Loss of DPC4 expression and its correlation with clinicopathological parameters in pancreatic carcinoma

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AIM: DPC4 is a tumor suppressor gene on chromosome 18q21.1 that has high mutant frequencies in pancreatic carcinogenesis. The purpose of this study was to investigate the role of DPC4 alterations in tumorigenesis and progression of pancreatic carcinomas.

METHODS: We studied the immunohistochemical markers of DPC4 in 34 adenocarcinomas and 16 nonmalignant specimens from the pancreas. The 16 nonmalignant specimens from the pancreas included 8 non-neoplastic cysts and 8 normal pancreatic tissues. The relationship between DPC4 alterations and various clinicopathological parameters was evaluated by chi-square test or Fisher’s exact test. Survivals were calculated using Kaplan-Meier method (by a log-rank test).

RESULTS: All the 16 nonmalignant cases of the pancreas showed expression of DPC4 gene. Loss of DPC4 expression was seen in 8 of 34 (23.5 %) pancreatic adenocarcinomas. The frequency of loss of DPC4 expression was higher in poorly differentiated adenocarcinoma (G3) than in well and moderately differentiated adenocarcinoma (G1 and G2) histologically (P=0.037). Loss of DPC4 expression of the patients at TNM stage IV was also higher than that of the patients at TNM stages I, II and III (60.0 % at stage IV, versus 14.3 % at stage I, 18.2 % at stage II, and 18.2 % at stage III) (P=0.0223). The mean and median survival in patients with DPC4 expression was longer than those in patients with loss of DPC4 expression. Kaplan-Meier survival analysis demonstrated patients with DPC4 expression had a higher survival rate than patients with loss of DPC4 expression, but the difference did not reach statistical significance (P =0.879).

CONCLUSION: This study suggests that DPC4 is involved in the development of pancreatic carcinoma and is a late event in pancreatic carcinogenesis, DPC4 expression may be a molecular prognostic marker for pancreatic carcinoma.

INTRODUCTION
The incidence of pancreatic carcinoma has increased in recent decades in the world, and this cancer has the lowest five-year survival rate among all cancers. The dismal survival of patients with pancreatic carcinomas is caused by the late diagnosis and low resection rates[12]. However, understanding the molecular pathogenesis of pancreatic carcinomas may be the foundation upon which to develop novel strategies for identifying genetic markers useful for the early diagnosis and treatment. An association has been demonstrated between pancreatic carcinomas and various genetic alterations including genes K-ras[3,4], Her-2/neu[5,6], p16[6] and p53[7]. Recently, DPC4 (deleted in pancreatic carcinoma, locus 4; Smad4) located on chromosome 18q21.1, has received special attention as its alterations may play a role in activation of pancreatic carcinogenesis[8].

DPC4 gene is a tumor suppressor gene, which has been shown to mediate the downstream effects of TGF-β superfamily signaling, resulting in growth inhibition[9]. Inactivation of DPC4 tumor-suppressor gene is relatively specific for pancreatic carcinoma, although it has been shown to occur in a small percentage of primary carcinomas of the esophagus[10,11], stomach[11,12], head and neck[13], breast, ovary[14], colon[15], and biliary tract[16]. DPC4 can be inactivated by one of the two identified mechanisms: intragenic mutation of one allele coupled with loss of the other allele, or deletion of both alleles (homozygous deletions). Both mutations and homozygous deletions of DPC4 gene have been observed in a high proportion of pancreatic carcinomas[9]. In contrast, the role of DPC4 in human pancreatic carcinoma remains less well defined. Recently, immunohistochemical labeling for the DPC4 gene product has become an extremely sensitive and specific marker for DPC4 gene alterations in pancreatic carcinomas, and has been shown to mirror the DPC4 genetic status of pancreatic carcinomas, because most mutations of DPC4 could result in a loss of the protein. Therefore, immunolabeling for DPC4 could provide a useful tool to examine genetic status in pancreatic adenocarcinomas[17,18].

In the present study, we examined DPC4 expression in 34 adenocarcinomas and 16 nonmalignant specimens from the pancreas using a monoclonal antibody to human DPC4 protein by means of immunohistochemistry and studied the relation between expression of DPC4 and various clinicopathological parameters in order to elucidate whether altered DPC4 expression played a role in the tumorigenesis and progression of pancreatic carcinomas.

MATERIALS AND METHODS

Patients and samples
Thirty-four specimens of pancreatic adenocarcinomas were retrieved from the pathology archives of China-Japan Friendship Hospital between 1984 and 2000. There were 22 males and 12 females with pancreatic carcinomas, and the average age of the patients was 55.18±11.29 years (mean±SD), with a range of 30-75 years. Twenty-eight patients were followed up until death or until the time of this study.
Histopathological grade and clinical staging were evaluated according to the criteria by Klöppel for pancreatic tumors and the International Union Against Cancer (UICC) TNM classification. Histopathologic examination revealed well-differentiated adenocarcinoma in 10 patients, moderately differentiated adenocarcinoma in 15 patients, and poorly differentiated adenocarcinoma in 9 patients. Seven patients were at UICC stages I, 11 at stages II, 11 at stage III, and 5 at stage IV. In addition, 16 nonmalignant specimens from the pancreas including 8 non-neoplastic cysts and 8 normal pancreatic tissues were used as controls.

**Immunohistochemistry**

Tissues were routinely fixed in neutral formalin, embedded in paraffin, and 5 µm thick consecutive sections were cut. After deparaffinized, the slides were placed in a solution of 3 % hydrogen peroxide (1:1) for 10 minutes to block the activity of endogenous peroxidase, and then heated in a microwave for 5 minutes at 100 °C. After the slides were cooled for 30 minutes, nonspecific binding was blocked with a protein solution for 10 minutes, and then each slide was labeled with a 1:100 dilution of monoclonal antibody to DPC4 (clone B8, Santa Cruz, CA). Anti-DPC4 antibody was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3,3’-diaminobenzidine. The sections were counterstained with hematoxylin. Positive cells were stained dark brown in the nuclei and/or cytoplasms, and the staining was graded into four categories: 0, no staining, 1+, weak staining, 2+, moderate staining, 3+, heavy staining. Positive staining was considered as expression of DPC4. Normal pancreatic ducts, islets of Langerhans, acini, lymphocytes, and stromal fibroblasts showing moderate to strong expression of DPC4 gene, served as positive internal controls for each section. For negative controls, the primary antibody was replaced with phosphate buffered solution (PBS).

**Statistical analysis**

The data were analyzed with chi-square test or Fisher’s exact test to compare the differences between the two subgroups of patients based on the results of DPC4 staining. All of the tests were two-tailed. Survivals were calculated using Kaplan-Meier method (by a log-rank test).

**RESULTS**

The results of DPC4 protein immunohistochemistry are summarized in Table 1, and typical examples of the positive and negative groups are shown in Figure 1 (A-E). It was observed that pancreatic carcinoma showed loss of DPC4 expression, whereas the adjacent normal pancreatic tissue had DPC4 expression (Figures 1B and C).

**Figure 1** Representative immunostaining results of DPC4 in pancreatic carcinoma (A-E). Positive cells were stained dark brown in the nuclei and/ or cytoplasms (A-D).
All the 16 nonmalignant cases of the pancreas showed expression of DPC4 gene products. Loss of DPC4 expression was seen in 8 of 34 (23.5 %) pancreatic adenocarcinomas. The results of immunostaining of DPC4 expression in 34 pancreatic carcinomas and the correlation with various clinicopathological parameters are shown in Table 2. A significant difference was found in the frequency of loss of DPC4 expression between well and moderately differentiated adenocarcinomas (G1 and G2) and poorly differentiated adenocarcinoma (G3) histologically (P = 0.037). Although loss of DPC4 expression in the patients at TNM staging IV was higher than that in those at stages I, II and III (60.0 % at stage IV, versus 14.3 % at stage I, 18.2 % at stage II, and 18.2 % at stage III), the difference did not reach any statistical significance (P = 0.223). In addition, a higher frequency of loss of DPC4 expression in patients with lymph node-metastasis was also revealed, however the difference was not significant (P = 0.228). The mean and median survival in patients with DPC4 expression was longer than that in patients with loss of DPC4 expression (Table 3). Kaplan-Meier survival analysis demonstrated patients with DPC4 expression had a higher survival rate than those with loss of DPC4 expression, but the difference did not reach any statistical significance (P = 0.879) (Figure 2).

**Table 1** Loss of DPC4 expression in pancreatic tissues (%)

| Tissues                      | n | Loss expression of DPC4 (%) |
|-----------------------------|---|----------------------------|
| Normal pancreas             | 8 | 0 (0)                      |
| Non-neoplastic cysts        | 8 | 0 (0)                      |
| Pancreatic carcinoma        | 34| 8 (23.5)                   |

**Table 2** Correlations between loss expression of DPC4 and clinicopathological parameters in pancreatic carcinoma

| Parameters                       | n | Loss expression of DPC4 (%) | P   |
|----------------------------------|---|-----------------------------|-----|
| Age (y)                          |   |                             |     |
| ≥ 60                             | 17| 2 (11.8)                    | 0.268|
| 45 ≤ x < 60                      | 11| 4 (36.4)                    | 0.842|
| < 45                             | 6 | 2 (33.3)                    |     |
| Sex                              |   |                             |     |
| Male                             | 22| 6 (27.3)                    | 0.681|
| Female                           | 12| 2 (16.7)                    |     |
| Pathological grade               |   |                             |     |
| G1+G2                            | 27| 4 (14.8)                    | 0.037|
| G3                               | 7 | 4 (57.1)                    |     |
| Tumor diameter                   |   |                             |     |
| ≤ 4.5 cm                         | 19| 4 (21.2)                    | 1.000|
| > 4.5 cm                         | 15| 4 (26.7)                    |     |
| Tumor location                   |   |                             |     |
| Head                             | 24| 6 (25.0)                    | 0.842|
| Body and tail                    | 10| 2 (20.0)                    |     |
| Lymph node                       |   |                             |     |
| Negative                         | 20| 3 (15.0)                    | 0.228|
| Positive                         | 14| 5 (35.7)                    |     |
| TNM staging                      |   |                             |     |
| I                                | 7 | 1 (14.3)                    | 0.223|
| II                               | 11| 2 (18.2)                    |     |
| III                              | 11| 2 (18.2)                    |     |
| IV                               | 5 | 3 (60.0)                    |     |

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

**Table 3** Mean and median survivals in pancreatic carcinomas

|                  | Mean survival (days) | Median survival (days) |
|------------------|----------------------|------------------------|
| DPC4 expression  | 329.94±41.54         | 319.00±30.32           |
| Loss of DPC4     | 300.00±61.88         | 206.00±88.39           |

**DISCUSSION**

This study aimed to clarify the role of DPC4 in the development of pancreatic carcinoma. The product of DPC4 gene belongs to the evolutionally conserved family of Smad proteins which are linked to TGF-β superfamily of cytokines. Smad proteins are involved in the regulation of cell differentiation as well as the inhibition of cell proliferation, and their alterations could confer resistance to TGF-β and thereby contribute to tumorigenesis.[21,22] DPC4 gene produces a 64-KD protein that influences gene transcription and growth arrest. In fact, DPC4 protein has three distinguishable domains, and mutations in each of these domains could lead to the loss of DPC4 function.[8,23,24]

There were different findings about the frequency of DPC4 alterations in pancreatic carcinomas in previous reports (9 %–55 %).[8,23] The discrepancies between studies might be due to differences in the study populations, techniques, or the statistical method. In our study, eight of the 34-pancreatic carcinoma specimens were immunohistochemically labeled for the loss of DPC4 protein (23.5 %). However, DPC4 immunohistochemical staining was found in all of the 16 nonmalignant specimens from the pancreas. This finding suggested that DPC4 might be involved in the tumorigenesis and development of pancreatic carcinoma.

Our study showed loss of DPC4 expression was correlated with the histological grade in patients with pancreatic carcinoma. Loss of DPC4 expression in those with poorly differentiated adenocarcinomas was significantly higher than that in those with well and moderately differentiated adenocarcinomas, which implied that DPC4 gene might preserve phenotypic characteristics under normal conditions and control the malignant progression of pancreatic carcinomas.

There was a trend toward a higher frequency of loss of DPC4 expression in patients at TNM staging IV in this study. When stratified by stage, the highest percentage of loss of DPC4 reactivity was found in carcinomas at stage IV (60.0 %), compared with 14.3 % at stage I, 18.2 % at stage II, and 18.2 % at stage III carcinomas. A higher frequency of loss of DPC4 expression in patients with lymph node-metastasis was also revealed. Survival analysis demonstrated patients with DPC4 expression had a higher survival rate than those with loss of DPC4 expression.

The data in our study were correlated fairly well with what has been reported. The results from Wilentz et al showed that DPC4 expression in duct lesions with a histologically low-grade (PanIN-1 and -2) was significantly higher than that in those with a histologically high-grade (PanIN-3).[25] Another study showed that DPC4 expression in PanIN could be...
predictive of DPC4 expression in the subsequent invasive ductal adenocarcinoma. Additionally, DPC4 expression could be used to differentiate recurrent or persistent adenocarcinomas from a second primary adenocarcinoma. A recent study found that survival of patients whose tumors expressed DPC4 protein was significantly longer (19.2 months) as compared with 14.7 months of those without DPC4 protein expression, and DPC4 expression was correlated with a better prognosis of pancreatic carcinomas[18]. Biankin et al also found DPC4/Smad4 expression had a potential as a prognostic indicator in patients with pancreatic cancer, and loss of DPC4 expression was associated with improved survival after resection, whereas resection did not improve the survival in patients whose tumor expressed DPC4.

These findings suggest that loss of DPC4 expression occurs biologically late in the neoplastic progression leading to the development of infiltrating pancreatic carcinoma, and indicates a poor prognosis for patients. It is reasonable to postulate that DPC4 plays a pivotal role in regulating all TGF-β superfamily signal pathways. Abrogation of DPC4 function might cause a breakdown in this signaling pathway and loss of transcription of genes critical to cell-cycle control. Cells might therefore become TGF-β resistant and escape from TGF-β-mediated growth control and apoptosis. Experimental evidences indicated that DPC4 could regulate an angiogenic switch by decreasing the expression of vascular endothelial growth factor (VEGF) and increasing the levels of angiogenesis inhibitor thrombospondin-1 (TSP-1).

In conclusion, our study shows that loss of DPC4 expression is involved in the carcinogenesis and development of pancreatic carcinoma, and DPC4 expression may be a molecular prognostic marker for pancreatic carcinoma.

ACKNOWLEDGEMENTS
We thank Dr. Gonghua Zhao for her critical suggestions regarding this work and Mr. Jing Zhang for helping perform the immunohistochemical staining.

REFERENCES
1 Bramhall SR, Allum WH, Jones AG, Allwood A, Cummins C, Neoptolemos JP. Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. Br J Surg 1995; 82: 111-115
2 Yeo CJ, Cameron JL. Prognostic factors in ductal pancreatic cancer. Langenbecks Arch Surg 1998; 383: 129-133
3 Hruban RH, van Mansfeld AD, Offerhaus GJ, van Weering DH, Allison DC, Goodman SN, Kinsler TW, Bose KK, Cameron JL, Bos JL. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allel-specific oligonucleotide hybridization. Am J Pathol 1993;143: 545-554
4 Robinson RA. K-ras mutations and the diagnosis of pancreatic carcinoma. Am J Clin Pathol 1996;105: 257-259
5 Safra H, Steinhoff M, Mangray S, Rathore R, King TC, Chai L, Berzin K, Moore T, Iannitti D, Reiss P, Pasquariello T, Akerman P, Quirk D, Mass R, Goldstein L, Tranvallyah U. Overexpression of the HER-2/neu oncogene in pancreatic adenocarcinoma. Am J Clin Oncol 2001; 24: 496-499
6 Hu YX, Watanabe H, Ohtsubo K, Yamaguchi Y, Ha A, Okai T, Sawabu N. Frequent loss of p16 expression and its correlation with clinicopathological parameters in pancreatic carcinoma. Clin Cancer Res 1997; 3: 1473-1477
7 Li Y, Bhuiyan M, Vaidkevicius VK, Sarkar FH. Molecular analysis of the p53 gene in pancreatic adenocarcinoma. Diagn Mol Pathol 1998; 7: 4-9
8 Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996; 271: 350-353
9 Dai JL, Tuncamycilu KK, Schutte M, Sugar AY, Kern SE. Dpc4 transcriptional activation and dysfunction in cancer cells. Cancer Res 1998; 58: 4592-4597
10 Maesawa C, Tanamura G, Nishizuka S, Iwaya T, Ogasawara S, Ishida K, Sakata K, Sato N, Ikeda K, Kimura Y, Saito K, Satodate R. MAD-related genes on 18q21.1, Smad2 and Smad4, are altered infrequently in esophageal squamous cell carcinoma. Jpn J Cancer Res 1997; 88: 340-343
11 Lej J, Zou TT, Shi YQ, Zhou X, Smolinski KN, Yin J, Souza RF, Appel R, Wang S, Cymes K, Chan O, Abraham JM, Harpaz N, Meltzer SJ. Infrequent DPC4 gene mutation in esophageal cancer, gastric cancer and ulcerative colitis-associated neoplasms. Oncogene 1996; 13: 2459-2462
12 Nishizuka S, Tanamura G, Maesawa C, Sakata K, Suzuki Y, Iwaya T, Terashima M, Saito K, Satodate R. Analysis of the DPC4 gene in gastric carcinoma. Jpn J Cancer Res 1997; 88: 335-339
13 Kim SF, Fan Y, Papadimtriakopoulou V, Clayman G, Hittelman WN, Hong WK, Lotan R, Mao L. DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. Cancer Res 1996; 56: 2519-2521
14 Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasy GM, Weinstein CL, Bova GS, Isaacs WB, Carrns P, Nawroz H, Sidersky D, Casero RA, Jr, Metzler PS, Hahn SA, Kern SE. DPC4 gene in various tumor types. Cancer Res 1996; 56: 2527-2530
15 Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, Shimokawa K, Saji S. Somatic alterations of the DPC4 gene in human colorectal cancers. Gastroenterology 1996; 111: 1369-1372
16 Hahn SA, Bartsch D, Schroers A, Galehardi H, Becker M, Ramaswamy A, Schwarte-Waldhoff I, Maschek H, Schmiegel W. Mutations of the DPC4/Smad4 gene in biliary tract carcinoma. Cancer Res 1998; 58: 1124-1126
17 Wilentz RE, Su GH, Dai JL, Sparks A, Argani P, Sohn TA, Yeo CJ, Kern SE, Hruban RH. Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas: a new marker of DPC4 inactivation. Am J Pathol 2000; 156: 37-43
18 Tasclar M, Skinner HG, Rosty C, Sohn T, Wilentz RE, Offerhaus GJ, Adsay V, Abrams RA, Cameron JL, Kern SE, Yeo CJ, Hruban RH, Goggins M. The Smad4/Dpc4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin Cancer Res 2001; 7: 4115-4121
19 Klöppel G. Pancreatic non-endocrine tumors. In: Klöppel G, Heitz PU ed. Pancreatic pathology. Edinburg: Churchill Livingstone 1984: 79-113
20 Hermanek P, Sobin LH. UICC TNM classification of malignant tumors. 4th ed. 2nd revision. Berlin: Springer-Verlag 1992: 71-73
21 Zhang Y, Feng X, We R, Derynick R. Receptor-associated Mad homologs synergize as effectors of the TGF-β response. Nature 1996; 383: 168-172
22 Chiao PJ, Hunt K, Grau AM, Abrami an, Flemming J, Zhang W, Breslin T, Abbrezzele J, Evans DB. Tumor suppressor gene Smad4/ DPC4, its downstream target genes, and regulation of cell cycle. Ann N Y Acad Sci 1999; 880: 31-37
23 Shi Y, Hata A, Lo RS, Massague J, Pavletich NP. A structural basis for mutational inactivation of the tumor suppressor Smad4. Nature 1997; 388: 87-93
24 De Caestecker MP, Hemmadi P, Larisch-Bloch S, Ajmera R, Roberts AB, Lechleider R. Characterization of functional domains of Smad4/Dpc4/Smad3. J Biol Chem 1997; 272: 13690-13696
25 Muppo PS, Orlandini S, Zamboni G, Capelli P, Rigaud G, Falconi M, Bassi C, Lemoine NR, Scarpa A. Pancreatic tumours: molecular pathways implicated in ductal cancer are involved in amphilary but not in exocrine nonductal or endocrine tumorigenesis. Br J Cancer 2001; 84: 253-262
26 Wilentz RE, Iacobuzio-Donahue CA, Argani P, McCarthy DM, Parsons JL, Yeo CJ, Kern SE, Hruban RH. Loss of expression of DPC4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res 2000; 60: 2002-2006

Edited by Zhu LH and Wang XL