Comparative Expression of mRNA and Protein of DPC4 in Relation to Ubiquitin Expression in Various Pancreatic Duct Lesions of Human Pancreatic Cancer Tissue

Tomoko Toga¹, Yoshinori Nio¹, Riruke Maruyama², Koji Hashimoto¹ and Tetsuya Higami¹

¹Department of Cardiovascular and Digestive Surgery, Shimane University School of Medicine, Izumo 693–8501, Japan

²Laboratory of Clinical Pathology, Shimane University School of Medicine, Izumo 693–8501, Japan

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The expression of Dpc4 protein (pDpc4) is immunohistologically demonstrated in various cancer cells, but the expression of its mRNA has yet to be defined. The present study was designed to assess the correlation between pDpc4 expression and DPC4-mRNA expression, and between pDpc4 and DPC4-mRNA expression and ubiquitin (Ub) expression in human pancreatic cancer tissue. In 76 pancreatic duct lesions of 21 specimens of pancreatic cancer tissues, the expression of DPC4-mRNA was assessed by *in situ* hybridization (ISH), and the expression of pDpc4 and Ub was assessed by immunohistochemistry (IHC). The pancreatic duct lesions were classified according to the pancreatic intraepithelial neoplasia (PanIN) criteria. In normal duct or early neoplastic lesions (PanIN-1), both DPC4-mRNA and pDPC4 were wide-

ly expressed, and Ub expression was low. In borderline or low-grade malignancy lesions (PanIN-2 or PanIN-3), DPC4-mRNA was highly expressed, but pDpc4 expression was low, and Ub expression was high. On the other hand, in high-grade malignancy (ductal carcinoma) lesions, expression of both DPC4-mRNA and pDpc4 was low, but Ub was highly expressed. Although DPC4-mRNA was widely expressed, pDpc4 expression decreased, while Ub expression increased along with the progression of pancreatic malignancy. These results suggest that the decreased expression of pDpc4 may be caused by its degradation by highly expressed Ub. In addition, the decreased expression of DPC4-mRNA in high-grade malignancy suggests a homozygous deletion of the DPC4 gene.

Key words: Smad4, immunohistochemistry, *in situ* hybridization, PanIN criteria, pancreatic carcinoma

I. Introduction

DPC4 (also called SMAD4) is a tumor suppressor gene located on 18q21.1. The locus was termed DPC4 for deleted in pancreatic carcinoma, locus 4 [6, 7]. It is similar in sequence to a *Drosophila melanogaster* gene (MAD) implicated in a transforming growth factor- β -like signaling pathway. The vast majority of pancreatic carcinomas have allelic loss of chromosome 18. Detailed analysis revealed a consensus region of homozygous deletion at 18q21.1 in one third of the

pancreatic carcinomas [6]. The DPC4 gene is located in this region, and is inactivated by intragenic mutations in another 20% of pancreatic carcinomas [6]. The intragenic mutations in one allele accompanied by the loss of a chromosomal region containing the other allele were termed loss of heterozygosity (LOH) [6]. To date, several authors have reported the loss of Dpc4 protein (pDpc4) in approximately 50% of pancreatic carcinomas using immunohistochemical (IHC) analysis [10, 23, 26, 27], and that it correlated with the prognosis of patients [16, 22, 23]. Some authors have also suggested that a loss of Dpc4 protein was caused by alterations of the DPC4 gene [23, 27], and that the mutated protein was degraded by a ubiquitin (Ub)-proteasome pathway [17, 28]. These reports suggest that DPC4 is an important gene correlating with the carcinogenesis and progression of pancreatic

Correspondence to: Tomoko Toga, Department of Cardiovascular and Digestive Surgery, Shimane University School of Medicine, 89-1 Enya-cho, Izumo, Shimane 693–8501, Japan.
E-mail: fsurgery@med.shimane-u.ac.jp

carcinoma.

The present study was designed to clarify the correlations that exist among the expression levels of Dpc4 protein, *DPC4*-mRNA and Ub by investigating their co-expression in serial slices of various human pancreatic duct lesions in pancreatic cancer tissues using *in situ* hybridization (ISH) and IHC methods. In the present study using ISH and IHC methods, we investigated the localization of *DPC4*-mRNA, Dpc4 protein and Ub visually and directly *in situ* in each lesion [2], and we determined that the expressions of *DPC4*-mRNA and Dpc4 protein were dissociated in invasive ductal carcinoma (IDC) lesions.

II. Materials and Methods

Pancreatic cancer specimens

Informed consent to the study on the genetic background of the patients was obtained from the patients or their family according to the recommendations of the ethics committee of our department that were documented in 1999.

Specimens were obtained from 21 patients who underwent resection of primary or metastatic lesions of pancreatic cancer between October 2001 and December 2002. The patients included 13 males and 8 females, and their ages ranged between 51 and 76 years. (mean $\pm$ SD, 69.9 $\pm$ 6.1 years). The clinical stage of the pancreatic cancer was classified according to the UICC TNM stage classification [11] as follows: stage I, n=1; II, n=1; III, n=9; IV, n=10. Histopathologically, all specimens were verified to be IDC of the pancreas.

To avoid degradation of the mRNA, the specimens were immediately immersed in cold 4% paraformaldehyde (PFA) solution after resection. After being fixed in 4% PFA for 24–36 hr, they were embedded in paraffin using routine procedures.

Next, after the procedures of both ISH and IHC using those specimens, the various pancreatic duct lesions in the specimens were classified according to the pancreatic intraepithelial neoplasia (PanIN) criteria, which were established at the National Cancer Institute-sponsored Pancreas Think Tank in September 1999 in Park City, Utah [9]. In the present study, the numbers of each pancreatic duct lesion were classified according to PanIN criteria as follows: normal duct, n=11; PanIN-1, n=11; PanIN-2, n=14; PanIN-3, n=19; IDC, n=21.

In situ hybridization

To detect *DPC4*-mRNA, we used digoxigenin (DIG)-labeled 40-mer oligonucleotide probes [14, 24]. Anti-sense and sense oligonucleotides for human *DPC4*-mRNA corresponded to bases 1322 to 1361 for human *DPC4*-mRNA (GenBank accession no. U44378). The selected sequences showed no significant similarity with known sequences recorded in GenBank and EMBL. They are located within exon-9 in *DPC4*-mRNA. This exon is known to have a non-sense mutation at codon-412 [7, 20] and the sequences used in the present study did not include this codon.

Four μ m-thick tissue sections were sliced from paraffin-embedded specimens. After deparaffinization, the sections were immersed in 10 mM sodium citrate buffer (pH 6) and autoclaved at 120°C for 20 min. To inhibit endogenous alkaline-phosphatase (AP) activity, the sections were treated with 0.2 N HCL for 20 min [13]. They were then hybridized with 1 μ g/ml DIG-labeled oligonucleotide probe in hybridization buffer (S3304, DakoCytomation, Kyoto, Japan) at 37°C overnight. During the above procedures, the sections were treated under RNase-free conditions. After washing in 2 $\times$ standard saline citrate (SSC) at 50°C for 30 min, to visualize the DIG-labeled probe, rabbit-F (ab') anti-DIG/AP (D5105, DakoCytomation) and BCIP/NBT (K0598, DakoCytomation) were used. Finally, the sections were counterstained with Nuclear Fast Red (S1963, DakoCytomation).

Immunohistochemistry

The specimens were primarily immunostained according to the labeled polymer method [18, 19]. The 4% PFA-fixed, paraffin-embedded specimens were cut into 4- μ m thick sections. The sections were deparaffinized in xylene for 5 min 3 times, hydrated in 100% and 95% ethanol and finally immersed in phosphate-buffered saline (PBS). To detect Dpc4 protein, the sections were treated with 10 mM sodium citrate buffer (pH 6) at 95°C for 40 min in a water bath (Yamato BM400, Japan) and cooled for 20 min at room temperature [12]. Next, the sections were incubated with a 1:100 dilution of anti-Dpc4 protein monoclonal antibody (Ab) (clone B8, Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) for 60 min at room temperature. To detect Ub, the sections were incubated with a 1:600 dilution of rabbit anti-Ub polyclonal Ab (Z0458, DakoCytomation) for 60 min at room temperature without any antigen retrieval procedures. Before incubation with both Abs, the sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity for 5 min. After incubation with the Abs, to visualize the antigens, we used labeled polymers: peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulin (K4000, DakoCytomation) for Dpc4, and peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulin (K4002, DakoCytomation) for Ub. Finally, the specimens were treated with a 0.05% 3,3'-diaminobenzidine solution for 5 min at room temperature. After washing in distilled water, the specimens were counterstained with hematoxylin.

Evaluation of ISH and IHC

For both ISH and IHC, the degree of expression was scored according to previous reports [23, 27]. The expression was scored as 0 when absolutely no cytoplasmic or nuclear staining was visible; scored as 1 when cells showed a very weak, barely perceptible expression that was not visible at low-power magnification but could be seen as faint expression upon very close inspection at high power; scored as 2 when the lesion contained two distinct populations of cells, that is those that were labeled with the antibodies or probe and those that were not; and scored as 3 when cells

were clearly positive with a staining intensity comparable to that of the surroundings. We further evaluated a score of 0 as negative expression (–), and a score of 1 to 3 as positive expression (+).

Statistical analysis

Student's t-test was used to compare the scoring values of ISH and IHC between the variables. Pearson's correlation analysis was applied to compare the correlations among the expression levels of Dpc4 protein, *DPC4*-mRNA and Ub. Significant differences were accepted at $p < 0.05$.

III. Results

Representative staining patterns for ISH of *DPC4*-mRNA and IHC for pDpc4 and Ub in pancreatic lesions are shown in Figures 1 and 2. The expression levels of *DPC4*-mRNA, pDpc4 and Ub are summarized in Table 1. Regarding a score of 0 as negative expression (–) and a score of 1 to 3 as positive expression (+), overall, *DPC4*-mRNA was

widely expressed in most pancreatic duct lesions (91%, 69/76), and negative expression was seen in only PanIN-3 and IDC lesions; pDpc4 was expressed in 55% (42/76) of all pancreatic duct lesions; and Ub was expressed in 64% (49/76) of all lesions. In normal duct or PanIN-1 lesions, pDpc4 was expressed in 100% (11/11) of the lesions, but Ub was expressed in 27% (3/11) and 36% (4/11) of these lesions ($p < 0.0001$ and $p = 0.0046$, respectively) (Table 2).

Comparative expressions of *DPC4*-mRNA, pDpc4 and Ub are summarized in Table 2. Overall, pDpc4 expression was significantly lower than *DPC4*-mRNA expression ($p = 0.0002$). This lower expression of pDpc4 than *DPC4*-mRNA was seen especially in PanIN-2, -3 and IDC lesions ($p = 0.0445$, $p < 0.0001$ and $p = 0.0239$, respectively). In IDC lesions, both *DPC4*-mRNA and pDpc4 expression levels were lower. The expression of pDpc4 decreased with the progression of the malignant grade of the duct lesions. In contrast, Ub expression increased with the progression of the malignant grade of the lesions. In PanIN-3 and IDC lesions, Ub expression was significantly higher than pDpc4 expres-

Fig. 1. A representative case of Category 2 (69-year-old man, well differentiated tubular adenocarcinoma). Original magnification $\times 100$. **A.** ISH demonstrated positive expression (stained reddish blue) of *DPC4*-mRNA by anti-sense probe in both carcinoma cells and stromal cells. **B.** ISH of the negative control using sense probe did not yield any specific signals. **C.** IHC for pDpc4 revealed no expression in carcinoma cells, while pDpc4 was expressed in stromal cells (stained brown). **D.** Ub was demonstrated in carcinoma cells (stained brown), but only rarely in stromal cells.

Fig. 2. Lesions with a score of 2 after ISH. (70-year-old man, adenocarcinoma metastasis to the liver). There are two distinct populations of cells, labeled cells (stained reddish blue) and unlabeled cells after ISH; neither labeled carcinoma cells nor unlabeled carcinoma cells expressed pDpc4 after IHC. Original magnification $\times 200$. **A.** ISH demonstrated positive expression of *DPC4*-mRNA (score 2). **B.** Negative control using a sense probe. **C.** pDpc4 (–) in carcinoma cells. **D.** Ub was stained brown in carcinoma cells.

sion ($p=0.0001$ and $p=0.0007$, respectively).

According to the present results, the co-expression patterns of *DPC4*-mRNA, pDpc4 and Ub were classified into three categories as follows: Category 1, *DPC4*-mRNA(+)/pDpc4(+); Category 2, *DPC4*-mRNA(+)/pDpc4(–); and Category 3, *DPC4*-mRNA(–)/pDpc4(–). Typical features are shown in Figure 1A–D for Category 2. The category classification of the co-expression patterns in pancreatic duct lesions is summarized in Table 3 as follows: all normal duct or non-malignant neoplasia (PanIN-1) lesions were classified as Category 1; most borderline (PanIN-2) or low grade malignancy (PanIN-3) lesions, were classified as Category 1 or Category 2: For IDC lesions, 24% were Category 1, 48% were Category 2, and 28% were Category 3. Notably 57% (4/7) of Category 3 had negative expression of Ub.

IV. Discussion

The present study demonstrated that pDpc4 expression decreased with the progression of malignancy, although *DPC4*-mRNA was widely expressed. This phenomenon was notable in the low-grade malignancy PanIN-3 lesions. In

addition, Category 3 (*DPC4*-mRNA(–)/pDpc4(–)) lesions were found in 28% of the high-grade malignancy IDC lesions. It was reported that pDpc4 expression was correlated with the status of all exons of the *DPC4* gene in 41 pancreatic carcinomas [27], and that the IHC matched the genetic data in 38 (93%) of the 41 cases; in 17 (94%) of 18 cases with wild-type *DPC4* genes, pDpc4 was labeled diffusely or focally positively, and in 21 (91%) of 23 cases with inactivated *DPC4* genes, pDpc4 was not labeled. Thus, IHC for pDpc4 was a highly sensitive (91%) and specific (94%) marker for genetic alterations. However, IHC does not differentiate between a mutant *DPC4* allele combined with loss of heterozygosity (LOH) and a homozygously deleted *DPC4* gene. Both cases show complete absence of pDpc4 labeling in the nucleus and the cytoplasm [27].

Thus, it was clarified that pDpc4 expression was correlated with the gene status of all exons of *DPC4* gene, but it is still unclear whether pDpc4 expression is directly correlated with the expression of *DPC4*-mRNA. The present study did not assess the *DPC4* gene status of the specimens, such as alterations, including homozygous deletions and LOH. However, using serial sections from the same paraffin block,

Table 1. Expression levels of *DPC4*-mRNA, *pDpc4*, and *Ub* in various pancreatic lesions

	Numbers (%) with positive expression				
Score	0	1	2	3	Overall
(I) <i>DPC4</i> -mRNA					
Normal duct	0	9 (81.8%)	2 (18.2%)	0	11 (100%)
PanIN-1	0	7 (63.6%)	4 (36.4%)	0	11 (100%)
PanIN-2	0	7 (50.0%)	6 (42.9%)	1 (7.1%)	14 (100%)
PanIN-3	1 (5.3%)	4 (21.1%)	13 (68.4%)	1 (5.3%)	19 (100%)
IDC	6 (28.6%)	8 (38.1%)	7 (33.3%)	0	21 (100%)
Overall	7 (9.2%)	35 (46.1%)	32 (42.1%)	2 (2.6%)	76 (100%)
(II) pDpc4					
Normal duct	0	6 (54.5%)	3 (27.3%)	2 (18.2%)	11 (100%)
PanIN-1	0	6 (54.5%)	5 (45.5%)	0	11 (100%)
PanIN-2	5 (35.7%)	5 (35.7%)	4 (28.6%)	0	14 (100%)
PanIN-3	13 (68.4%)	3 (15.8%)	3 (15.8%)	0	19 (100%)
IDC	16 (76.2%)	2 (9.5%)	2 (9.5%)	1 (4.8%)	21 (100%)
Overall	34 (44.7%)	22 (28.9%)	17 (22.4%)	3 (3.9%)	76 (100%)
(III) Ub					
Normal duct	8 (72.7%)	3 (27.3%)	0	0	11 (100%)
PanIN-1	7 (63.6%)	3 (27.3%)	0	1 (9.1%)	11 (100%)
PanIN-2	6 (42.9%)	3 (21.4%)	3 (21.4%)	2 (14.3%)	14 (100%)
PanIN-3	2 (10.5%)	6 (31.6%)	6 (31.6%)	5 (26.3%)	19 (100%)
IDC	4 (19.0%)	7 (33.3%)	5 (23.8%)	5 (23.8%)	21 (100%)
Overall	27 (35.5%)	22 (28.9%)	14 (18.4%)	13 (17.1%)	76 (100%)

pDpc4: DPC4 protein; *Ub*: Ubiquitin; IDC: Invasive ductal carcinoma.

Table 2. Comparison of *DPC4*-mRNA, *pDpc4* and *Ub* expression levels

Lesions	Expression score (mean±SD)*				<i>Ub</i>
	<i>DPC4</i> -mRNA	p value**	<i>pDpc4</i>	p value***	
Normal duct	1.18±0.41	0.1377	1.64±0.81	<0.0001	0.27±0.47
PanIN-1	1.36±0.51	0.6761	1.46±0.52	0.0046	0.55±0.93
PanIN-2	1.57±0.65	0.0445	0.93±0.83	0.6714	1.07±1.14
PanIN-3	1.74±0.65	<0.0001	0.47±0.77	0.0001	1.73±0.99
IDC	1.05±0.80	0.0239	0.43±0.87	0.0007	1.52±1.10
Overall	1.38±0.69	0.0002	0.86±0.91	0.0669	1.17±1.10

pDpc4: DPC4 protein; *Ub*: Ubiquitin; IDC: Invasive ductal carcinoma.

* Scoring criteria are explained in Materials and Methods (Evaluation of ISH and IHC).

** *DPC4*-mRNA vs. *pDpc4*.

*** *pDpc4* vs. *Ub*.

ISH and IHC could be used to compare *DPC4*-mRNA expression with *pDpc4* expression directly in each lesion. The present study indicated that the expression of *DPC4*-mRNA and *pDpc4* was dissociated in PanIN-2, PanIN-3 and IDC lesions. In studies using cell lines, it was reported that the cell lines, which showed no *pDpc4* in Western blot analysis, expressed *DPC4*-mRNA in RT-PCR [1]. Other authors also reported that the level of expression of *DPC4*-mRNA was relatively steady and was not always correlated with the amount of *pDpc4* in the cell lines [5]. Therefore, the present study may be the first to demonstrate the dissociation between the expression of *pDpc4* and the expression of

DPC4-mRNA in human pancreatic cancer tissue using IHC and ISH analyses.

The mechanisms responsible for this dissociated expression between *DPC4*-mRNA and DPC4 protein are not clear. We hypothesize that some protein degradation system may play a role.

It was reported that a nonsense mutation generating a C-terminal truncation of 38 amino acids in *pDpc4* has been identified in pancreatic adenocarcinoma [7]. Deletion of the C-terminal 38 amino acids of *DPC4* results in the removal of α -helix and β -strand 11 and half of the L3 loop [21]. Loss of β -strand 11 is likely to severely disrupt the overall structure

Table 3. Comparative expression of *DPC4*-mRNA and pDpc4 in combination with Ub expression in various pancreatic lesions

Ub expression score	Numbers (%) expressing Ub							
	<i>DPC4</i> -mRNA(+)/pDpc4(+)		<i>DPC4</i> -mRNA(+)/pDpc4(−)		<i>DPC4</i> -mRNA(−)/pDpc4(−)		Overall	
	0	1–3	0	1–3	0	1–3	0	1–3
Normal duct	8 (73%)	3 (27%)	—	—	—	—	8 (73%)	3 (27%)
PanIN-1	7 (64%)	4 (36%)	—	—	—	—	7 (64%)	4 (36%)
PanIN-2	4 (50%)	4 (50%)	2 (33%)	4 (67%)	—	—	6 (43%)	8 (57%)
PanIN-3	1 (17%)	5 (83%)	1 (8%)	11 (92%)	0 (0%)	1 (100%)	2 (11%)	17 (89%)
IDC	0 (0%)	5 (100%)	0 (0%)	10 (100%)	4 (67%)	2 (33%)	4 (19%)	17 (81%)
Total	20 (49%)	21 (51%)	3 (11%)	25 (89%)	4 (57%)	3 (43%)	27 (36%)	49 (64%)

Ub: Ubiquitin; pDpc4: DPC4 protein; IDC: invasive ductal carcinoma.

of the pDpc4 MH2 domain, because it is the part of the β -sandwich that makes up the hydrophobic core of the molecule [21] leading to an unstable protein [17]. Therefore, the mutant pDpc4 is highly unstable compared with wild type pDpc4 and is rapidly degraded through a Ub-proteasome pathway [17, 28].

Ub, a small polypeptide consisting of 76 highly conserved amino acids (8.5 kDa), occurs in all eukaryotic cells. Ub plays a very important role in the regulation of non-lysosomal ATP-dependent protein degradation. The protein to be degraded is conjugated with Ub, and the ubiquitinated protein is then selectively degraded through a 26S-complex multi-catalytic pathway. This complex process is thought to be of great importance for regulating numerous cellular processes [3, 4, 8, 15, 25]. The present results of IHC for Ub suggest that the Ub-proteasome pathway may be a major route for degrading mutant pDpc4 in pancreatic carcinoma cells. Certainly, the Ub-proteasome pathway is involved in the degradation of mutant pDpc4, and other proteins can also be targets of Ub [25].

Based on the present results, we classified the co-expression patterns of *DPC4*-mRNA and pDpc4 into 3 categories (Table 3), and all normal duct or non-malignant neoplasia (PanIN-1) lesions were classified as Category 1. Most borderline (PanIN-2) or low-grade malignancy (PanIN-3) lesions were classified as Category 1 or Category 2. However, with respect to IDC lesions, 24% were Category 1, 48% were Category 2, 28% were Category 3, and about half of the Category 3 had negative expression of Ub.

These results lead us to hypothesize that the status of Category 2 (*DPC4*-mRNA(+)/pDpc4(-)) may be caused by a mutant *DPC4* allele combined with LOH. The high expression of Ub in the lesions suggested that mutant pDpc4, which was synthesized based on information from intragenic mutated mRNA genes, was degraded by a Ub-proteasome pathway. In addition, the findings for Category 3 (*DPC4*-mRNA(-)/pDpc4(-)) suggested that *DPC4* gene may be homozygously deleted, and that the Ub-proteasome pathway may not be involved for Category 3 lesions. However, the Ub(+) status found for Category 3 is difficult to explain, and it is possible that the Ub-proteasome pathway may work against the various mutant products of other genes.

The present study demonstrates that pDpc4 expression is lost with the progression of pancreatic duct lesion malignancy, although *DPC4*-mRNA is widely expressed. On the other hand, Ub expression increases with the progression of malignancy. These results suggest that decreased expression of pDpc4 may be caused by its degradation by highly expressed Ub. In addition, the decreased expression of *DPC4*-mRNA in high-grade malignancy suggests a homozygous deletion of the *DPC4* gene. During replication, each of the double strands of DNA is used as a template for the formation of a complementary DNA strand. Therefore, in the case of LOH (which is an intragenic mutation in one allele accompanied by a loss of a chromosomal region containing the other allele), a homozygous deletion may arise in the next replication. ISH study showed two distinct populations in IDC lesions, labeled cells and unlabeled cells (Fig. 2A). We hypothesize that, in ISH study of *DPC4*-mRNA, the labeled cells in IDC lesions may reflect this alteration in the gene status of LOH. According to this hypothesis, labeled cells develop into unlabeled cells due to a homozygous deletion of the *DPC4* gene as malignancy progresses.

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VI. References

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