

The Dissociated Expression of Protein and Messenger RNA of DPC4 in Human Invasive Ductal Carcinoma of the Pancreas and their Implication for Patient Outcome

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Abstract. *Background:* The loss of expression of Dpc4 protein (pDpc4) has been demonstrated in about half of invasive ductal carcinoma (IDC) of the pancreas, but the expression of DPC4-mRNA remains to be evaluated. The present study assessed the comparative expression of pDpc4 and DPC4-mRNA in pancreatic IDC and their implication for patient outcome. *Materials and Methods:* In the freshly separated specimens of 21 IDCs and the paraffin-embedded specimens of 88 resectable IDCs, the expression of mRNA was assessed by *in situ* hybridization and the expression of pDpc4 was assessed by immunohistochemistry. *Results:* In the freshly separated specimens, DPC4-mRNA was expressed in 71% of the IDC, but pDpc4 expression was lost in 76% of the IDC. In 88 resectable IDCs, pDpc4 expression was lost in 75 (85.2%) and loss of pDpc4 expression was significantly correlated with the grade of nodal involvement ($p=0.0265$). Furthermore, the survival rate of the pDpc4 (-) group was significantly lower than that of the pDpc4 (+) group ($p=0.0391$). Adjuvant chemotherapy (ACT) significantly improved the survival rate and, in the ACT group, pDpc4 (-) patients had a significantly lower survival rate than the pDpc4 (+) patients. *Conclusion:* In human pancreatic IDC, although DPC4-mRNA was usually expressed, the expression of pDpc4 was lost. The expression of pDpc4 is an indicator of better prognosis and response to ACT.

DPC4 (also called SMAD-4) is a tumor suppressor gene located on 18q21.1. The locus was termed DPC4 for deleted

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in pancreatic carcinoma, locus 4 (1,2). 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 with a homozygous deletion at 18q21.1 in one-third of pancreatic carcinomas (1). The DPC4 gene is located in this region, and is inactivated by intragenic mutations in another 20% of pancreatic carcinomas (1). The intragenic mutations in one allele, which is accompanied by the loss of a chromosomal region containing the other allele, are described as loss of heterozygosity (LOH) mutations (1). To date, several authors have reported that the loss of Dpc4 protein (pDpc4) was seen in approximately 50% of pancreatic carcinomas, using immunohistochemical (IHC) analysis (3-6), and that this loss was correlated with the prognosis of patients (7,8). Some authors suggested that the loss of pDpc4 was caused by alterations in the DPC4 gene (4,5). These reports also suggested that DPC4 is an important gene correlated with the carcinogenesis and progression of pancreatic carcinoma.

The present study was designed to clarify the correlations between the expression of pDpc4 and DPC4-mRNA in invasive ductal carcinoma (IDC) of the pancreas. It is, however, difficult to separate cancer cells from non-malignant cells completely, because cancer tissues usually contain malignant cells as well as normal cells, which have a different genetic status. Therefore, it is not easy to demonstrate the inactivation of various genes in cancer tissues using RT-PCR or the Northern blot method. In the present study, this problem was solved by investigating the co-expression of DPC4-mRNA and pDpc4 in serial slices of individual lesions using ISH and IHC methods.

In addition, the present study assessed the implication of pDpc4 expression in the outcome of the 88 patients with resectable IDC. To our knowledge, there have been only two studies which reported the effects of the loss of pDpc4

Table I. *pDpc4* expression and clinicopathological characteristics.

| Feature | No. of patients | No.(%) of pDpc4 | | Correlation coefficient (p-value) |
|-----------------------|-----------------|-----------------|-----------|-----------------------------------|
| | | (-) | (+) | |
| Overall | 88 | 75(85%) | 13(15%) | |
| Age (y.o.): mean | 65.9±9.5 | 66.4 ± 9.1 | 61.9±11.7 | r=-0.052 (p=0.6318) |
| Gender: Male | 43(49%) | 37(49%) | 6(46%) | r=-0.023 (p=0.8351) |
| Female | 45(51%) | 38(51%) | 7(54%) | |
| Histological grade: 1 | 37(42%) | 32(43%) | 5(39%) | r=0.084 (p=0.4388) |
| 2 | 45(51%) | 39(52%) | 6(46%) | |
| 3 | 6(7%) | 4(5%) | 2(15%) | |
| pTNM Stage: I | 12(14%) | 8(11%) | 4(31%) | r=-0.162 (p=0.1319) |
| II | 5(6%) | 4(5%) | 1(7%) | |
| III | 43(49%) | 39(52%) | 4(31%) | |
| IV | 28(32%) | 24(32%) | 4(31%) | |
| pT: 1 | 8(9%) | 6(8%) | 2(15%) | r=-0.093 (p=0.3886) |
| 2 | 29(33%) | 24(32%) | 5(39%) | |
| 3 | 28(32%) | 25(33%) | 3(23%) | |
| 4 | 23(26%) | 20(27%) | 3(23%) | |
| pN: 0 | 10(11%) | 6(8%) | 4(31%) | r=-0.252 (p=0.0175) |
| 1a | 34(39%) | 29(39%) | 5(38%) | |
| 1b | 44(50%) | 40(53%) | 4(31%) | |
| M: 0 | 84(95%) | 72(96%) | 12(92%) | r=0.063 (p=0.5615) |
| 1 | 4(5%) | 3(4%) | 1(8%) | |

expression on the prognosis of pancreatic IDC, (4,8), and the present study may be the first report on the clinicopathological significance of DPC4 in Japanese pancreatic IDC.

Materials and Methods

Pancreatic cancer specimens. Informed consent to study the genetic background of the patients was obtained from the patients or their family, according to the recommendations of the ethical committee of our department as documented in 1999.

Fresh specimens of IDC were obtained from 21 patients who underwent resection of primary or metastatic lesions of pancreatic IDC between October 2001 and December 2002. Histopathologically, all specimens were verified to be IDC of the pancreas. To avoid degradation of the mRNA, the specimens were immediately bathed in 4% PFA (paraformaldehyde) cold solution after resection. After being fixed in 4% PFA for 24 - 36 hours, they were embedded in paraffin using routine procedures. Furthermore, 88 formalin-fixed, paraffin-embedded specimens, which were resected between 1982 and 2002, were also employed. The profiles of these patients are summarized in Table I.

Thirty patients received surgery alone and 58 received adjuvant chemotherapy (ACT) after surgery. ACTs included UFT alone (n=23), UFT and cyclophosphamide (UC therapy, n=26) and UC + gemcitabine (UCG therapy, n=5), and 4 others.

The clinical stage of the pancreatic cancer was classified according to the post-surgical TNM stage classification of the International Union Against Cancer (UICC).

In situ hybridization. To detect *DPC4*-mRNA, we used digoxigenin (Dig)-labeled 40-mer oligonucleotide probes (9,10). 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 show 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 nonsense mutation at codon-412 (2,11); the sequences used in the present study did not include this codon.

Four-µm-thick tissue sections were sliced from paraffin-embedded specimens. After deparaffinization, the sections were bathed in 10mM sodium citrate buffer (pH6) and autoclaved at 120°C for 20 min. To inhibit the endogenous alkaline-phosphatase (AP) activity, the sections were treated with 0.2N HCl for 20 min (12), then hybridized with 1 µg/ml Dig-labeled oligonucleotide probe in hybridization buffer (S3304, DakoCytomation Co. Ltd., Kyoto, Japan) at 37°C overnight. During the above procedures, the sections were treated under RNase-free conditions. After washing in 2 x 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(5-Bromo-4-Chloro-3-Indoxyl Phosphate and Nitro Blue Tetrazolium Chloride) solution (K0598, DakoCytomation) were used. Finally, the sections were counter-stained with Nuclear Fast Red (S1963, DakoCytomation).

Immunohistochemistry. The specimens were immuno-stained primarily according to the labeled polymer method (13,14). Formalin-fixed, paraffin-embedded specimens were cut into 4-µm sections. The sections were deparaffinized in xylene for 5 min 3 times, hydrated in 100% and 95% ethanol and finally bathed in phosphate-buffered saline (PBS). To detect pDpc4, the sections were treated with 10mM sodium citrate buffer (pH6) at 95°C for 40 min in a water bath (Yamato BM400, Japan) and cooled for 20 min at room temperature. 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

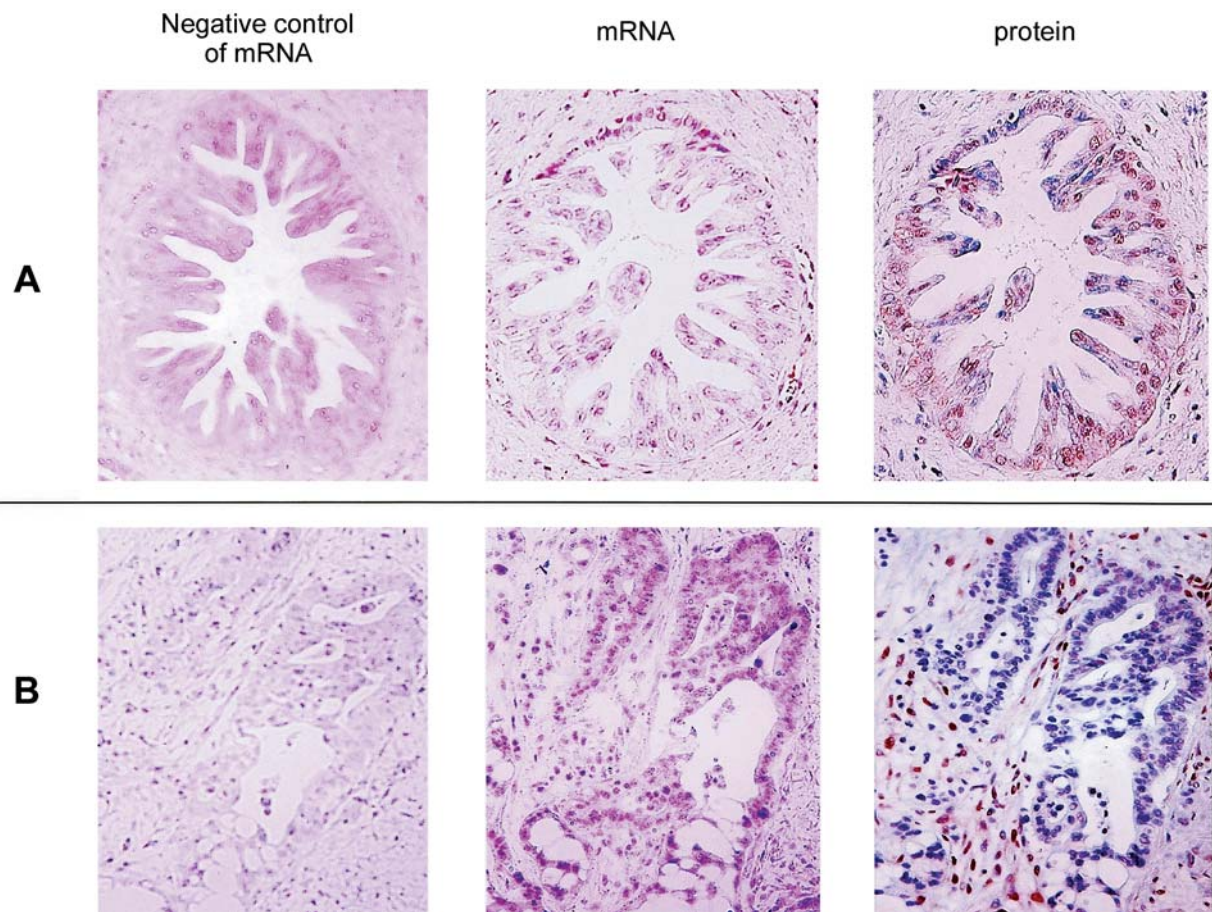


Figure 1. Representative ISH staining for DPC4-mRNA and IHC staining for pDpc4 . Original magnification 200x. Fig. 1A and 1B represent serially sliced sections of the same paraffin-embedded specimens. 1A, a 73-year-old man, a well-differentiated tubular adenocarcinoma: both mRNA and protein of DPC4 were positively stained in the nuclei of carcinoma cells. 1B, a 69-year-old man, a well-to moderately-differentiated tubular adenocarcinoma: mRNA was positively expressed in reddish blue in the nuclei of carcinoma cells, while protein was not stained in the carcinoma cells, but was clearly stained in brown in the nuclei of surrounding stromal cells.

at room temperature. Before incubation with Ab, the sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity for 5 min. After incubation with the Ab, to visualize the antigen, we used labeled polymer: peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins (K4000, DakoCytomation). Finally, the specimens were treated with a 0.05% 3,3'-diaminobenzidine solution for 10 min at room temperature. After washing in distilled water, the specimens were counter-stained with hematoxylin.

Evaluation of ISH and IHC. Staining was considered positive when nuclear staining of more than 10% of the tumor cells were observed. Those cases with only faint staining were regarded as negative.

Statistical analysis. The Mann-Whitney *U*-test was used to compare patient backgrounds among the groups. The cumulative survival rates were calculated according to the Kaplan-Meier method and were compared by the Cox-Mantel test. A multivariate analysis of

Table II. Comparative expression of DPC4-mRNA and Dpc4 protein (pDpc4) in freshly separated specimens of invasive ductal carcinoma (IDC) of the pancreas.

| | pDpc4 | | Total |
|-----------|----------------|-----------|------------|
| | (-) | (+) | |
| DPC4-mRNA | (-) 6 (28.6%) | 0 (0%) | 6 (28.6%) |
| | (+) 10 (47.6%) | 5 (23.8%) | 15 (71.4%) |
| Total | 16 (76.2%) | 5 (23.8%) | 21 (100%) |

Cox's proportional hazard risk model was used to obtain the conditional risk of pancreatic cancer-related death. Statistically significant differences were defined at $p < 0.05$. The statistical analysis was carried out using SAS computer software.

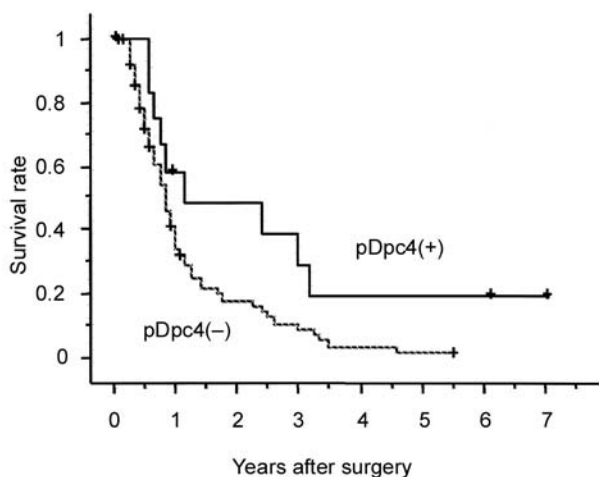


Figure 2. The survival curves grouped by the pDpc4 expression. pDpc4(-) (n = 75) vs. pDpc4(+) (n=13), p=0.0391.

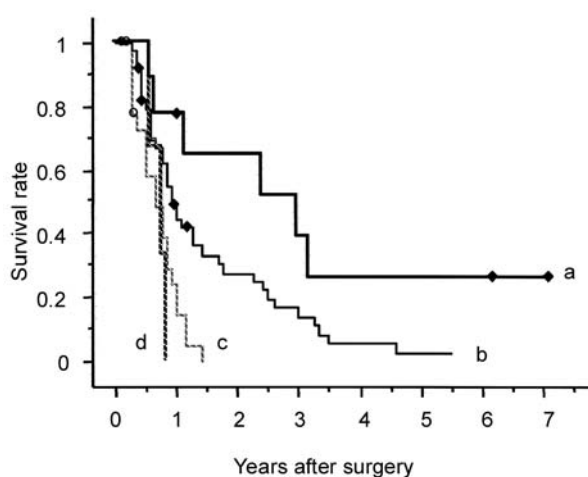


Figure 4. Effects of pDpc4 expression on the efficacy of adjuvant chemotherapy (ACT). a, pDpc4(+)/ACT (+) (n=9); b, pDpc4(-)/ACT (+) (n=49); c, pDpc4(+)/ACT (-) (n=4); d, pDpc4(-)/ACT (-) (n=26). a vs. b, p=0.0450.

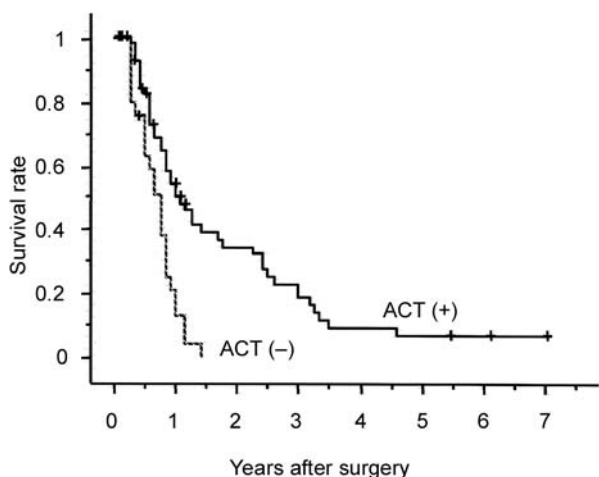


Figure 3. Effects of adjuvant chemotherapy (ACT) on survival after surgery. ACT (+) (n=58) vs. ACT (-) (n=30), p<0.0001.

Results

Representative ISH staining for *DPC4*-mRNA and IHC staining for pDpc4 in pancreatic lesions are shown in Fig. 1A and 1B, which shows the typical co-expression patterns in pancreatic IDC as follows: both *DPC4*-mRNA and pDpc4 were expressed in the nucleus of the IDC cells (Figure 1A), by contrast *DPC4*-mRNA was expressed in the nucleus of the ductal carcinoma cells, while pDpc4 was not expressed in the ductal carcinoma cells but, in the stromal cells surrounding carcinoma cells, stable expression of pDpc4 was

demonstrated (Figure 1B). The expression of *DPC4*-mRNA and pDpc4 are summarized in Table II. *DPC4*-mRNA was expressed in 71% of the IDCs, but pDpc4 expression was lost in 76% of the IDCs. Furthermore, both positive were seen in only 24% of IDC lesions and in an about half of IDCs, *DPC4*-mRNA was expressed, but pDpc4 expression was lost. Furthermore, 28.6% of IDC lesions were both negative.

In 88 resectable IDCs, the expression of pDpc4 was assessed and its impact on the clinicopathological factors and patient outcome were analyzed. The patient profile and the results of the analyses are summarized in Table I. The pDpc4 expression was lost in 75 out of 88 IDCs (85.2%) and the loss of pDpc4 expression was significantly correlated with the grade of nodal involvement (p=0.0265). Furthermore, the survival rate of the patients with pDpc4 (-) IDC was significantly lower than that of those with pDpc4 (+) IDC (p=0.0391) (Figure 2).

The adjuvant chemotherapy (ACT) showed a significant effect on the prognosis, and the survival rate of the ACT (+) group was significantly higher than that of the ACT (-) group (p<0.0001) (Figure 3). In the ACT (+) group, pDpc4 (+) patients had significantly higher survival rate than the pDpc4 (-) patients, but in the ACT (-) group there was no difference in survival rate between the pDpc4(+) and (-) groups (Figure 4).

The implication of pDpc4 expression was assessed by multivariate analysis, which demonstrated that ACT and stage were the significant prognostic variables, but pDpc4 expression was not a significant variable (Table III). Furthermore, in subgroup analyses, pDpc4 expression was not a significant variable in either the ACT group or surgery alone group.

Table III. Multivariate analysis by Cox's proportional hazard risk model.*

| Variables | Conditional risk ratio (95% confidence limit) | p-value (Chi-square) |
|---------------------------------|---|-------------------------|
| (I) Overall patients | | |
| Clinical stage | 1.685 (1.241 - 2.288) | 0.0008 |
| Adjuvant chemotherapy | 0.388 (0.220 - 0.683) | 0.0011 |
| Histological grade | 1.537 (0.979 - 2.412) | 0.0615 |
| pDpc4 expression | 0.651 (0.315 - 1.344) | 0.2460 |
| Gender | 1.229 (0.721 - 2.094) | 0.4478 |
| Age | 0.998 (0.971 - 1.025) | 0.8638 |
| (II) Adjuvant chemotherapy (+) | | |
| Clinical stage | 1.612 (1.109 - 2.343) | 0.0123 |
| Histological grade | 1.951 (1.078 - 3.529) | 0.0272 |
| pDpc4 expression | 1.580 (0.590 - 4.234) | 0.3629 |
| Age | 0.992 (0.960 - 1.026) | 0.6576 |
| Gender | 1.110 (0.571 - 2.157) | 0.7588 |
| (III) Adjuvant chemotherapy (-) | | |
| Clinical stage | 2.664 (1.091 - 6.506) | 0.0315 |
| Gender | 1.635 (0.617 - 4.330) | 0.3229 |
| pDpc4 expression | 1.579 (0.382 - 6.523) | 0.5277 |
| Age | 1.013 (0.958 - 1.072) | 0.6429 |
| Histological grade | 1.151 (0.550 - 2.413) | 0.7086 |

*dependent variable = month , censoring variable = death due to pancreatic cancer

Discussion

The present study demonstrated that pDpc4 expression decreased in IDC, although *DPC4*-mRNA was relatively expressed. In addition, about 50% of IDC was mRNA(+)/protein(-) and, in 29% of IDC lesions, neither mRNA nor pDpc4 expression were found.

The present study demonstrated that the intracellular pDpc4 is located in the nucleus, although previous studies reported that the subcellular localization of the pDpc4 was mainly cytoplasmic (3,5), with or without expression of pDpc4 in the nuclei. However, in the present study, we found that localization of pDpc4 is primarily in the nucleus and not in cytoplasm. pDpc4 has been reported to work as a transcriptional activator that binds specific DNA sequences and whose nuclear localization is induced after exposure to type β transforming growth factor-like cytokines (15). The previous reports utilized a biotin-avidin method for IHC, but, in the past, we had experienced false-positive expression due to endogenous biotin with IHC methods based on biotin-avidin interactions. In the present study, in the IHC techniques using the SAB (Streptavidin Biotin) method to detect pDpc4, some tissue samples showed stronger expression in the cytoplasm than in the nucleus. To avoid the influence of endogenous biotin in pancreatic tissue that has a lot of biotin (16), we chose

the labeled polymer method for detecting pDpc4. As a result, it was found that pDpc4 was not expressed in 76% of fresh IDC specimens and in 85% of paraffin-embedded specimens, whereas previous studies using the biotin-avidin method indicated that pDpc4 was expressed in about 50% of IDC lesions. Accordingly, we believe that pDpc4 is more frequently deleted in pancreatic cancer than previously reported.

The previous studies reported that pDpc4 expression was correlated with the status of all exons of the *DPC4* gene in pancreatic carcinomas by IHC (5): in 17 (94%) out of 18 cases with wild-type *DPC4* genes, pDpc4 was labeled diffusely or focally positively and in 21 (91%) out of 23 cases with inactivated *DPC4* genes, pDpc4 was not labeled. Thus, it was concluded that IHC for pDpc4 was a highly sensitive (91%) and specific (94%) marker for genetic alterations (5). However, these results were based on the IHC using the biotin-avidin method. As described above, pDpc4 is more frequently deleted in pancreatic cancer in the present study (76 ~ %) using the labeled polymer method than in the previous studies using the biotin-avidin method (about 50%). Accordingly, previous studies that reported pDpc4 expression was correlated with the gene status of all exons of the *DPC4* gene are questionable. Furthermore, it was reported that in some cell lines there was a dissociation between the expression of pDpc4 in Western blot analysis and that of *DPC4*-mRNA in RT-PCR analysis (17), while other authors also reported that the expression of *DPC4*-mRNA was relatively constant and was not always correlated with the amount of pDpc4 in the cell lines (18). Although the present study did not assess the *DPC4* gene status of the specimens, such as alterations, including homozygous deletions and LOH, by using serial sections from the same paraffin block, ISH and ICH could compare *DPC4*-mRNA expression with pDpc4 expression directly in each lesion. Therefore, the present study may be the first report demonstrating the dissociation between the expression of pDpc4 and the expression of *DPC4*-mRNA in human pancreatic IDC.

The present study demonstrated that about half of IDCs were *DPC4*-mRNA(+)/protein(-). If *DPC4*-mRNA(+)/protein(+) is a normal genetic state, a quarter of IDC may possess a normal status of *DPC4* gene. Another quarter may possess mRNA(-)/protein(-). The status of which suggested a transcriptional disorder including gene alterations, especially a homozygous deletion of the *DPC4* gene. About half of IDC were *DPC4*-mRNA(+)/protein(-). The mechanisms responsible for this dissociated expression between mRNA and protein are not clear. We hypothesize several mechanisms are responsible. One is a possibility that translation may be inhibited by a disorder of the ribosome including peptidyl-transferase abnormality; however, in this condition, cells cannot survive. Another possibility is the *DPC4*-mRNA(+)/pDpc4(-) status may be caused by a mutant *DPC4* allele combined with LOH. It was reported that a nonsense mutation generating a C-terminal

truncation of 38 amino acids in pDpc4 has been identified in pancreatic adenocarcinoma (2). Deletion of the C-terminal 38 amino acids of *DPC4* results in the removal of the alpha-helix and beta-strand 11 and half of the L3 loop (19). Loss of the beta-strand 11 is likely to severely disrupt the overall structure of the Dpc4 MH2 domain, because it is the part of the beta-sandwich that makes up the hydrophobic core of the molecule (19) and disruption would lead to an unstable protein (20). Therefore, the mutant pDpc4 is highly unstable compared with wild-type pDpc4 and may be rapidly degraded through a protease system, such as an Ub-proteasome pathway (20,21).

To our knowledge, there have been only two studies which reported the effects of the loss of pDpc4 expression on the prognosis of pancreatic IDC and they reported that the loss of pDpc4 expression in pancreatic IDC was significantly correlated with the poorer prognosis (4,8). In addition, the present study may be the first report on the clinicopathological significance of *DPC4* in Japanese pancreatic IDC. In the present study, the survival rate of the pDpc4(-) IDC was significantly lower than that of pDpc4(+) IDC, which was compatible with the previous studies. Furthermore, the present study may be the first report analyzing the effect of the loss of pDpc4 expression on the efficacy of ACT. However, to clarify the true clinicopathological significance of *DPC4* in human pancreatic IDC and to draw a conclusion, studies and results should be further replicated by other working groups.

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