consent at Saga University Hospital. The gastric tumor cell lines MKN1, MKN7, MKN28, MKN45, MKN74 and KATOIII were obtained from RIKEN Bioresource Center (Tsukuba, Japan) while HSC-45 and HSC-57 (39) were gifts from Dr K. Yanagihara (Department of Life Sciences, Yasuda Women's University Faculty of Pharmacy, Hiroshima, Japan). All of the cell lines were maintained in RPMI-1640 medium containing 10% FBS.

Reagents. An anti-PDCD4 antibody was prepared by immunizing rabbits with a synthetic peptide corresponding to the N-terminal amino acid sequence (13). Anti-β-catenin, anti-E-cadherin, anti-Akt, anti-p44/42 MAP kinase and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). An anti-PCNA antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Table I. Clinicopathological characteristics of the subjects.

<table>
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<th>(n=21)</th>
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<tr>
<td>Age</td>
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<td>Mean (range)</td>
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<td>Gender</td>
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<tr>
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<td>12 (57.1%)</td>
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<tr>
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<tr>
<td>M1</td>
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<td>Histological grade</td>
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<td>Adenocarcinoma</td>
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<tr>
<td>Well</td>
<td>7</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
</tr>
<tr>
<td>Poor</td>
<td>7</td>
</tr>
<tr>
<td>Signet ring cell carcinoma</td>
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Immunohistochemistry. After deparaffinization, 4-µm sections of the tumor tissues were treated with 1 mM EDTA for 5 min in a pressure cooker, and then incubated with a primary antibody. The bound antibody was visualized using a Universal Envision kit with DAB as the chromogen (Dako, Glostrup, Denmark) according to the manufacturer's protocol.

Evaluation of specimens. Semiquantitative analyses of the immunohistochemical staining were performed. The expression levels of PDCD4 and/or β-catenin were evaluated on scales of one to four or five as follows: PDCD4 in the nucleus: 5, very strong, normal cell level; 4, frequent and strong; 3, frequent but weak; 2, less frequent and weak; 1, negative, staining level with non-immune serum; PDCD4 in the cytoplasm: 4, strong; 3, moderate; 2, weak; 1, negative, staining level with non-immune serum; expression of β-catenin: 4, strong; 3, moderate; 2, weak; 1, negative, staining level with non-immune serum. All the specimens were evaluated by an experienced pathologist (S.T.) who was unaware of the clinical conditions of the patients.

Western blotting. Cell extracts were prepared by sonication in an SDS buffer (2% SDS, 0.05 M Tris-HCl, pH 6.8, 1 mM PMSF) followed by centrifugation at 10,000 x g for 10 min. The normal stomach tissue was homogenized in the SDS buffer using a Potter homogenizer and centrifuged at 10,000 x g.
An aliquot (10 µg protein) of each sample was separated by SDS-polyacrylamide gel electrophoresis and transblotted onto a Sequi-Blot PVDF membrane (Bio-Rad, Hercules, CA). After blocking with 5% skim milk at 4°C overnight, the membrane was incubated with a primary antibody followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. Specific bands were visualized using an ECL kit (Amersham, Buckinghamshire, UK) according to the manufacturer's protocol. The intensities of the bands were determined using Image J version 1.41o software (NIH, Bethesda, MD).

**Real-time RT-PCR.** Total RNA was extracted using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Quantitative analyses were performed using a real-time RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PDCD4 primer HS00205438_ml (Applied Biosystems) was used together with the GAPDH primer HS99999905_ml (Applied Biosystems) as a control.

**Statistical analysis.** Statistical summarization and analysis of the correlation between PDCD4 and β-catenin were performed with Dr. SPSS II software (SPSS Japan Co., Ltd., Tokyo, Japan). Differences were considered significant at P<0.05.

### Results

**Clinical characteristics of the subjects.** The clinicopathological characteristics of the 21 subjects enrolled in this study are summarized in Table I. The pathological grade, as the degree of differentiation of the gastric adenocarcinoma, was well in seven subjects, moderate in five subjects, poor in seven subjects and signet ring cell in two subjects.

**Expression patterns of PDCD4 in gastric adenocarcinoma.** The pathological features of the samples are summarized in Table II. PDCD4 was strongly expressed in the nuclei with weak expression in the cytoplasm in the normal gastric tissues (Fig. 1Bb). PDCD4-positive cells were localized in the deep mucosae overlapping with the PCNA-positive cell area in the normal tissues (Fig. 1Bb). In the gastric adenocarcinoma tissues, PDCD4 protein was localized mostly in the cytoplasm and less frequently and more weakly in the nuclei, compared with the normal tissues (Fig. 1). A comparison of the staining patterns in the differentiated types of carcinoma tissues with those in the poorly differentiated tissues revealed that the frequencies of nuclear staining of grade 2 or more were highest in the well-differentiated tissues (6/7, 86%) and decreased according to the differentiation grade (3/5, 60% and 3/7, 43% for moderately and poorly differenti-
Figure 1. Expression patterns of PDCD4 in adenocarcinoma tissues. (A) Positive expression patterns of PDCD4. Serial sections of a well differentiated gastric carcinoma tissue specimen (sample 2 in Table II) were stained with anti-PCNA (a) and anti-PDCD4 (b) antibodies. Original magnification, x40. (c) Higher magnification image of the field marked with a square in panel b. PDCD4 is still localized in the nuclei of the tumor cells but less frequently compared with the normal tissues. Original magnification, x400. Serial sections of a moderately differentiated carcinoma tissue specimen (sample 8 in Table II) were stained with anti-PCNA (d) and anti-PDCD4 (e) antibodies. The cytoplasm is well stained and some nuclei are also PDCD4-positive. Original magnification, x400. Serial sections of a poorly differentiated carcinoma tissue specimen (sample 13 in Table II) were stained with anti-PCNA (f) and anti-PDCD4 (g) antibodies. (h) Higher magnification image of the field marked with a square in panel g. PDCD4 protein is localized mostly in the cytoplasm and occasionally in the nuclei. Original magnification, f and g, x40; h, x200. L, lymphoid nodule. (B) Poor expression patterns of PDCD4. Serial sections of sample 7 (Table II) were stained with anti-PCNA (a) and anti-PDCD4 (b) antibodies. Insert in panel b, higher magnification image of the area indicated by an arrow. N, normal cell area; T, tumor cell area; L, lymphoid nodules. It should be noted that the inside PCNA-positive cells are PDCD4-negative while the outside PCNA-negative cells are PDCD4-positive in the nodules and that PDCD4 is localized in the cytoplasm of lymphocytes. Serial sections of sample 11 (Table II) stained with anti-PCNA (c) and anti-PDCD4 (d) antibodies. Serial sections of a poorly differentiated carcinoma tissue specimen (sample 16 in Table II) stained with anti-PCNA (e) and anti-PDCD4 (f) antibodies. Original magnification, x40.
ated cancer tissues, respectively). However, the staining in the cytoplasm was similar for all differentiation grades, or somewhat increased in the poorly differentiated grade (Table II).

Expression patterns of PDCD4 in gastric signet ring cell carcinoma. We examined two samples of signet ring cell carcinoma tissues from two patients. The staining patterns of PDCD4 were similar in both carcinoma tissue specimens, but very different from the patterns in the adenocarcinoma tissues described above. PDCD4-positive nuclei were localized as a belt in the middle of the epithelial layer in the signet ring cell carcinoma tissues (Fig. 2a and b). The cell nuclei of the signet ring cells were often PDCD4-positive, while the cytoplasm was poorly stained (Fig. 2c). Invasion was not observed in either tissue specimen.

PDCD4 expression is correlated with β-catenin expression in gastric adenocarcinoma cells. It has been shown that PDCD4 suppression decreases E-cadherin expression followed by release of β-catenin from the membrane, thereby activating c-myc through the transcription factor β-catenin/Tcf in colon cancer cell line (35,36). To examine the correlation between PDCD4 and β-catenin expression, sections of the gastric carcinoma tissues were stained with an anti-β-catenin antibody. β-catenin was localized in the cell membrane of normal gastric cells (Fig. 3a), consistent with a previous study (40) and PDCD4 was strongly expressed in the nuclei of these cells (Fig. 3b). The staining patterns of β-catenin in the carcinoma tissues were mostly in the cytoplasm, but occasionally included cell membrane staining (Fig. 3c and e; Table II). Five of 12 well/moderately and two of seven poorly differentiated carcinoma tissues showed β-catenin staining in the cell membrane, and six of the seven samples with β-catenin staining in the

Figure 2. Expression patterns of PDCD4 in a gastric signet-ring cell carcinoma. Immunohistological images of a gastric signet ring carcinoma stained with anti-PDCD4 (a) and anti-PCNA (b) antibodies. (c) Higher magnification image of the field marked with a square in panel a. The arrows show PDCD4-positive nuclei in the signet ring cells. The PDCD4-positive nuclei are localized as a belt in the middle of the epithelium (a). L, lymphoid nodule. Original magnification, a and b, x100; c, x400.

Figure 3. Immunohistological staining patterns of gastric adenocarcinoma tissues with an anti-β-catenin antibody. (a) Normal gastric tissue stained with an anti-β-catenin antibody. β-catenin is mainly localized in the cell membrane. (b) Serial section to that shown in panel a stained with an anti-PDCD4 antibody. Samples 1 (c), 4 (d), 17 (e) and 9 (f) in Table II were stained with an anti-β-catenin antibody. The membranous β-catenin-positive specimens shown in panels c and e, an well differentiated adenocarcinoma (sample 1) and a poorly differentiated adenocarcinoma (sample 17) were nuclear PDCD4-positive as shown in Table II. Original magnification, x400.
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Cell membrane contained PDCD4-positive nuclei, with the exception of sample number 7 (Table II). In addition, the correlation between the expression grades of PDCD4 in the nuclei and β-catenin in the membrane was statistically significant (r=0.618, p=0.004, 95% CI=0.227-0.837; Fig. 4). Nuclear staining of β-catenin (Fig. 3d and f) was observed in two of seven well differentiated and two of five moderately differentiated carcinoma tissues, but not in the poorly differentiated tissues (Table II).

To further investigate the correlation between PDCD4 and β-catenin, the two proteins were analyzed in gastric carcinoma cell lines by Western blotting together with E-cadherin, which is the binding partner of β-catenin. Akt and ErK were also examined, because they function to control the PDCD4 levels by participating in the degradation of PDCD4 protein (11,12). Among them, only β-catenin expression was significantly correlated with PDCD4 expression (Fig. 5B-D).

**PDCD4 expression is mostly controlled at the transcription level in the gastric carcinoma cell lines.** The PDCD4 protein levels were generally suppressed in tumor cells compared with the corresponding normal cells. However, the mechanisms of the protein suppression varied between suppression at the transcription level and/or the post-transcription levels depending on the cell types or species involved. As shown in Fig. 5A and C, the PDCD4 protein and mRNA levels were both suppressed in gastric carcinoma cell lines compared with the normal gastric tissues and the PDCD4 protein levels were well correlated with the mRNA levels (Fig. 5D). These findings indicate that the PDCD4 levels may be mainly controlled at the transcription level in the gastric carcinoma cell lines.

**Discussion**

In the present study, we observed that PDCD4 protein was mostly localized in the nuclei with much smaller amounts in the cytoplasm in the normal gastric cells. The expression in the nuclei was largely suppressed, while that in the cytoplasm was retained or somewhat increased in the gastric carcinoma cells, indicating that loss of PDCD4 protein from the nuclei may be important in carcinogenesis of gastric cells. Fassen et al (41) recently reported that loss of nuclear PDCD4 was inversely correlated with the miR-21 level in colon carcinogenesis. In gastric carcinomas, suppression of PDCD4 was shown to be correlated with clinicopathological parameters such as size,
PDCD4 in the nuclei in gastric cancer tissues. These findings in the cell membrane was associated with the localization of β-catenin in gastric cancer cells, similarly to colon cancer. However, PDCD4 expression was correlated with β-catenin expression, while E-cadherin expression was not correlated with either PDCD4 or β-catenin expression in gastric carcinoma cell lines. Therefore, the control mechanisms of E-cadherin and β-catenin by PDCD4 may be not simple in the carcinogenesis of gastric cells. More investigations are necessary in the future.

Wang et al (48,49) demonstrated that PDCD4 mediates the sensitivity of gastric cancer cells to tumor necrosis factor-related apoptosis-induced ligand (TRAIL)-induced apoptosis via down-regulation of FADD-like interleukin-1β-converting enzyme (FLICE) inhibitor (FLIP).

In conclusion, PDCD4 was mostly localized in the nuclei in normal gastric cells and the nuclear PDCD4 was decreased in gastric carcinoma tissues. The loss of nuclear PDCD4 was correlated with the loss of membranous β-catenin, indicating that nuclear PDCD4 may function to suppress gastric carcinogenesis at least partly by inhibiting the activation of β-catenin as the mechanism of colon carcinogenesis.

References


