SPANDIDOS PUBLICATIONS

Overexpression of connexin 26 in carcinoma of the pancreas

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Abstract. Contrary to the previously purported role of gap junction (GJ) associated-protein connexin 26 (Cx26) as a tumor suppressor, increased expression of Cx26 has recently been demonstrated in several human malignancies. Surprisingly, this high expression is reportedly related to poor prognosis in squamous cell lung carcinoma and breast cancer. In this study, we examined levels of Cx26 in various human gastrointestinal (GI) carcinomas, with a focus on pancreatic carcinomas, using immunohistochemistry. Many GI carcinomas displayed abundant Cx26 expression, predominantly in the cytoplasm. Cx26 was detected in 5/8 gastric cancers (62.5%), 6/8 squamous cell carcinomas of the esophagus (75.0%), 7/8 pancreatic cancers (87.5%) and 7/8 colon cancer cases (87.5%). However, Cx26 expression was not present in hepatocellular carcinoma (HCC, 0/8). Extensive immunohistochemical examination was performed on pancreatic carcinomas, revealing strong expression of Cx26 protein in 30/43 cases (70%), weak expression in 6/43 (14%) and no expression in 7/43 (16%). The present study demonstrated up-regulated Cx26 expression in a considerable percentage of GI carcinomas, with the exception of HCC. Our findings suggest that Cx26 may be involved in some of the malignant processes of GI cancers, and especially in pancreatic carcinomas.

Introduction

Gap junctions (GJs) mediate intracellular communication and regulate cell proliferation and differentiation by allowing small molecules and inorganic ions, such as metabolites, to pass from cell to adjacent cell (1,2). A GJ channel is composed of two membrane-integrated hemichannels supplied by two adjacent cells, each hemichannel consisting of a hexameric complex of connexin proteins (3).

Neoplastic transformation is frequently associated with a loss of GJ intercellular communication and with the reduced expression of connexins in various types of tumors (4,5). Conversely, forced expression of Cxs in Cx-deficient cell lines results in the inhibition of tumor growth and the induction of apoptosis *in vitro* and prevention of tumor formation *in vivo* (6,7). Thus, the multi-gene family of Cxs may act as a tumor suppressor by restoring cellular communication and reverting the phenotype of transformed cells.

Accumulating evidence, however, indicates that connexin 26 (Cx26), a connexin family member, is overexpressed in carcinomas of the head and neck, colon and prostate, as well as in keratinocyte-derived skin tumors (8-11). Interestingly, increased Cx26 expression was observed in invasive breast carcinomas and metastatic lymph nodes, but not in the epithelial cells of benign lesions (12,13). As well, Cx26 expression was detected at the invasive front of a malignant melanoma of the skin (14). Furthermore, recent reports demonstrate that high Cx26 expression is associated with poor prognosis in lung squamous cell and breast carcinoma (15,16). Together, these strands of evidence appear to contradict the conventionally held view of the role of connexins as tumor suppressors.

Pancreatic carcinoma is one of the most aggressive human malignancies. Incidences of carcinoma of the pancreas are associated with high mortality rates. In the United States, 5-year overall survival increased from 1% in 1961 to only 3-5% in 1991. Several factors have been implicated in the recent rise in frequency of pancreatic carcinoma, including cigarette smoking, gallstones, a diet high in animal fat and chronic calcific pancreatitis (17). Although there have been a few studies suggesting possible roles for K-*ras* oncogenes, tumor suppressor genes (p16, p53 and DPC4) and growth factors (epidermal growth factor, basic fibroblast growth factor and insulin-like growth factor I) in carcinoma of the pancreas, the exact pathogenic mechanisms and progression of this neoplasm remain obscure (18-21).

Little is known about the expression of Cx26 in human pancreatic carcinoma. In the present study, we examined the expression of Cx26 protein by immunohistochemistry in

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Abbreviations: Cx26, connexin 26; GI, gastrointestinal; GJ, gap junction; HCC, hepatocellular carcinoma

Key words: connexin 26, pancreatic cancer, gastrointestinal cancer

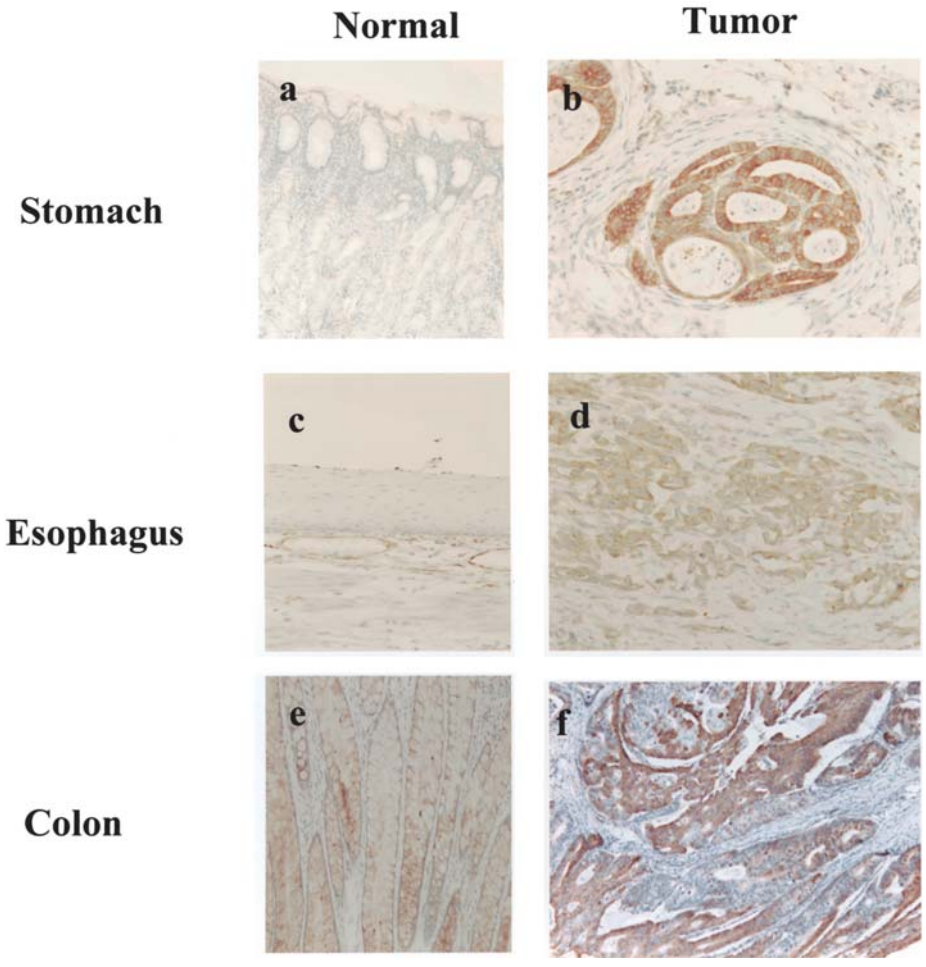

Figure 1. Immunostaining for Cx26 in normal and tumor tissues of the stomach, esophagus and colon. (a) Normal gastric epithelium; (b) gastric carcinoma; (c) normal esophageal epithelium; (d) esophageal squamous cell carcinoma; (e) normal colon mucosa; (f) colon carcinoma. The normal gastric epithelium (a) and esophageal epithelium (c) scarcely expressed Cx26, while the colon mucosa (e) expressed Cx26 in the plasma membrane. Relatively large-sized vessels in the submucosa of the esophageal epithelium expressed Cx26 (c). Carcinoma tissues often expressed abundant Cx26 in the plasma membrane and/or cytoplasm (b,d and f). Magnification: a and c, x40; e, x80; b, d and f, x100.

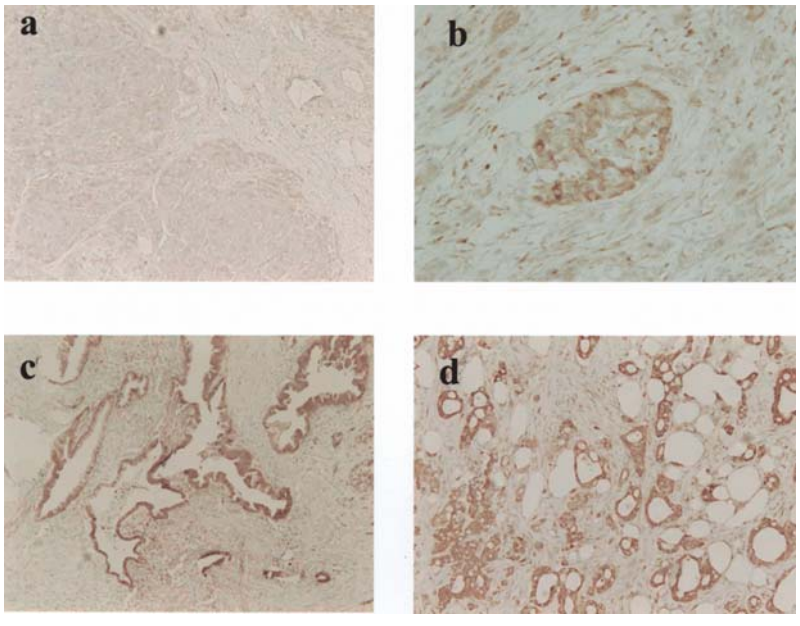

Figure 2. Immunostaining for Cx26 in normal and tumor tissues of the pancreas. (a) Acinar cells negative for Cx26. (b) Islet cells displaying Cx26 staining. (c) Well-differentiated and (d) poorly-differentiated carcinomas displaying strong Cx26 expression, predominantly in the cytoplasm. Magnification: a, c and d, x100; b, x200.

ncreatic tumors as compared to other human gastrointestinal (GI) cancers.

Materials and methods

Tissue samples. Tissue samples were obtained from patients who underwent surgery at the Department of Surgery, Osaka University Hospital, between 1995 and 2003. Samples were fixed in 10% neutral buffered formalin, processed through graded ethanol solutions and embedded in paraffin. Use of the samples was approved by the ethics committee.

Antibodies. Mouse anti-Cx26 monoclonal antibody 13-8100, which recognizes the human Cx26 protein (molecular weight, 26.5 kDa), was obtained from Zymed Laboratories Inc. (Invitrogen Corporation, Carlsbad, CA).

Hematoxylin and eosin (H&E) staining and immunohistochemistry. Tissue sections (4-μm thick) were deparaffinized in xylene, rehydrated and stained with H&E solution for histological diagnosis. The tissue sections were autoclaved at 121˚C for 20 min for antigen retrieval and then processed for immunohistochemistry using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA), as previously described (22). Slides were incubated overnight at 4˚C with the appropriate primary antibodies, and Cx26 antibody was used at a dilution of 1:500. Non-immunized mouse IgG (Vector Laboratories) was used as a negative control and a substitute for the primary antibody to exclude possible false-positive responses from the secondary antibody or from the non-specific binding of IgG.

Immunohistochemical assessment. All immunostained tissue sections were evaluated by N.K. and K.E. in a coded manner without their knowing the clinical and pathological backgrounds of the patients. In each section, five high-power fields were randomly selected and at least 1,000 cells were evaluated. For the assessment of Cx26 expression, the percentage of clearly stained cells was calculated.

Statistical analysis. Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts Inc., Berkeley, CA). Associations between discrete variables were assessed using the χ^2 test. Mean values were compared using the Mann-Whitney test. All data were expressed as the mean ± SD. P-values of <0.05 were accepted as statistically significant.

Results

Cx26 expression in GI carcinomas. We first examined Cx26 expression in a subset of GI cancers. Cx26 was scarcely expressed in the normal epithelium of the stomach and esophagus, expressed in the plasma membrane of the normal colonic mucosa (Fig. 1a, c and e) and abundantly expressed in the plasma membrane and/or cytoplasm of most carcinoma tissues (Fig. 1b, d and f). In the epithelial cells of normal pancreatic ducts and normal acinar cells, only negligible expression of Cx26 was noted (Fig. 2a). Pancreatic islets expressed Cx26 to moderate extent (Fig. 2b), while pancreatic carcinoma tissues expressed abundant Cx26, predominantly in the cytoplasm (Fig. 2c and d). Hepatocellular carcinoma (HCC) and adjacent non-tumor liver tissues did not express Cx26 at all (data not shown).

Cx26 was detected in 5/8 gastric cancer cases (62.5%), 6/8 squamous cell carcinomas of the esophagus (75.0%), 7/8 pancreatic carcinomas (87.5%) and 7/8 colon cancers (87.5%). HCC did not express Cx26 (0/8 HCCs).

Cx26 expression in pancreatic carcinomas. Since pancreatic carcinomas often displayed intense expression of Cx26, we conducted an extensive immunohistochemical examination of Cx26 expression in a series of 43 pancreatic carcinomas. We classified the tumors into three groups according to staining intensity: strong, weak, and none. Strong expression was found in 30/43 carcinomas (70%), weak in 6 (14%) and none in 7 (16%). Staining intensity was mainly correlated to the incidence of Cx26-positive tumor cells. Thus, 80-100% cells were positive for Cx26 when the staining intensity was strong, and 50-80% were positive when it was weak,. Using this classification, we did not find significant differences between the two groups (strong vs. weak and none) based on various clinicopathological parameters such as gender, tumor location, T factor, nodal involvement, stage (UICC, the International Union Against Cancer classification) and histological grade (Table I).

Table I. Cx26 expression and clinicopathological parameters in pancreatic carcinomas.

	Cx26 expression		
	Strong	Weak	None
Gender			
Male	16	2	4
Female	14	4	3
Localization			
Head	26	4	4
Body/tail	4	2	3
T factor			
T1, 2	0	1	1
T3, 4	30	5	6
Lymph node metastasis			
Positive	22	2	5
Negative	8	4	2
Stage			
I, II	5	3	1
III, IV	25	3	6
Histological type and grade			
Adenocarcinoma			
Well-differentiated	9	1	2
Moderately-differentiated	17	4	4
Poorly-differentiated	4	0	0
Papillary adenocarcinoma	0	1	1

No distant metastasis was observed.

Discussion

GJs and connexin subunits have been shown to be down-regulated in various cancers (4,5), but accumulating evidence indicates that the connexin Cx26 in particular is increased in various carcinomas (8-14). Our preliminarily study showed that Cx26 expression was induced in a considerable percentage of GI malignancies, including colon cancer, gastric cancer, squamous esophageal cancer and pancreatic cancer, but not in HCC. These findings suggest that Cx26 function in human malignancies is complex, and appear to contradict previous views of its role as a tumor suppressor gene (4-7).

Among the GI carcinomas tested, pancreatic carcinomas exhibited the most striking Cx26 expression. We therefore performed an extensive immunohistochemical examination of this type of malignancy and found that approximately 70% of the carcinomas displayed strong Cx26 expression. Although a clinical and pathological survey revealed no correlation between Cx26 expression and several clinical parameters, Cx26 was expressed in various histological types of carcinomas, i.e., poorly-differentiated as well as moderately to well-differentiated adenocarcinomas. Furthermore, our data revealed that Cx26 expression was not affected by several parameters associated with tumor progression, such as tumor size, lymph node metastasis and clinical stage. These findings suggest that Cx26 may be a marker for the malignant potential of pancreatic carcinomas, although its exact function remains to be clarified.

Although Cx26 is initially localized in the plasma membrane, we often observed its cytoplasmic accumulation in GI carcinomas. This indicated that Cx26 translocates from the plasma membrane to the cytoplasm in tumor cells, as previously observed (9,12-13,23-24). Although the precise function of cytoplasmic Cx26 is as yet unclear, one possibility is that the cytoplasmic accumulation of Cx26 may be a prerequisite for the execution of its role in the plasma membrane, contributing to GJ formation as needed.

Many studies have shown that the introduction of the Cx26 gene into tumor cells usually causes growth inhibition *in vitro* and *in vivo* (6,7), and it is postulated that the inhibitory effect of GJ formation on tumor growth may suppress initial tumor formation. However, once malignant formation is completed, the growth arrest of cancer cells may be essential to their extravasation at a later stage of metastasis (14,25). Cell growth and invasion may also be differentially regulated by GJs, as GJ inhibitors decrease the invasion of prostate cancer cells (10).

In conclusion, we have found that Cx26 is aberrantly expressed in a considerable percentage of pancreatic and other GI carcinomas, with the exception of HCC. Our data suggest important clinical implications for Cx26 being a potential therapeutic target against pancreatic carcinoma. MI-18 and MI-22, selective Cx26 inhibitor oleamide derivatives which both drastically suppressed spontaneous lung metastasis of melanoma cells, are already feasible as clinically important prototypes (26,27).

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