Prognostic Significance of Activated Akt Expression in Pancreatic Ductal Adenocarcinoma

Shinji Yamamoto,[1] Yasuhiko Tomita,[2] Yoshihiko Hoshida,[2] Takaya Morooka,[2] Hiroaki Nagano,[1] Keizo Dono,[1] Koji Umeshita,[1] Masato Sakon,[1] Osamu Ishikawa,[3] Hiroaki Ohigashi,[3] Shoji Nakamori,[1] Morito Monden,[1] and Katsuyuki Aozasa[2]

Departments of [1]Surgery and Clinical Oncology and [2]Pathology, Osaka University Graduate School of Medicine, Osaka, and [3]Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

ABSTRACT

Purpose: **Akt is a serine/threonine kinase that plays a central role in tumorigenesis. Among the members of Akt family, Akt2 is associated with the development of human cancers. The present study was designed to clarify the prognostic significance of Akt2 and activated Akt expression in pancreatic ductal adenocarcinoma (PDAC). In addition, activated extracellular signal-regulated kinase 1 and 2 (ERK1/2) and the proliferation activity of tumor cells detected by Ki-67 immunohistochemistry were examined.**

Experimental Design: **Immunohistochemical analysis was performed on paraffin-embedded specimens from 65 patients with PDAC; 36 males and 29 females with ages ranging from 48 to 79 years (median, 66 years) of age. Expression levels of Akt2, phosphorylated Akt (*p*-Akt), and phosphorylated ERK 1/2 (*p*-ERK 1/2) were categorized as either weaker (low intensity) or equal to stronger (high intensity) compared with those in the endothelial cells of the same specimens. For Ki-67 immunohistochemistry, cases were divided into two groups: level 1, Ki-67 labeling index (LI), <20%; level 2, Ki-67 LI, ≥20%.**

Results: **Twenty-six (42.6%), 28 (45.9%), 39 (63.9%), and 46 (75.4%) of the tumors showed high intensity of Akt2, *p*-Akt, and *p*-ERK 1/2 expression, and Ki-67 LI level 2, respectively. A significant positive correlation was observed between Akt2 and *p*-Akt expression ($P < 0.01$). Multivariate analysis revealed that *p*-Akt expression, Ki-67 LI, and histological differentiation are independent prognosticators for PDAC.**

Conclusions: **p-Akt expression is a significant prognostic indicator for PDAC. Inhibition of Akt is a possible molecular approach for treatment of PDAC.**

INTRODUCTION

Three variations of the serine/threonine kinase Akt are present in the human genome, thus designated as *Akt1*, *Akt2*, and *Akt3* (1, 2). Akt is activated by various growth factors and plays a role in tumorigenesis by inhibiting apoptosis and mediating cell proliferation (1–3). Phosphorylation of Thr^{308} is necessary for Akt activation, and phosphorylation of Ser^{473} results in maximum activity (4).

Pancreatic ductal adenocarcinoma (PDAC) usually has a poor prognosis (5–7). The altered expression of genes such as the *K-ras* oncogene frequently occurs during the development of PDAC (8). However, specific patterns of gene alternations responsible for PDAC have not been identified (9).

The detection of Akt activation is a useful method for predicting the progression and prognosis of PDAC because it represents the signaling of various growth factors that playing key roles in tumorigenesis (1). Among the members of the Akt family, Akt2 is associated with the development of human cancers (10). Overexpression of the *Akt2* gene is observed in ~20% of all cases of PDAC (11). Transfection of antisense *Akt2* RNA into PDAC cell lines inhibits its tumorigenicity (11). Therefore, an association between the activation or overexpression of Akt, and the clinicopathological behavior of PDAC is postulated.

In the present study, the expression of Akt was immunohistochemically examined in 65 patients with PDAC, and its relationship with clinicopathological factors and the patient survival rate was evaluated. In addition, the activation status of extracellular signal-regulated kinase 1 and 2 (ERK1/2), another serine/threonine kinase that plays a role in cell proliferation and differentiation (12, 13), and the proliferation activity of tumor cells seen by using Ki-67 immunohistochemistry (14) was examined.

MATERIALS AND METHODS

Patients. Sixty-five patients who underwent curative resection of primary PDAC were selected for the study. They underwent surgery at the Gastroenterological Surgery Division, Osaka University Hospital and Division of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases from June 1982 to August 2001. The patients consisted of 36 males and 29 females ranging from 48 to 79 years (median, 66 years) of age. The stage of the disease was classified according to the pathological Tumor-Node-Metastasis staging system (15).

Surgically resected specimens were macroscopically examined to determine the location and size of the tumors. Then, tissue samples were fixed in 10% formalin and routinely processed for paraffin embedding. Histological sections cut with a

Received 11/23/02; revised 11/17/03; accepted 12/10/03.
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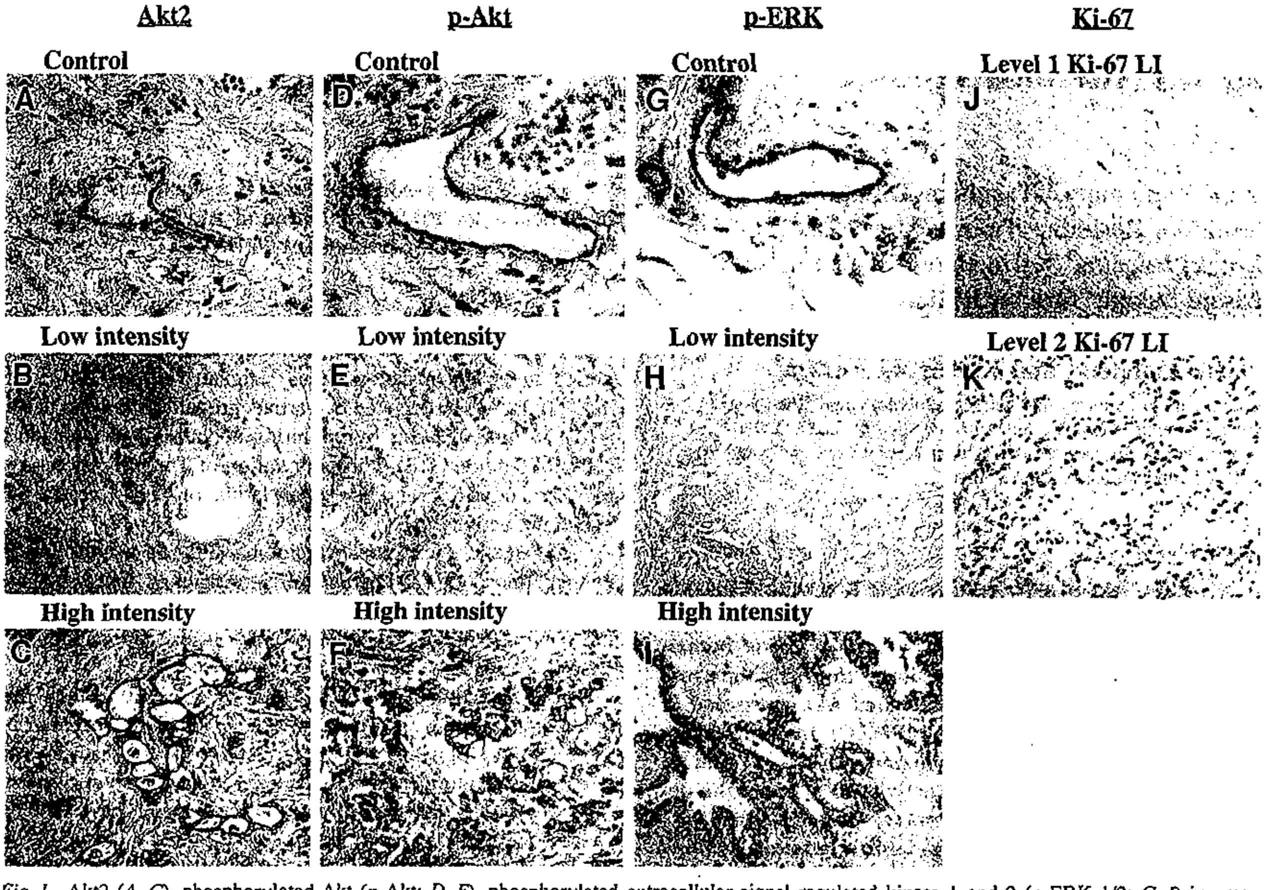

Fig. 1 Akt2 (*A–C*), phosphorylated-Akt (*p*-Akt; *D–F*), phosphorylated extracellular signal regulated kinase 1 and 2 (*p*-ERK 1/2; *G–I*) immunostaining. *A*, *D*, and *G*, internal controls for Akt2, *p*-Akt, and *p*-ERK 1/2 staining. The cytoplasm of endothelial cells is positively stained (magnification, ×50). *B*, *E*, and *H*, low intensity staining of Akt2, *p*-Akt, and *p*-ERK 1/2. Tumor cells are weakly stained compared with that of endothelial cells (magnification, ×25). *C*, *F*, and *I*, high intensity staining of Akt2, *p*-Akt, and *p*-ERK 1/2. Tumor cells are strongly stained similar to that of endothelial cells (magnification, ×25). *J*, Ki-67 labeling index (LI) level 1. Less than 20% of the tumor cells show nuclear staining (magnification, ×25). *K*, Ki-67 LI level 2. More than 20% of the tumor cells show nuclear staining (magnification, ×25).

4-μm thickness were stained with H&E and reviewed by one of the authors (Y. Hoshida) to determine histological differentiation and the existence of metastasis in the lymph nodes. All tumors were adenocarcinomas with well- (31 cases), moderately (24 cases), and poorly differentiated (10 cases) morphologies.

After surgery, serum CA 19-9 level was measured, and ultrasonography and computed tomography scan were performed at 3-month intervals. The patients were followed-up until March 31, 2002: the follow-up periods for survivors ranged from 3.4 to 105.5 months (median, 23.6 months) after surgery.

Immunohistochemical Analysis. The specificity of antiphosphorylated Akt (*p*-Akt; Thr^{308}) polyclonal antibody (Cell Signaling Technology, Beverly, MA) was examined by Western blot analysis using extracts from Jurkat cells, which showed a positive band after platelet-derived growth factor treatment. Antibody preincubated with antigen peptide was used for a negative control test and gave a uniformly negative result.

The immunoperoxidase procedure (avidin-biotin-complex method) was performed on the paraffin-embedded sections. Antigen retrieval was carried out by heating the sections in 10 mM citrate buffer for 5 min. Anti-Akt2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-*p*-Akt polyclonal antibody, anti-*p*-ERK1/2 (Thr^{202}/Tyr^{204}) monoclonal antibody (Cell Signaling Technology), and anti-Ki-67 (clone MIB 1) monoclonal antibody (Dako Cytomation A/S, Copenhagen, Denmark) were used as primary antibodies at dilutions of 1:400, 1:100, 1:100, and 1:50, respectively. Sections were counterstained lightly with methyl green. For negative controls, nonimmunized mouse or rabbit IgG serum (Vector Laboratories, Burlingame, CA) was used as primary antibodies. Positive intracytoplasmic staining of endothelial cells in the noncancerous areas in each specimen was used as an internal positive control. Stained sections were blindly evaluated without prior knowledge of the clinicopathological parameters. The staining intensity in the cytoplasm of the tumor cells was categorized as follows: weaker (low intensity) or equal to stronger (high intensity) than that of the endothelial cells. Cases with negative endothelial cell staining were regarded as having underwent poor antigen preservation and excluded from additional analyses. For Ki-67 immunohistochemistry, cells showing intranuclear staining were judged as Ki-67 positive. The Ki-67-positive cells among 200 tumor cells were counted, and the percentage was

Table 1 Correlation between Akt2, *p*-Akt[a], *p*-ERK 1/2[a], and Ki-67 expression and clinicopathological factors in 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	Total no. of patients	Patients with *high-intensity* Akt2 ($n = 26$)	P	Patients with *high-intensity* *p*-Akt ($n = 28$)	P	Patients with high-intensity *p*-ERK 1/2 ($n = 39$)	P	Patients with level 2 Ki-67 LI ($n = 46$)	P
Age (years)	1: ≤60	15	6 (40.0%)	N.S.	7 (46.7%)	N.S.	9 (60.0%)	N.S.	10 (66.7%)	N.S.
	2: >60	46	20 (43.5%)		21 (45.7%)		30 (65.2%)		36 (78.3%)	
Gender	1: Male	34	13 (38.2%)	N.S.	12 (35.3%)	N.S.	20 (58.8%)	N.S.	28 (82.4%)	N.S.
	2: Female	27	13 (48.1%)		16 (59.3%)		19 (70.4%)		18 (66.7%)	
Location of tumor	1: Head	50	23 (46.0%)	N.S.	24 (48.0%)	N.S.	31 (62.0%)	N.S.	36 (72.0%)	N.S.
	2: Body/Tail	11	3 (27.3%)		4 (36.4%)		8 (72.7%)		10 (90.9%)	
T (pTNM)	1: T_1	4	1 (25.0%)	N.S.	1 (25.0%)	N.S.	2 (50.0%)	N.S.	3 (75.0%)	N.S.
	2: T_2	7	3 (42.9%)		3 (42.9%)		5 (71.4%)		3 (42.9%)	
	3: T_3	31	13 (41.9%)		15 (48.4%)		20 (64.5%)		23 (74.2%)	
	4: T_4	19	9 (52.6%)		9 (52.6%)		12 (63.2%)		17 (89.5%)	
Histological differentiation	1: Well	29	13 (44.8%)	N.S.	14 (48.3%)	N.S.	19 (65.5%)	N.S.	25 (86.2%)	N.S.
	2: Moderately	23	8 (34.8%)		11 (47.8%)		14 (60.9%)		14 (60.9%)	
	3: Poorly	9	5 (55.6%)		3 (33.3%)		6 (66.7%)		7 (77.8%)	
Lymph node metastasis	1: Present	36	15 (41.7%)	N.S.	17 (47.2%)	N.S.	24 (66.7%)	N.S.	28 (77.8%)	N.S.
	2: Absent	25	11 (44.0%)		11 (44.0%)		15 (60.0%)		18 (72.0%)	
Stage (pTNM)	1: I	4	1 (25.0%)	N.S.	1 (25.0%)	N.S.	2 (50.0%)	N.S.	2 (50.0%)	N.S.
	2: II	38	16 (42.1%)		18 (47.4%)		25 (65.8%)		27 (71.1%)	
	3: III	19	9 (47.4%)		9 (47.4%)		12 (63.2%)		17 (89.5%)	
Akt2 expression	1: Low intensity	35			11 (31.4%)	<0.01	21 (60.0%)	N.S.	25 (71.4%)	N.S.
	2: High intensity	26			17 (65.4%)		18 (69.2%)		21 (80.8%)	
p-Akt expression	1: Low intensity	33	9 (27.3%)	<0.01			20 (60.6%)	N.S.	26 (78.8%)	N.S.
	2: High intensity	28	17 (60.7%)				19 (67.9%)		20 (71.4%)	
p-ERK 1/2 expression	1: Low intensity	22	8 (36.4%)	N.S.	9 (40.9%)	N.S.			16 (34.8%)	N.S.
	2: High intensity	39	18 (46.2%)		19 (48.7%)				30 (76.9%)	
Ki-67 LI	1: Level 1 (<20%)	15	5 (33.3%)	N.S.	8 (53.3%)	N.S.	9 (60.0%)	N.S.		
	2: Level 2 (≥20%)	46	21 (45.7%)		20 (43.5%)		30 (65.2%)			

[a] *p*-Akt, phosphorylated Akt; *p*-ERK 1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; Ki-67 LI, Ki-67 labeling index; N.S., nonsignificant; pTNM, pathological Tumor-Node-Metastasis.

used as the Ki-67 labeling index (LI). Cases were divided into two groups: level 1, Ki-67 LI, <20%; level 2, Ki-67 LI, ≥20%.

Statistics. Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). χ^2 test and Fisher's exact probability test were used to analyze the correlation between Akt overexpression or activation and clinicopathological features. The Kaplan-Meier method was used to calculate the overall patient survival rate, and the difference in survival curves was evaluated using a log-rank test (16). Cox's proportional hazards regression model with stepwise manner was used to analyze the independent prognostic factors (17). $P < 0.05$ was considered to be statistically significant.

RESULTS

Akt2, *p*-Akt, and *p*-ERK Expression and Ki-67 LI in PDACs. Four (6.1%) of the 65 cases that did not show endothelial staining for Akt2, *p*-Akt, or *p*-ERK were regarded as having undergone poor antigen preservation and were excluded from additional analyses. Twenty-six, 28, and 39 cases showed high intensity staining for Akt2, *p*-Akt, and *p*-ERK, respectively, and 46 cases showed Ki-67 LI level 2 (Fig. 1).

Univariate and Multivariate Analyses for Prognostic Factors for PDAC. A significant positive correlation was observed between Akt2 and *p*-Akt staining patterns ($P < 0.01$; Table 1). Correlation between other combinations was not observed.

The 5-year overall survival rate for the 61 patients was 32.3%. Patients with low intensity of *p*-Akt expression showed a significantly better 5-year survival rate (57.0%) than those with high-intensity expression (14.1%; $P < 0.05$, Fig. 2). Akt2 and *p*-ERK expression were not significant prognosticators. A univariate analysis revealed that *p*-Akt expression, Ki-67 LI, histological differentiation, and T factor of the pathological Tumor-Node-Metastasis staging system were significant factors of the overall survival rate (Table 2). A subsequent multivariate analysis revealed that *p*-Akt expression, Ki-67 LI, and histological differentiation were independent prognosticators (Table 3).

DISCUSSION

To establish proper therapeutic modalities for PDAC, an accurate assessment of the factors affecting tumor progression and patient prognosis is critical. Although the conventional Tumor-Node-Metastasis staging system, which is defined by tumor size, tumor progression, lymph node involvement, and distant metastasis (15), is useful for PDAC classification, the outcome is poor for patients even in the low-stage (I and II) groups (5). Therefore, the prognostic use of several molecular markers for PDAC classification have been investigated (8), although none proved useful for predicting patient prognosis (9). We undertook the present study to determine whether Akt2, *p*-Akt, and *p*-ERK expression and the proliferation activity seen by Ki-67 LI are valid biological indicators of the aggressiveness of PDAC.

The constitutive activation of Akt in endothelial cells is

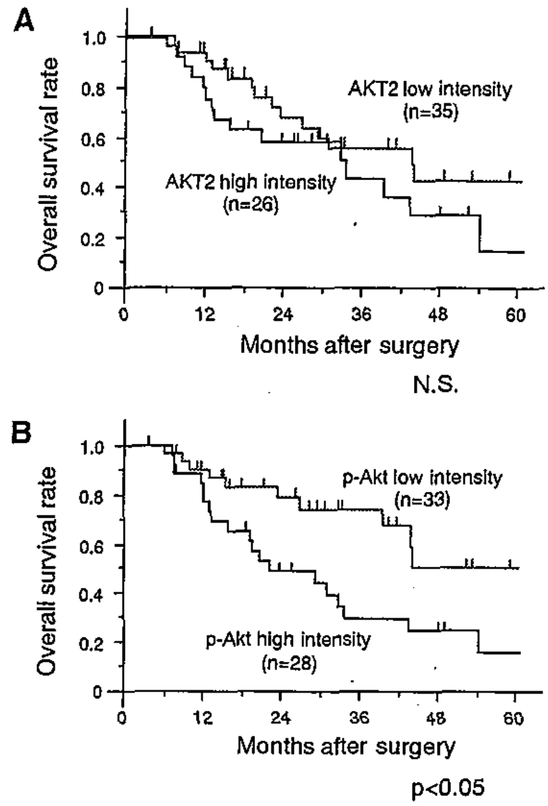

Fig. 2 Overall survival rate of pancreatic ductal adenocarcinoma patients with high and low intensity Akt2 and *p*-Akt expression. A significant difference was observed between the two groups for *p*-Akt expression (B; $P < 0.05$) but not for Akt2 expression [A; nonsignificant (N.S.)].

essential for the maintenance of endothelial cell-extracellular matrix attachment (18). In the present study, Akt2 and *p*-Akt expressions in endothelial cells were used as an internal positive control for each specimen, which showed a consistent moderate staining intensity. However, the staining intensity of nontumorous pancreatic tissues varied among the samples (data not shown). This is because of the different conditions of pancreatic tissue frequently affected by varying degrees of inflammation and fibrosis.

The patients characteristics such as gender, age, and 5-year survival rates were similar to those in the previous studies from Japan (5, 6) and Western countries (7). The present uni- and multivariate analyses confirmed the prognostic significance of tumor differentiation and T-factor for the pathological Tumor-Node-Metastasis classification of PDAC, as previously reported (5, 7). Therefore, the results of the present cases are generally applicable to PDAC.

The present study showed a significant correlation between Akt2 and *p*-Akt for PDAC ($P < 0.01$), which implies a close association between increased Akt activity and overexpression. Additional investigation is necessary to clarify the mechanism that controls Akt expression and activation.

Uni- and multivariate analyses showed that activated Akt expression is an independent prognosticator for PDAC patients together with histological differentiation and Ki-67 LI. The cause for poor prognosis of PDAC is local recurrence (5–7). Pleiotropic Akt activities such as antiapoptosis (3), proliferation (3), invasiveness (19), and angiogenesis (20) provide PDAC a microinvasiveness at the resected margin and promotes the recurrence of cancer. These findings show that activated Akt expression is a sign of poor prognosis for PDAC.

The phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) is a tumor suppressor gene that negatively

Table 2 Univariate analysis of clinicopathological factors for the overall survival rate of 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	No. of patients	5-year overall survival rate (%)	*P*
Age (years)	1: ≤60	15	71.1	N.S.
	2: >60	46	25.2	
Gender	1: Male	34	34.9	N.S.
	2: Female	27	30.5	
Location of tumor	1: Head	50	37.9	N.S.
	2: Body/Tail	11	0	
T(pTNM)	1: T_1	4	66.7	<0.05[b]
	2: T_2	7	55.6	
	3: T_3	31	32.2	
	4: T_4	19	18.4	
Histological differentiation	1: Well/Moderately	52	37.6	<0.01
	2: Poorly	9	0	
Lymph node metastasis	1: Present	36	19.6	N.S.
	2: Absent	25	44.9	
Akt2 expression	1: Low intensity	35	43.6	N.S.
	2: High intensity	26	14.6	
p-Akt expression	1: Low intensity	33	50.6	<0.05
	2: High intensity	28	16.4	
p-ERK 1/2 expression	1: Low intensity	22	45.1	N.S.
	2: High intensity	39	28.4	
Ki-67 LI	1: Level 1 (<20%)	15	66.3	<0.05
	2: Level 2 (≥20%)	46	18.8	

[a] N.S., nonsignificant; pTNM, pathological Tumor-Node-Metastasis; *p*-Akt, phosphorylated Akt; *p*-ERK 1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; Ki-67 LI, Ki-67 labeling index.
[b] 1 and 2 *versus* 3 and 4.

Table 3 Multivariate analysis of clinicopathological factors for overall survival rate of 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	Relative risk	95% confidence interval	χ^2 value	*P*
Histological differentiaton	1: Well/Moderately 0: Poorly	3.69	1.19–2.94	6.67	0.0098
p-Akt[a] expression	1: Low intensity 0: High intensity	3.44	1.28–2.80	10.04	0.0015
Ki-67 LI	1: Level 1 (< 20%) 0: Level 2 (≥ 20%)	3.92	1.20–3.72	7.70	0.0055

[a] *p*-Akt, phosphorylated Akt; Ki-67 LI, Ki-67 labeling index.

regulates the Akt pathway, and its mutations are often associatedwith an aggressive tumor phenotype (21). Somatic deletions or mutations of this gene have been identified in a large proportion of tumors, including glioblastomas, and endometrial and prostate cancers, thus placing *PTEN* among the most commonly mutated genes in human cancers (21). The decreased expression of PTEN for PDAC occurs at the mRNA and protein levels, whereas deletions or mutations are not frequently observed (22).

Constitutive activation of the Akt pathway occurs in many human PDAC cell lines (23). Inhibition of the phosphatidylinositol 3′-kinase/Akt pathway by inhibitors of phosphatidylinositol 3′-kinase (LY294002) induces apoptosis in tumor cells *in vitro* and inhibits the growth of tumor xenografts *in vivo* without excessive toxicity in nude mice (23). *In vitro* studies showed that Akt activation inhibits gemcitabine-induced apoptosis, and the addition of Akt inhibitors diversely enhances apoptosis (24). The Akt activation pathway could be a target for the treatment of PDAC.

In conclusion, activated Akt expression determined by immunohistochemistry is a new prognosticator for PDAC and is a new way to explore effective modalities for the treatment of PDAC. The inhibition of Akt might be a possible molecular target for the treatment of PDAC.

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[CANCER RESEARCH 64, 3215–3222, May 1, 2004]

Radiation to Stromal Fibroblasts Increases Invasiveness of Pancreatic Cancer Cells through Tumor-Stromal Interactions[1]

Kenoki Ohuchida,[1] Kazuhiro Mizumoto,[1] Mitsuhiko Murakami,[1] Li-Wu Qian,[1] Norihiro Sato,[1] Eishi Nagai,[1] Kunio Matsumoto,[2] Toshikazu Nakamura,[2] and Masao Tanaka[1]

[1]*Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and* [2]*Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medical Science, Suita, Osaka, Japan*

ABSTRACT

Radiotherapy represents a major treatment option for patients with pancreatic cancer, but recent evidence suggests that radiation can promote invasion and metastasis of cancer cells. Interactions between cancer cells and surrounding stromal cells may play an important role in aggressive tumor progression. In the present study, we investigated the invasive phenotype of pancreatic cancer cells in response to coculture with irradiated fibroblasts. Using *in vitro* invasion assay, we demonstrated that coculture with nonirradiated fibroblasts significantly increased the invasive ability of pancreatic cancer cells and, surprisingly, the increased invasiveness was further accelerated when they were cocultured with irradiated fibroblasts. The hepatocyte growth factor (HGF) secretion from fibroblasts remained unchanged after irradiation, whereas exposure of pancreatic cancer cells to supernatant from irradiated fibroblasts resulted in increased phosphorylation of c-Met (HGF receptor) and mitogen-activated protein kinase activity, possibly or partially via increased expression of c-Met. We also demonstrated that scattering of pancreatic cancer cells was accelerated by the supernatant from irradiated fibroblasts. The enhanced invasiveness of pancreatic cancer cells induced by coculture with irradiated fibroblasts was completely blocked by NK4, a specific antagonist of HGF. These data suggest that invasive potential of certain pancreatic cancer cells is enhanced by soluble mediator(s) released from irradiated fibroblasts possibly through up-regulation of c-Met expression/phosphorylation and mitogen-activated protein kinase activity in pancreatic cancer cells. Our present findings further support the potential use of NK4 during radiotherapy for patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a leading cause of cancer-related deaths in the industrialized countries (1, 2). A vast majority of patients with pancreatic cancer have poor outcomes because of difficulties in early diagnosis and limited efficiency of conventional therapeutics such as surgical resection, chemotherapy, and radiotherapy (3).

Radiotherapy is one of the major adjuvant treatments for many malignant tumors and has been frequently applied for patients with pancreatic cancer. The rationale for radiotherapy is based on the findings that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro* and inhibit tumor growth *in vivo* (4). Recently, however, several lines of evidence have shown that irradiation promotes malignant behaviors of cancer cells *in vitro* and *in vivo* by activating several pathways involved in tumor invasion and metastasis (5–9). We have previously reported that irradiation enhances invasive potential of pancreatic cancer cells via an increased expression of matrix metalloproteinase (MMP)-2 or c-Met/hepatocyte growth factor (HGF) receptor in cancer cells (10, 11).

Interactions between cancer cells and surrounding stromal fibroblasts have been suggested to play a critical role in tumor invasion and metastasis (12, 13). For example, invasive growth of certain cancer cells is drastically enhanced by tumor-stromal interactions involving the HGF/c-Met (HGF receptor) pathway (14–16). HGF, which was originally identified as a potent mitogen for hepatocytes (17, 18), is primarily secreted from stromal cells, and the secreted HGF can promote motility and invasion of various cancer cells that express c-Met in a paracrine manner (19–21). Binding of HGF to c-Met leads to receptor phosphorylation and activation of Ras/mitogen-activated protein kinase (MAPK) signaling pathway, thereby enhancing malignant behaviors of cancer cells (22, 23).

Infiltrating ductal adenocarcinoma of the pancreas is often characterized by an abundant desmoplastic stroma. Nevertheless, the effects of radiation to the stromal components have not been studied. We hypothesized that irradiation to stromal cells as well as to cancer cells themselves could affect the invasive phenotype of pancreatic cancer through modifying tumor-stromal interactions. To test this hypothesis, we investigated the invasive behavior of pancreatic cancer cells in response to coculture with irradiated fibroblasts. We also determined the concentration of a panel of growth factors including HGF in culture supernatant from fibroblasts after irradiation and expression/phosphorylation of c-Met and MAPK activity in pancreatic cancer cells exposed to the supernatant from irradiated fibroblasts.

MATERIALS AND METHODS

Cells and Reagents. Three human pancreatic cancer cell lines [Suit-2, generously provided by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan) and Capan-1 and SW1990, purchased from American Type Culture Collection (Rockville, MD)], one human fibroblast cell line [MRC5, from Riken (Tokyo, Japan)], and primary pancreatic fibroblasts derived from a patient with invasive pancreatic adenocarcinoma were used in this study. Cells were maintained in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C with humidified 90% air and 10% CO_2. Human recombinant NK4 was purified from the conditioned medium of Chinese hamster ovary cells transfected with expression vector for human NK4 cDNA as described previously (24). Polyclonal anti-HGF antibody was prepared as described previously (25). Anti-fibroblast growth factor (FGF) basic antibody was purchased from R & D Systems (Minneapolis, MN).

Radiation. Cells were irradiated with a dose of 5 or 10 Gy at room temperature using a ^{137}Cs source (Gamma Cell 40; Atomic Energy of Canada, Ltd., Ontario, Canada) with a delivering rate of 1.0 Gy/min. MRC5 fibroblasts were seeded at a density of 1.5×10^6 cells in 90-mm dishes and irradiated at 5 or 10 Gy. After cultivation for 24 h, the supernatant of these fibroblasts were harvested, centrifuged to remove debris, and then used to stimulate pancreatic cancer cells.

Invasion Assay. Invasion of pancreatic cancer cells was measured by the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ) as reported previously (26). In brief, transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well; Becton Dickinson). Fibroblasts were initially seeded on a 24-well plate at a density of $2.5 \times 10^4/cm^2$ and cultured in DMEM supplemented with 10% FBS for 24 h.

Received 8/15/03; revised 1/13/04; accepted 2/18/04.

Grant support: Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and Kyushu University Interdisciplinary Programs in Education and Projects in Research Development.

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Requests for reprints: Kazuhiro Mizumoto, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5440; Fax: 81-92-642-5458; E-mail: mizumoto@med.kyushu-u.ac.jp.

These fibroblasts were then irradiated at 5 or 10 Gy, and the medium was immediately replaced with 750 μl of fresh medium. Cancer cells were seeded at a density of 1×10^5/cm^2 in the upper chambers with 250 μl of DMEM supplemented with 10% FBS with or without human recombinant NK4, anti-HGF antibody, or anti-FGF basic antibody at an indicated final concentration. After 24 h of incubation, cells that had invaded to lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five randomly selected fields under a light microscope.

Immunoblot Analysis for c-Met and Its Phosphorylated Type (p-Met). Pancreatic cancer cells were cultured with supernatant of irradiated or nonirradiated MRC5 fibroblasts for the indicated hours. For c-Met detection, cells cultured in 90-mm dishes were directly lysed in a lysis buffer (900 μl) composed of 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 30 mM sodium PP_i, 50 mM NaF, 0.5 μg/ml leupeptin, 1 mM phenylmethyl sulfonylfluoride, and 1% Triton X-100. The whole cell lysates were fractionated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with 1:500–1,000 dilutions of antihuman c-Met polyclonal IgG specific for the COOH terminus of c-Met p140 (C-12; Santa Cruz Biotechnology, Santa Cruz, CA) and then probed with antirabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology).

For c-Met tyrosine phosphorylation, the cells were washed with PBS and lysed in an ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM phenylmethyl sulfonylfluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin (pH 7.4)]. The cell lysates were separated by 8% SDS-PAGE under reducing conditions. The phosphorylated Met was probed by a polyclonal IgG recognizable for phosphorylation site in amino acids 1231–1240 of human c-Met (Y1234 and Y1235; Upstate Biotechnology, Lake Placid, NY). Immunoblots were detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) and developed by Hyperfilm (Amersham Biosciences). The relative density of each band was determined with Image Gauge software (version 3.01; Fuji Photo Film Co., Ltd. and Kohshin Graphic Systems, Inc., Tokyo, Japan).

p42/p44 MAP Kinase Enzyme Assay. MAPK activity was assayed using the peptide phosphorylation assay kit (code RPN 84; Amersham Biosciences). The assay used as substrate a peptide containing a single phosphorylation site corresponding to the MAPK phosphorylation consensus sequence, Pro-Leu-Thr-Pro, found in epidermal growth factor receptor at Thr^{669}. Cells were grown subconfluent in 6-well plates and then cultured with the supernatant of irradiated or nonirradiated fibroblasts for the indicated hours. Cells were washed in ice-cold PBS and lysed in 0.3 ml of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM phenylmethyl sulfonylfluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. After centrifugation of the cell lysate at 16,000 rpm for 30 min, 15-μl aliquots were used for the assay. MAP kinase activity was determined as the amount of ^{32}P incorporation, which was quantitatively read by a liquid scintillation system (LSC-3500; Aloka, Tokyo, Japan). At least four measurements were done for each sample.

Scattering Assay. Pancreatic cancer cells were plated at a density of 250 cells/well in DMEM containing 10% FBS into 6-well plates. After cultivation for 5 days, cells were stimulated by the supernatant of irradiated or nonirradiated MRC5 cells. Scattering of cells was evaluated under a phase-contrast microscope (DIAHOT; Nikon, Inc., Tokyo, Japan) at 24 h after stimulation.

Laser-Scanning Confocal Microscopy for Immunofluorescence Staining of c-Met. Pancreatic cancer cells were plated on 24-well chamber slides (Lab-Ted Nunc, Inc., Naperville, IL) and were stimulated by the supernatant from irradiated or nonirradiated MRC5 fibroblasts. The cancer cells were then incubated for 8 h, fixed with cold methanol for 5 min, blocked with a blocking solution (10% normal goat serum, 3% BSA, and 0.5% gelatin in PBS) and incubated with the c-Met antibody (C-12; Santa Cruz Biotechnology) for 30 min at 37°C. The cells were then incubated for 30 min with Alexa 488-conjugated antirabbit IgG (Molecular Probes, Eugene, OR). Nuclear DNA was counterstained with propidium iodide. Slides were mounted with Gel/Mount (Biomeda Corporation, Foster City, CA) and visualized under a laser-scanning confocal fluorescent microscope (LSM-GB200 System; Olympus Optical Corp., Tokyo, Japan).

ELISA. ELISA was performed using a human HGF enzyme immunoassay kit (Special Immune Institute, Tokyo, Japan) for HGF, Biotrak human ELISA (Amersham Biosciences) for vascular endothelial growth factor, and a Quantikine kit (R & D Systems) for epidermal growth factor, FGF, and transforming growth factor β1 according to the manufacturer's instructions. MRC5 cells and primary fibroblasts were seeded at a density of 2×10^5/well on 6-well plates in 2 ml of DMEM containing 10% FBS and cultured for 24 h. Culture supernatants were harvested from irradiated or nonirradiated fibroblasts after 24 h of incubation and subjected to the measurements for each growth factor.

Reverse Transcription-PCR for HGF and c-met mRNA Expression. Total RNA was extracted from cells using an Isogen-based method (Nippongene, Inc., Toyama, Japan). The reverse-transcription from RNA to cDNA and PCR amplification were performed using an RNA PCR kit (TaKaRa Biomedicals, Kyoto, Japan), according to the manufacturer's instructions. Primers for amplification of the c-met gene and HGF gene were designed as described previously (27). Thirty-five cycles were performed, each consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. An aliquot of the reaction was then analyzed by 2% agarose gel electrophoresis. The sizes of the predicted products should be 523 bp for HGF and 222 bp for c-Met.

***Ex vivo* Model.** MRC5 cells and pancreatic fibroblasts were irradiated at 10 or 5 Gy, respectively, just before implantation. Suit-2 cells (1×10^6) and 1×10^6 irradiated or nonirradiated fibroblasts were mixed well and implanted into the pancreas of 6-week-old female nude mice (BALB/c nu/nu; Kyoudo Co., Ltd., Saga, Japan). Three mice were used in each group. Seven days after implantation, tumors were resected with the surrounding tissue. Tissues were fixed in 10% neutral formalin for 24 h and embedded in paraffin according to standard histological procedures. These tissue sections were washed in PBS and stained with H&E for histopathological examinations. To semiquantitate the invasiveness of the implanted pancreatic cancer, we defined the invasion score based on histological observations as previously reported (24): score 0, invasion was undetectable, and the tumor was surrounded by a capsule; score 1, invasion was undetectable, but the tumor was not surrounded by a capsule; score 2, invasion was partial; score 3, invasion was extensive, and normal pancreatic and tumor regions could not be distinguished.

Immunohistochemical Studies. Fixed tumor tissue sections were embedded in paraffin. These tissue sections were quenched with 3% hydrogen peroxide in PBS for 5 min, washed in PBS, and treated with 0.1% trypsin at room temperature for 5 min. The sections were exposed for 30 min to 10% normal goat serum and incubated with rabbit anti-c-Met phosphospecific antibodies (diluted 1:50; BIOSOURCE) overnight at 4°C. Next, the sections were incubated with biotinylated peroxidase-conjugated goat antirabbit IgG antibodies (DAKO Co., Carpinteria, CA) for 60 min. The reaction was observed by incubating the sections with substrate solution containing diaminobenzidine and hydrogen peroxide. The sections were then washed in PBS and counterstained with hematoxylin.

Statistical Analysis. Values were expressed as the mean ± SD. Comparisons between three or more groups were done by one-way ANOVA and by the Student's *t* test for comparison between two groups. The level of statistical significance was set at $P < 0.01$.

RESULTS

Irradiated Fibroblasts Promote the Invasiveness of Nonirradiated Pancreatic Cancer Cells. Before investigating the effect of irradiation on invasiveness of cancer cells, we examined the effect of 5-Gy or 10-Gy irradiation on proliferation of cancer cells (Suit-2 cells) and fibroblasts (MRC5 cells and pancreatic fibroblasts) using propidium iodide assay as described in our previous report (10). The cells were not cytolytic 24 h after radiation and cytostatic *in a* dose-dependent manner. The proliferation of cancer cells was completely inhibited at the dose of 10 Gy, whereas that of fibroblasts was only partially inhibited at the same dose.

Using *in vitro* invasion assay, we examined the invasiveness of pancreatic cancer cells cocultured with MRC5 fibroblasts that were nonirradiated, irradiated at 5 Gy, or irradiated at 10 Gy. Only a small number of Suit-2 pancreatic cancer cells invaded through Matrigel when they were cultured alone, whereas coculture with MRC5 fibroblasts increased the invasive ability of Suit-2 cells, which is consistent with our previous data (28). Surprisingly, coculture with MRC5 cells after irradiation further enhanced the invasiveness of Suit-2 cells in a

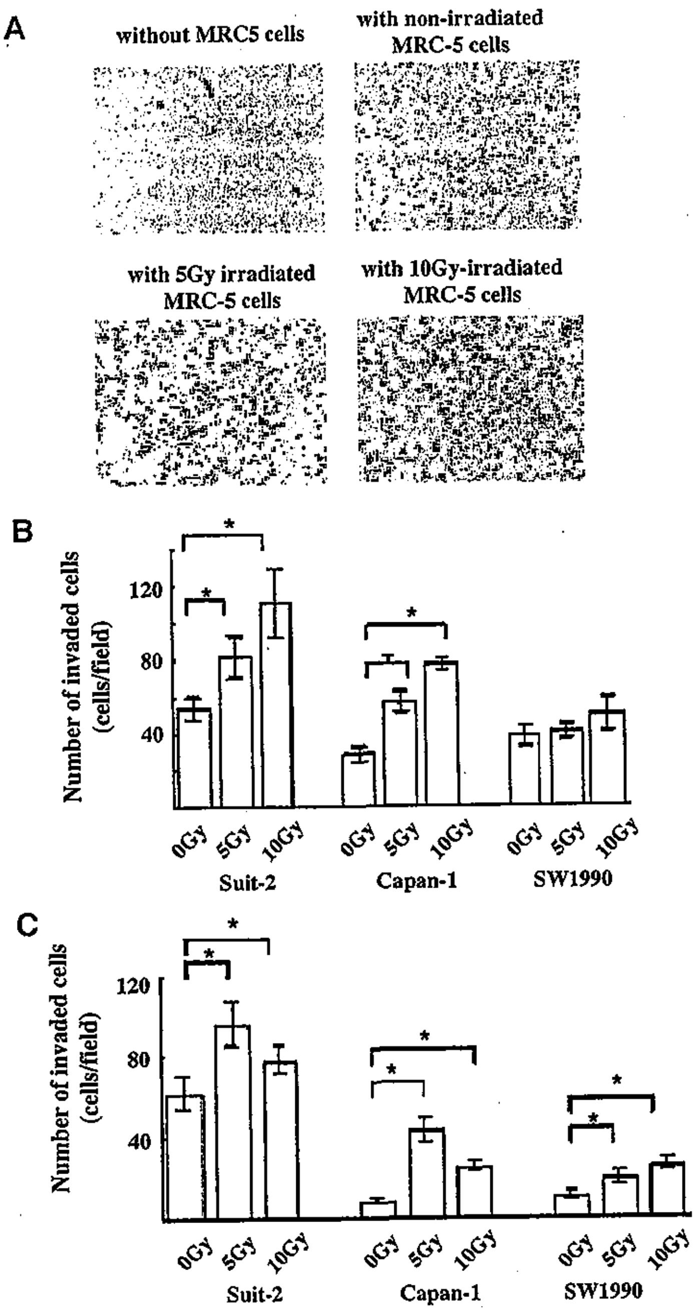

Fig. 1. Effects of coculture with irradiated fibroblasts on the invasiveness of human pancreatic cancer cells. *A*, photomicrographs of *in vitro* invasion assay in Suit-2 cells cocultured with nonirradiated or irradiated MRC5 fibroblasts (at 5 or 10 Gy). H&E stain, ×40. *B*, invasive potential (as determined by the number of invaded cells) of three pancreatic cancer cell lines (*Suit-2, Capan-1,* and *SW1990*) cocultured with irradiated MRC5 fibroblasts. *C*, invasive potential of three pancreatic cancer cell lines cocultured with irradiated primary pancreatic fibroblasts. Each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

dose-dependent manner (Fig. 1*A*). The number of invading Suit-2 cells cocultured with MRC5 cells irradiated at 10 Gy was 2.1-fold greater than the number of invading Suit-2 cells cocultured with nonirradiated MRC5 cells (Fig. 1*B*). Similar results were obtained in Capan-1 cells but not in SW1990 cells; irradiation to MRC5 fibroblasts further accelerated the invasion of Capan-1 in a dose-dependent manner in a coculture system but not of SW1990 (Fig. 1*B*).

To rule out the possibility that the enhanced invasiveness by irradiation to fibroblasts was a specific event for MRC5 cells, we next tested the invasiveness of pancreatic cancer cell lines cocultured with primary pancreatic fibroblasts derived from resected specimen from a patient with invasive pancreatic adenocarcinoma. Coculture with irradiated primary pancreatic fibroblasts (at 5 and 10 Gy) showed significantly larger number of invading cells compared with coculture with nonirradiated counterparts in all three pancreatic cancer cell lines tested (Suit-2, Capan-1, and SW1990; Fig. 1*C*).

Irradiated Fibroblasts Further Promote the Invasiveness of Irradiated Pancreatic Cancer Cells. We previously documented that the invasiveness of human pancreatic cancer cells including AsPC1 and Panc1 was enhanced after irradiation (10). We therefore investigated the invasiveness of irradiated or nonirradiated Suit-2 cells in coculture with irradiated or nonirradiated fibroblasts. Suit-2 cells irradiated at 5 Gy showed a slight increase in the number of invading cells when they were cocultured with nonirradiated MRC5 cells (Fig. 2). In contrast, coculture of nonirradiated Suit-2 cells with MRC5 cells that had received the same dose (5 Gy) of irradiation increased the number of invading Suit-2 cells. Coculture of irradiated Suit-2 and irradiated MRC5 cells showed the largest number of invading cells, but the increase in the number of invading cells was modest compared with coculture of nonirradiated Suit-2 and irradiated MRC5 (Fig. 2*B*). These results suggest that radiation to fibroblasts rather than to pan-

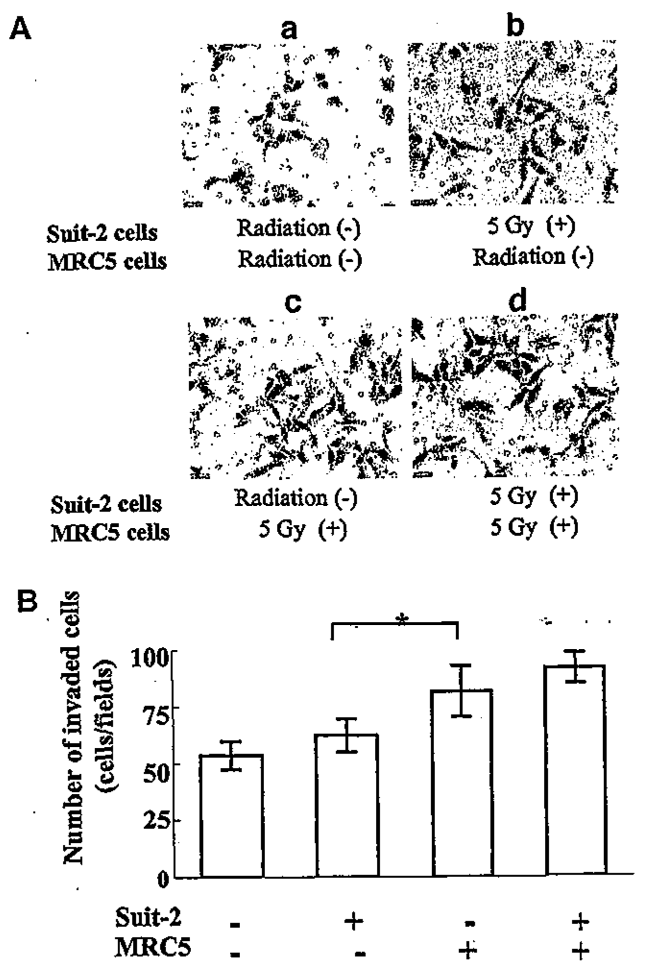

Fig. 2. Effects of coculture of nonirradiated or irradiated pancreatic cancer cells and nonirradiated or irradiated fibroblasts on the invasion of pancreatic cancer cells. *A*, photomicrographs of *in vitro* invasion assay in nonirradiated or irradiated (5 Gy) Suit-2 cells cocultured with either nonirradiated or irradiated (5 Gy) MRC5 fibroblasts. H&E stain, ×200. *B*, invasive potential (as determined by the number of invaded cells) of nonirradiated or irradiated (5 Gy) Suit-2 cells cocultured with either nonirradiated or irradiated (5 Gy) MRC5 fibroblasts. Each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

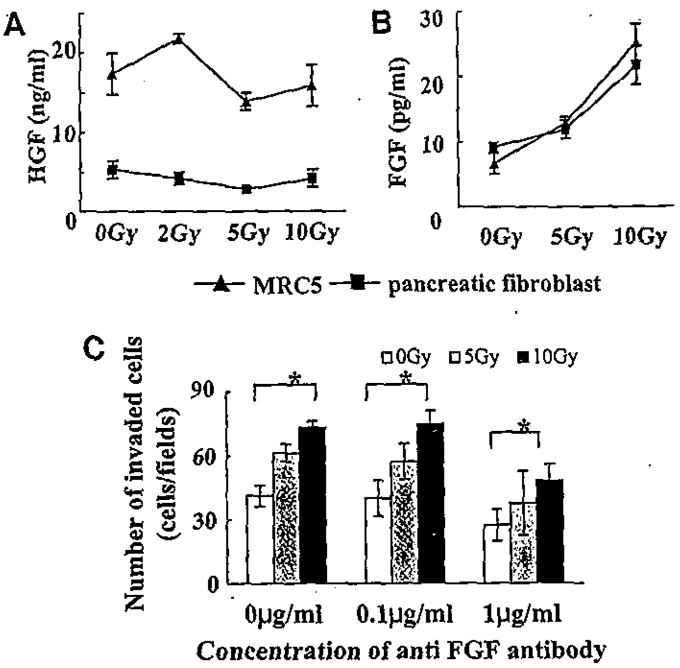

Fig. 3. The concentration of *HGF* (*A*) and *basic-FGF* (*B*) in the supernatant of fibroblasts (MRC5 or primary pancreatic fibroblasts) after irradiation. Fibroblasts were irradiated at 2, 5, or 10 Gy and cultured for 24 h in a fresh serum-containing medium, and the supernatant was subjected to *ELISA to measure the concentration* of these growth factors. *C*, the number of invaded Suit-2 cells cocultured with MRC5 cells in the absence or presence of anti-FGF basic antibody at a concentration of 0.1 or 1 μg/ml. Each value represents the mean ± *SD of triplicate measurements*. *, $P < 0.01$

creatic cancer cells themselves strongly enhances the invasive behaviors of pancreatic cancer cells in a coculture system.

Changes in the Concentration of Growth Factors and the Expression/Activity of Major Proteolytic Systems in the Supernatant from Irradiated Fibroblasts. To elucidate the mechanism by which coculture with irradiated fibroblasts enhances the invasiveness of pancreatic cancer cells, we measured the concentration of a panel of growth factors (HGF, basic-FGF, transforming growth factor $\beta 1$, vascular endothelial growth factor, and epidermal growth factor) in the supernatant from fibroblasts after irradiation. The concentration of HGF remained unchanged after irradiation in both MRC5 cells and primary pancreatic fibroblasts (Fig. 3*A*). The concentration of basic FGF in the supernatant of irradiated MRC5 cells and primary pancreatic fibroblasts increased significantly after irradiation (Fig. 3*B*). To confirm whether the modestly increased basic FGF expression is a responsible factor to enhance the invasion potential of cancer cells with a coculture after radiation, the anti-FGF basic antibody was used for invasion assay. As shown in Fig. 3*C*, the anti-FGF basic antibody showed the modest inhibitory effect on the invasion potential independently of irradiation, but the radiation-enhanced invasion potential of Suit-2 cells was still maintained in the presence of 1 μg/ml anti basic FGF antibody, which is sufficient to inhibit bioactivity of 500 pg/ml basic FGF completely. These data suggest that the increase of basic FGF is not critical to induce radiation-enhanced invasiveness in a coculture system, although the presence of FGF is involved in invasion potential.

The concentrations of other growth factors (transforming growth factor $\beta 1$, vascular endothelial growth factor, and epidermal growth factor) were relatively low in nonirradiated fibroblast cultures (29–31) and did not show any changes after irradiation (data not shown), suggesting that these growth factors may not be involved in the increased invasiveness of cocultured pancreatic cancer cells. We also examined the supernatant from irradiated fibroblasts for the expression of two major proteolytic systems including matrix MMPs and urokinase-type plasminogen activator. We used gelatin zymography to determine the activity and the expression of MMP-2 and MMP-9 and immunoblot analysis to examine the expression of urokinase-type plasminogen activator. The expressions of MMP-2 and MMP-9 were detectable in the fibroblast supernatant, but their expression levels and activities were not changed 8, 16, and 24 h after irradiation (data not shown). The expression of urokinase-type plasminogen activator was not detected in both MRC5 cells and pancreatic fibroblasts before and after irradiation (data not shown).

Change in MAPK Activity in Suit-2 Cells Exposed to the Supernatant of Irradiated Fibroblasts. MAPK is known to be activated by various growth factors including HGF and basic FGF (32), and activation of this signaling pathway has been shown to play a crucial role in the acquisition of invasive and metastatic phenotype of cancer cells (22, 23). We therefore measured the MAPK activity in Suit-2 cells after exposure to the supernatant from either irradiated or nonirradiated MRC5 fibroblasts. The MAPK activity kinetics in Suit-2 after exposure to the supernatant from nonirradiated MRC5 cells showed an increased activity as a single peak at ~30 min after the beginning of exposure (Fig. 4). In contrast, the MAPK activity kinetics in Suit-2 cells exposed to the supernatant of irradiated MRC5 cells showed a biphasic pattern of increase identified as two distinct peaks at ~30 min and ~8 h after exposure (Fig. 4). The first peaks were always observed in all experiments, but the degree of these peaks was not reproducible. However, the second peaks observed only in irradiated experiments were always reproducible. Notably, the MAPK activity in Suit-2 exposed to the supernatant from irradiated fibroblasts reached to ~4-fold at the second peak compared with the activity observed in Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts. Thus, the increase in the MAPK activity at the late phase may be associated with the increased invasion of Suit-2 cells cocultured with irradiated fibroblasts.

Changes in c-Met and p-Met Expression in Suit-2 Cells Exposed to the Supernatant of Irradiated MRC5 Cells. Because interactions between growth factors and their receptors (such as the interaction of HGF and its receptor c-Met) can activate the Ras-Raf-mitogen-activated protein/extracellular signal-regulated kinase kinase-MAPK signal transduction pathway (29, 33, 34), we examined the expression of c-Met and the phosphorylated form of c-Met (p-Met) in Suit-2 cells after exposure to the supernatant from irradiated or nonirradiated fibroblasts. The expression of c-Met in Suit-2 cells increased after exposure to the supernatant from irradiated fibroblasts in a time-dependent manner and reached to approximately 2-fold at 8 h after exposure (Fig. 5). In contrast, the c-Met expression in Suit-2

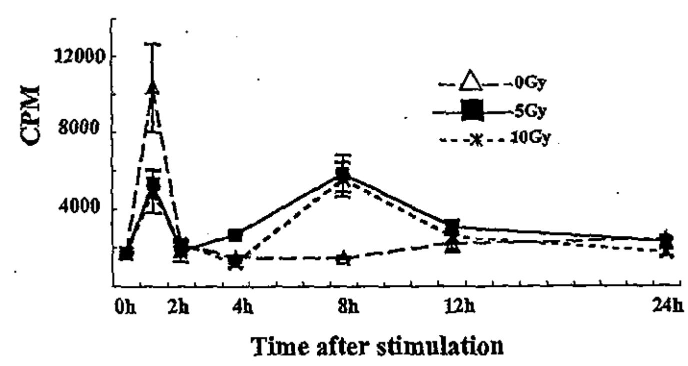

Fig. 4. p44/p42 MAPK(extracellular signal-regulated kinase 1/2) enzyme activity in Suit-2 pancreatic cancer cells exposed to the supernatant from irradiated or nonirradiated MRC5 fibroblasts. A biphasic increase in MAPK activity *is observed in Suit-2 cells after exposure to the supernatant* from irradiated MRC5 cells, in contrast to the activity of Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts showing a single peak. Suit-2 cells were seeded and cultured for 24 h.

cells exposed to the supernatant from nonirradiated fibroblasts showed a minimal increase at 1 h after stimulation. The expression of c-Met and especially its phosphorylated form (p-Met) was markedly augmented in Suit-2 cells after exposure to the supernatant from irradiated fibroblasts, and the relative intensity of p-Met expression induced by irradiated MRC5 cells increased in a dose-dependent manner to 2.9-fold (at 5 Gy) and 3.6-fold (at 10 Gy) as compared with that induced by nonirradiated MRC5 cells (Fig. 6*A*). The relative intensity of p-Met expression induced by irradiated pancreatic fibroblasts increased to 4.2-fold (at 5 Gy) and 2.4-fold (at 10 Gy) as compared with that induced by nonirradiated pancreatic fibroblasts (Fig. 6*B*). These changes of p-Met were well correlated with the change in the invasion potential observed in invasion assay using both of MRC5 cells and pancreatic fibroblasts (Fig. 1, *B* and *C*). These data strongly support the involvement of phospho-Met in the promoting effect of coculture with irradiated fibroblasts on invasion of cancer cells.

To confirm the increased expression of c-Met, we used indirect fluorescent microscopy to examine the *in situ* expression of c-Met in Suit-2 cells after exposure to the supernatant of irradiated or nonirradiated MRC5 cells. Compared with Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts, the c-Met fluorescence intensity was increased specifically in the cell membrane of Suit-2 at 8 h after exposure to the supernatant of irradiated MRC5 cells (Fig. 7).

The Supernatant from Irradiated MRC5 Cells Promotes Scattering of Suit-2 Cells. We then examined the effect of the supernatant derived from irradiated fibroblasts on cell scattering, which is a well-characterized behavior activated by HGF/c-Met system (35). Exposure to the supernatant from irradiated fibroblasts accelerated the scattering of Suit-2 cells in a dose-dependent manner (Fig. 8).

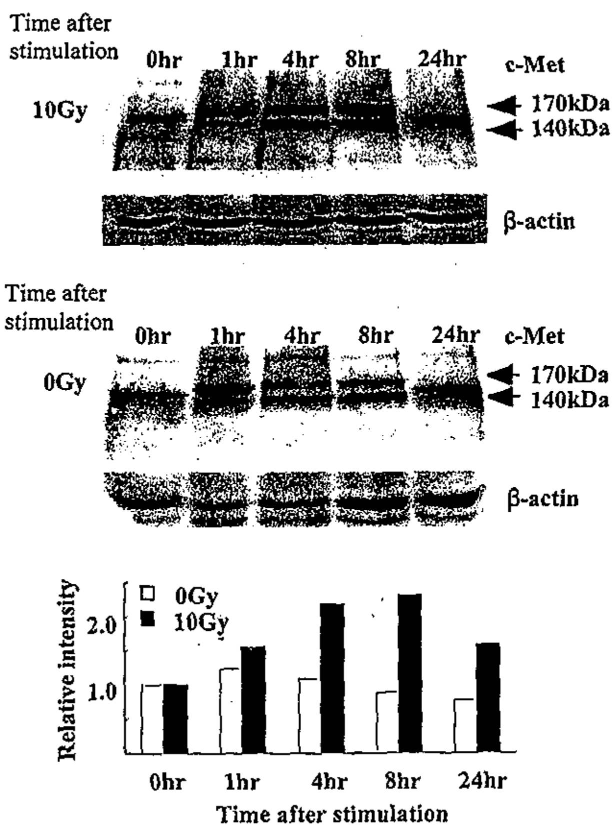

Fig. 5. Immunoblot analysis of c-Met expression in Suit-2 cells after exposure to the supernatants from either 10 Gy irradiated or nonirradiated MRC5 cells. The value in bar graph represents a band intensity of c-Met relative to the intensity at 0 h after normalization by the corresponding β-actin expression.

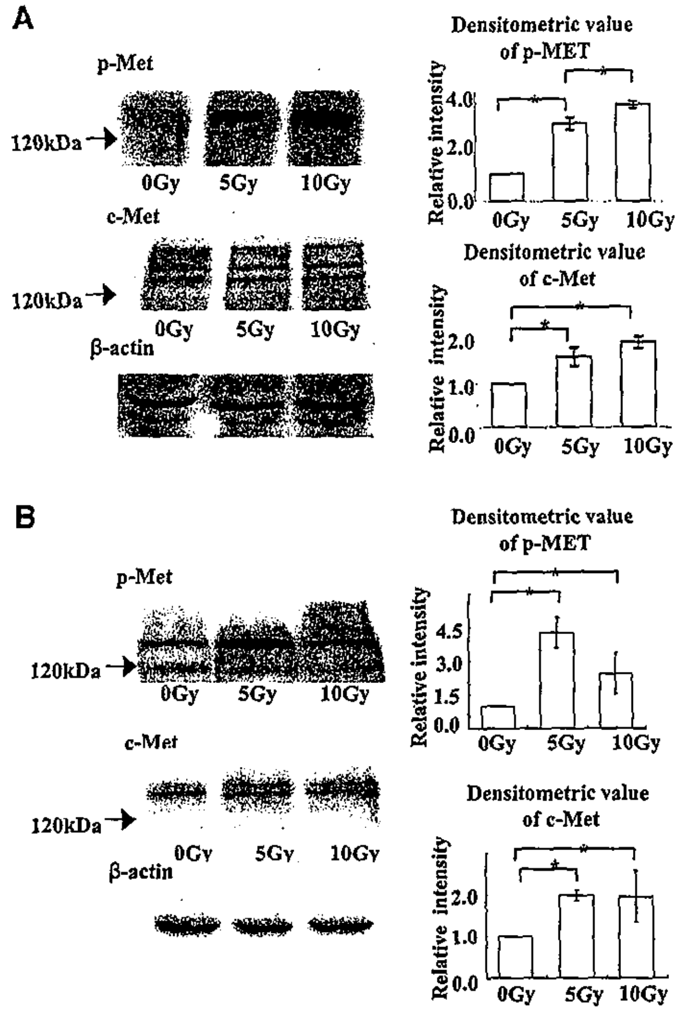

Fig. 6. Immunoblot analysis of c-Met and p-Met expression in Suit-2 cells at 8 h after exposure to the supernatant from either nonirradiated or irradiated (5 or 10 Gy) MRC5 cells (*A*) and pancreatic fibroblasts (*B*). The value in *bar graph* represents a band intensity of p-Met or c-Met (means and SD of three independent experiments) relative to the intensity at 0 Gy after normalization by the corresponding β-actin expression. *, $P < 0.01$

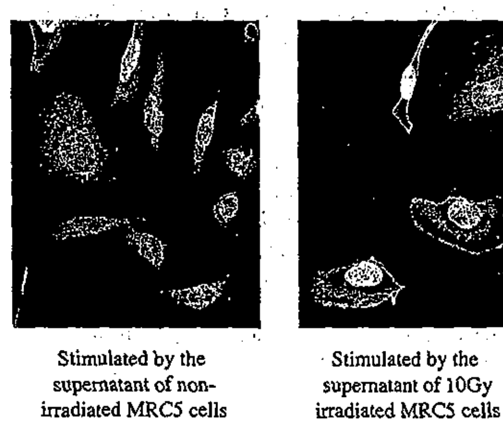

Fig. 7. Laser-scanning confocal microscopy of Suit-2 pancreatic cancer cells stained with an anti-c-Met antibody (*green*) showing an enhanced fluorescent intensity of c-Met on the cell membrane after exposure to the supernatant from 10 Gy-irradiated MRC5 cells.

NK4 Inhibits the Accelerated Invasiveness of Suit-2 Cells Cocultured with Irradiated Fibroblasts. NK4 is composed of 447 amino acids, which is an intramolecular region of HGF and includes a specific domain binding to c-Met receptor (36). We and other investigators (24, 25, 37, 38) have shown that NK4 acts as a specific

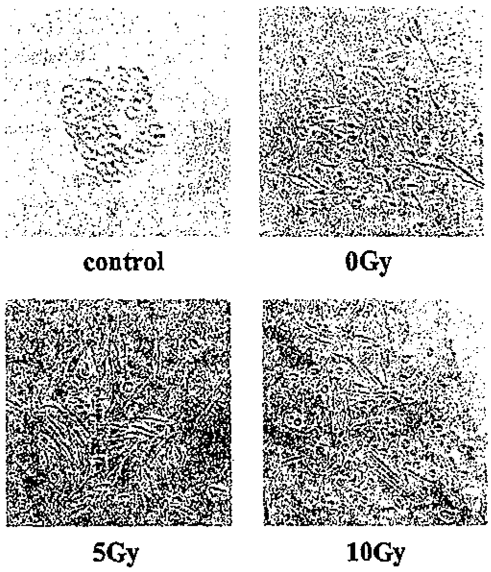

Fig. 8. Scattering of Suit-2 cells before and after exposure to the supernatant from nonirradiated (0 Gy) or irradiated *MRC5 cells at 5 or 10 Gy.*

HGF antagonist and inhibits the malignant behaviors of various cancer cells induced by the HGF/c-Met pathway. To further confirm the role of HGF/c-Met pathway in the increased invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts, we performed coculture invasion assay in the presence or absence of NK4 or anti-HGF antibody. The invasion of Suit-2 cells was accelerated when they were cocultured with irradiated fibroblasts, but the increased invasiveness was completely blocked in the presence of NK4 (at a concentration of 5 or 10 μg/ml; Fig. 9). The anti-HGF antibody showed the same inhibitory effect at almost the same dose as NK4 (data not shown). Furthermore, NK4 (10 μg/ml) inhibited the increased expression of c-Met/p-Met and the biphasic activation of MAPK in suit-2 cells exposed to the supernatant from irradiated fibroblasts (data not shown). These results indicate that HGF/c-Met pathway is a possible critical factor to induce invasiveness of Suit-2 cells in response to coculture with irradiated fibroblasts.

To rule out the effect of the change of basic FGF on phosphorylation of c-Met, we used the anti-FGF basic antibody in the same supernatant model and found that the anti-FGF antibody did not affect the phosphorylation of c-Met (data not shown).

The mRNA Expression of HGF and c-met in Cancer Cells and Fibroblasts. Although HGF is known to be secreted primarily from stromal cells (*e.g.*, *fibroblasts*), there is a possibility that HGF produced from cancer cells in an autocrine manner participates in the activation of HGF/c-Met and MAPK pathway and subsequent increase in the invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts. To rule out this possibility, we performed reverse transcription-PCR to examine the mRNA expression of HGF and c-Met in a panel of pancreatic cancer cell lines and fibroblasts. Reverse transcription-PCR showed no detectable expression of HGF in Suit-2 and Capanl, whereas the expression was detected in SW1990 and fibroblasts (MRC5 and primary pancreatic fibroblasts; Fig. 10). Expression of c-met mRNA was detectable in all of the cell lines and fibroblasts tested (Fig. 10). These results raise the possibility that autocrine loop of HGF might play a role in the increased invasiveness of SW1990 but not of Suit-2 and Capan-1 in response to coculture with irradiated fibroblasts.

Irradiated Fibroblasts Promote the Invasion of Pancreatic Cancer in *ex Vivo* Models. To support the *in vitro* data, we evaluated the effect of irradiated fibroblasts on the invasion of pancreatic cancers in *ex vivo* models. As shown in Fig. 11*A*, the tumor derived from Suit-2 cells and 10 Gy-irradiated MRC5 cells invaded into normal pancreatic tissues without forming a capsule, whereas the tumor cells derived from Suit-2 cells and nonirradiated MRC5 cells were encapsulated by multiple layers of stromal cells. Also, the same result was found in pancreatic fibroblasts. Histological estimation of tumor invasion indicated that the invasive score in groups with irradiated fibroblasts was significantly higher than that with nonirradiated fibroblasts (MRC5 and pancreatic fibroblasts; Fig. 11*B*; $P = 0.013$ and 0.038, respectively).

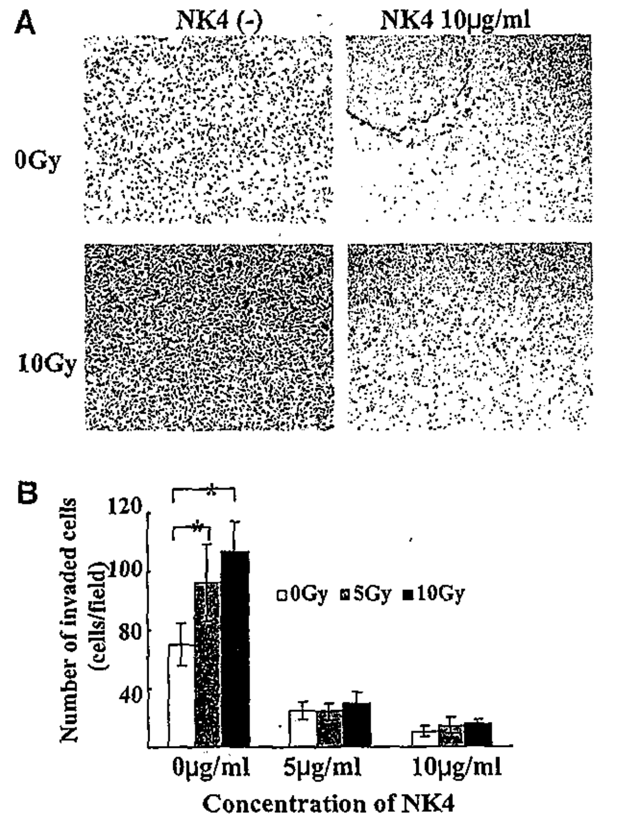

Fig. 9. NK4 inhibits the enhanced invasion of Suit-2 cells cocultured with irradiated MRC5 cells. Photomicrographs of *in vitro* invasion assay in Suit-2 cells cocultured with nonirradiated or 10-Gy irradiated MRC5 cells in the presence or absence of *10 μg/ml* NK4. H&E stain, ×40. *A*, the number of invaded Suit-2 cells cocultured with MRC5 cells in the absence or presence of NK4 at a concentration of 5 or 10 μg/ml. *B*, each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

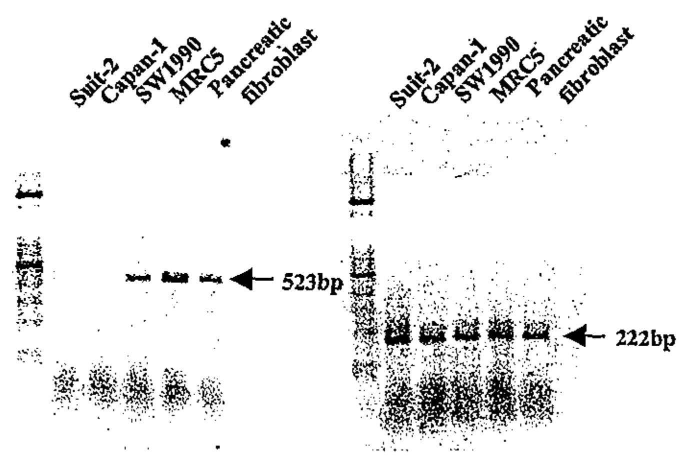

Fig. 10. Reverse transcription-PCR analysis of HGF and c-met mRNA expression in three pancreatic cancer cell lines (*Suit-2, Capan-1,* and *SW 1990*), *MRC5* fibroblasts, and primary *pancreatic fibroblasts*. The HGF expression is detectable as a 532-bp product in SW1990, MRC5, and pancreatic fibroblasts, whereas the c-met expression is detectable as a 222-bp product in all of the cell lines and fibroblasts tested.

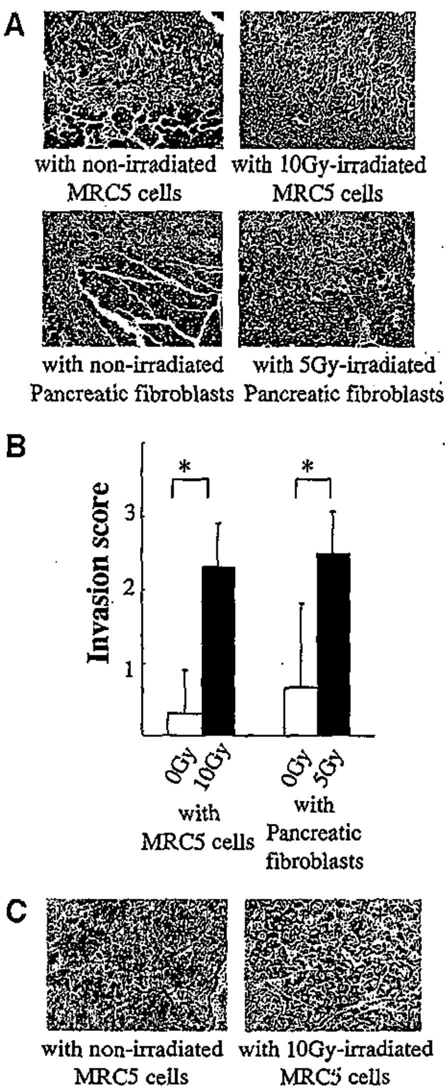

Fig. 11. MRC5 cells and pancreatic fibroblasts were irradiated at 10 or 5 Gy, respectively, just before implantation. Suit-2 cells (1×10^6) and 1×10^6 irradiated or nonirradiated fibroblasts were mixed well and implanted into the pancreas of mice. Three or four mice were used in each group. Seven days after implantation, tumors were resected with the surrounding tissues. Tissues were stained with H&E ×100 (*A*) or anti-c-Met phosphospecific antibodies ×400 (*C*) and evaluated for the invasiveness as described in "Materials and Methods" (*B*). *, $P < 0.01$

Furthermore, we evaluated the effect of irradiated fibroblasts on p-Met expression in tumors using immunohistochemical studies. As shown in Fig. 11*C*, clearly enhanced expression of p-Met was found in tumors with irradiated MRC5 cells as compared with that in tumors with nonirradiated MRC 5 cells. Also, similar results were observed in the same model using pancreatic fibroblasts.

DISCUSSION

Despite the fact that infiltrating adenocarcinoma of the pancreas is often characterized by an abundant desmoplastic stroma and that tumor-stromal interactions play a critical role in tumor invasion and metastasis, the relationship between radiation to stromal cells and the invasive phenotype of pancreatic cancer is unknown. In the present study, we demonstrate, for the first time, that coculture with irradiated fibroblasts significantly accelerates the invasion of certain pancreatic cancer cells through enhanced tumor-stromal interactions including activation of the HGF/c-Met system. Our present findings raise the possibility that radiation to stromal components as well as to neoplastic cells themselves could affect the progression of pancreatic cancer and therefore may have important clinical implications.

There are some clinical data suggesting that local radiation to tumors or normal tissue leads to the increase in metastasis. Fisher *et al.* (39) reviewed some prior clinical observation regarding enhanced metastasis after radiation therapy for breast cancer. Stjernsward (40) implicated routine postoperative radiation therapy as leading to increased mortality in early-stage cancers, presumably because of increased distant metastasis. Anderson *et al.* (41) demonstrated that local control failures after radiation therapy of cervix cancer was associated with increased distant metastasis. In addition, some investigators reported that metastasis occasionally appeared initially or preferentially in previously irradiated tissue in a various of tumors, such as melanoma (42), nasopharynx carcinoma (43), breast carcinoma (40), and uterine carcinoma (44).

Also, in experimental models, previous studies have implicated the relationship between radiation to the stromal tissues and the metastatic phenotype of malignant tumors. For example, radiation to the tumor bed stroma enhances the metastatic spread of carcinomas and sarcomas in mice (45). It has also been shown that metastasis of squamous cell carcinoma was more frequently observed in mice in which tumors developed in previously irradiated tissue than in mice with tumors in nonirradiated tissue (46). Our present study using *in vitro* coculture system and *ex vivo* models supports these *in vivo* findings and suggests that the accelerated metastasis in these irradiated tissues may be mediated by tumor-stromal interactions.

Recent evidence has shown that in several experimental systems, irradiation can promote invasion and metastasis of cancer cells by activating several genes/pathways important for tumor invasion and metastasis. For example, sublethal doses of irradiation enhance the migration and invasiveness of malignant glioma cells (9). In addition, radiation to a primary Lewis lung carcinoma results in accelerated growth of previously dormant lung metastases in mice (8). We have previously reported that radiation to pancreatic cancer cells enhances their invasive ability via up-regulated expression of c-Met and increased activity of MMP (10, 11). In the present study, we also observed that irradiation to pancreatic cancer cells resulted in an increase in their invasion ability when they were cocultured with nonirradiated fibroblasts, but the increase was modest. By contrast, the invasiveness of nonirradiated pancreatic cancer cells was markedly increased when they were cocultured with irradiated fibroblasts. These findings suggest that radiation to stromal fibroblasts could lead to an increased invasiveness of pancreatic cancer cells indirectly through enhanced tumor-stromal interactions even if the neoplastic cells themselves do not receive irradiation. In this respect, current radiation strategies (especially the setting of target radiation field) need to be carefully reevaluated.

To gain insights into the mechanisms by which coculture with irradiated fibroblasts increases the invasiveness of pancreatic cancer cells, we investigated the expression of HGF/c-Met system, one of the major pathways involved in tumor-stromal interactions, in our experimental models. We found that the increased invasiveness in response to coculture with irradiated fibroblasts was associated with phosphorylation of c-Met, possibly via increased expression of c-Met. These findings suggest that a soluble mediator(s) released from irradiated fibroblasts may trigger the activation of c-Met in pancreatic cancer cells. It has been shown that c-Met expression is induced by several growth factors and cytokines including HGF and basic-FGF (47, 48). In the present study, however, the secretion of HGF from fibroblasts was not increased after irradiation. We did observe an increase in the expression of basic FGF, a potent inducer of c-Met (49), in the supernatant from irradiated fibroblasts, although the concentration (~30 pg/ml) was insufficient to up-regulate p-Met because anti-FGF antibody did not inhibit the expression of p-Met. In addition, under the complete blockade of basic FGF, radiation-enhanced invasion potential was maintained. These findings suggest that increased basic FGF is not a main factor responsible to induce enhancement of the invasion potential in coculture with irradiated fibroblasts, although it may be partially involved in the change of the invasion potential, being independent of phosphorylation of c-Met.

We also demonstrated that NK4, a specific antagonist of HGF, completely inhibited the increased invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts. Notably, NK4 blocked the increased expression of p-Met and activation of MAPK in Suit-2 cells exposed to the supernatant from irradiated fibroblasts. These results further support the involvement of the HGF/c-Met system in the enhanced invasiveness of pancreatic cancer cells induced by irradiated fibroblasts. We and other investigators (28, 38, 50–52) have shown that NK4 inhibits growth, invasion, and metastasis of a variety of human cancers *in vivo* as well as *in vitro*. Our present results further support the therapeutic efficacy of NK4 to inhibit the invasion of cancer cells and suggest that simultaneous administration of NK4 during radiotherapy could be a novel approach to improve the treatment efficacy for patients with pancreatic cancer.

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Intraductal Papillary Mucinous Neoplasm of the Pancreas

Diagnosis and Treatment

Masao Tanaka, MD, PhD

Abstract: Intraductal papillary mucinous neoplasm (IPMN) is characterized by cystic dilatation of the main and/or branch pancreatic duct. Only one-third of all patients are symptomatic, and others are diagnosed by chance. IPMNs are classified into 3 types: main duct, branch duct, and mixed IPMN. Most branch-type IPMNs are benign, while the other 2 types are frequently malignant. The presence of large mural nodules increases the possibility of malignancy in all types. Presence of a large branch-type IPMN and marked dilatation of the main duct indicate, at the very least, the existence of adenoma. Ultrasonography, endosonography, and intraductal ultrasonography clearly demonstrate ductal dilatation and mural nodules, and magnetic resonance pancreatography best visualizes the entire outline of IPMN. Not infrequently, synchronous or metachronous malignancy develops in various organs, including the pancreas. Prognosis is excellent after complete resection of benign and noninvasive malignant IPMNs. Asymptomatic branch-type IPMNs without mural nodules may be followed up without resection. Malignant IPMNs displaying acquired aggressiveness after parenchymal invasion require adequate lymph node dissection. Total pancreatectomy is needed for some IPMNs; its benefits, however, must be balanced against operative and postoperative risks because most IPMNs are slow growing and affect elderly people, and prognosis is favorable for IPMN patients with even malignant neoplasms.

Key Words: intraductal papillary mucinous neoplasm, pancreatic cancer

(*Pancreas* 2004;28:282–288)

The intraductal papillary mucinous neoplasm (IPMN) is characterized by cystic dilatation of the main and/or branch pancreatic duct. This neoplasm is often found in elderly men in the head of the pancreas. Histologically, the IPMN is composed of intraductal papillary growth of mucin-producing columnar epithelia that show a wide spectrum of histologic differentiation, from hyperplasia, adenoma, and borderline neoplasm to carcinoma, according to the degree of epithelial dysplasia.[1,2] It is documented that the development of IPMN includes the so-called adenoma-carcinoma sequence, where the ultimate form of malignant progression is invasive carcinoma.[1,3,5]

IPMN accounts for 0.5% of all pancreatic neoplasms found at autopsy, 7.5% of clinically diagnosed pancreatic neoplasms, and 16.3% of surgically resected pancreatic neoplasms.[6] In a collective review of 259 patients from Japan and Western countries with mucin-producing neoplasm of the pancreas, Kimura et al[7] demonstrated a distinct male preponderance (M, 177; F, 82; M:F = 2.2) and a mean age of 65.5 years (range, 30–94). Our personal series showed similar age and gender distribution.[8]

Excessive secretion of mucin produces cystic dilatation of the pancreatic ducts, hence, the name ductectatic tumors of the pancreas.[6] Granted, there is some confusion over the nomenclature because there exist many other terms that represent the unique features of IPMN, including intraductal papilloma; papillary adenoma[9]; villous adenoma[10,11]; intraductal cystadenocarcinoma[12]; cystic adenocarcinoma[13]; diffuse papillomatosis; diffuse intraductal papillary adenocarcinoma[14]; intraductal mucin-hypersecreting neoplasm[15]; intraductal mucin-producing tumor[16]; mucinous ductal ectasia[17–19]; mucinous pancreatic duct ectasia[20]; mucin-producing tumor[3,21]; mucus-hypersecreting tumor[2,22]; and intraductal papillary neoplasm.[23] Some investigators believe these lesions to be precursors of ductal carcinoma,[14,24,25] while others regard the lesions as variants of mucinous cystic neoplasm.[12,26,27]

The distinction between IPMN and the mucinous cystic neoplasm of the pancreas remains to clarified; however, the definition and classification proposed by the World Health Organization[28] and the Armed Forces Institute of Pathology[29] have provided considerable guidance. Although both the IPMN and mucinous cystic neoplasm produce large amounts of mucin and there have been cases that are frequently indistinguishable, IPMN and mucinous cystic neoplasm are certainly 2 distinct entities.

CLINICAL SYMPTOMS

The IPMN often presents with acute pancreatitis of mild to moderate severity. It has been reported that approximately

Received for publication October 31, 2003; accepted January 7, 2004.
From the Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.
Reprints: Masao Tanaka, MD, PhD, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Fukuoka 812-8582, Japan (e-mail: masaotan@med.kyushu-u.ac.jp).

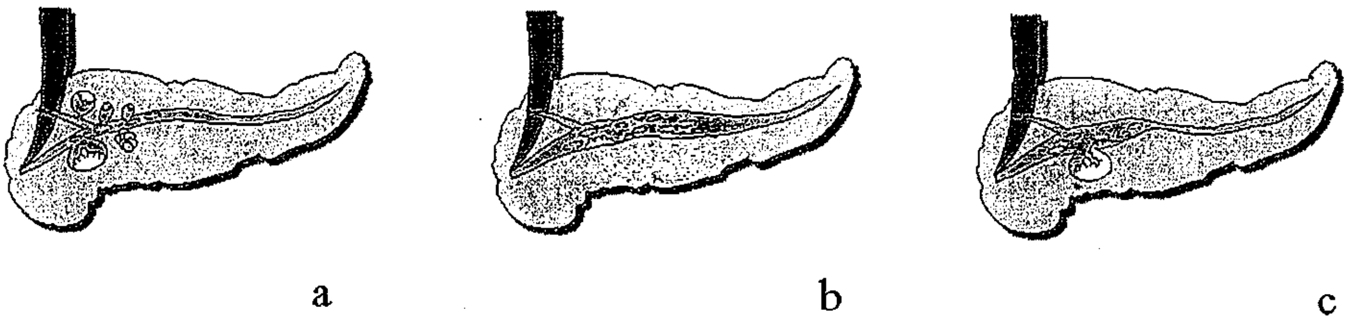

FIGURE 1. Schematic representation of the 3 types of IPMN. A: A variety of branch-type IPMNs. B: Main duct–type IPMNs. C: Mixed-type IPMN.

one-fourth of patients with IPMN experience symptoms including epigastric discomfort and/or pain and backache.[30,31] In addition, hyperamylasemia is often present for many years.[10,31] These acute pancreatitis symptoms are due to temporary partial or complete occlusion of the main pancreatic duct with viscid mucin. Persistent occlusion may result in pancreatic insufficiency, presenting with diabetes, steatorrhea, or both. Jaundice may ensue when viscid mucin obliterates the ampulla, when the IPMN is large enough to cause compression of the common bile duct, or when benign or malignant mural nodules involve the common bile duct and/or ampulla. However, two-thirds of patients with IPMN do not display any symptoms due to relatively inactive production of mucin and/or the location of the tumor in the tail portion of the pancreas. In cases like these, IPMNs may be discovered during routine workup for other diseases or at autopsy.

MACROSCOPIC FEATURES

IPMNs are usually classified into 3 types: main duct, branch duct, and mixed, according to the site and extent of involvement (Fig. 1).[32,33] The majority of IPMNs occur in the head of the pancreas. In our series of 58 patients, 35 (60%) tumors were located in the head of the pancreas, 9 (16%) in the body, and 14 (24%) in the tail.[8] Most, if not all, branch-type IPMNs are benign, while main duct– and mixed-type IPMNs are frequently malignant.

Branch Type

The branch-type IPMN affects one or more branches of the pancreatic duct, which consequently show cystic dilatation (Fig. 2). On inspection, a dilated branch in the periphery of the pancreas appears as a gray-colored, grapelike cluster. The dilated branch duct may contain solitary or multiple tumors and/or viscid mucin. The presence of large and high mural nodules indicates an increased likelihood of malignancy. In an analysis of 39 patients with IPMN who underwent endosonography and subsequent resection, Maguchi et al[33] reported that a branch-type IPMN should be excised when it is ≥25 mm in size and has mural nodules ≥6 mm in diameter and/or the main pancreatic duct is dilated to ≥7 mm. When the IPMN is large enough to cause compression of the main pancreatic duct, obstructive pancreatitis may result, and jaundice may develop when the compression affects the common bile duct. Especially when malignant, an IPMN in the head of the pancreas may cause a fistula in the duodenum or common bile duct. Likewise, a malignant IPMN in the body and/or tail of the pancreas may cause a fistula in the stomach and colon. Some pa-

FIGURE 2. Endoscopic retrograde pancreatogram of a branch-type IPMN originating from the Santorini duct.

tients have displayed multiple branch-type IPMNs distributed throughout the entire pancreas (Fig. 3).

Main Duct Type

A main duct–type IPMN is characterized by a diffusely or partially dilated main pancreatic duct filled with excessive mucin (Fig. 4). This type of IPMN occurs predominantly in the head of the pancreas and only occasionally in the tail. The inner surface of the dilated duct frequently contains mural nodules. Patients with marked dilatation of the main pancreatic duct (>1 cm) and mural nodules (>1 cm) face probable diagnosis of malignant IPMN.[22,30] Several years of main duct obstruction with viscid mucin or mural nodules may result in chronic pancreatitis, in which case the entire pancreas is markedly fibrotic.

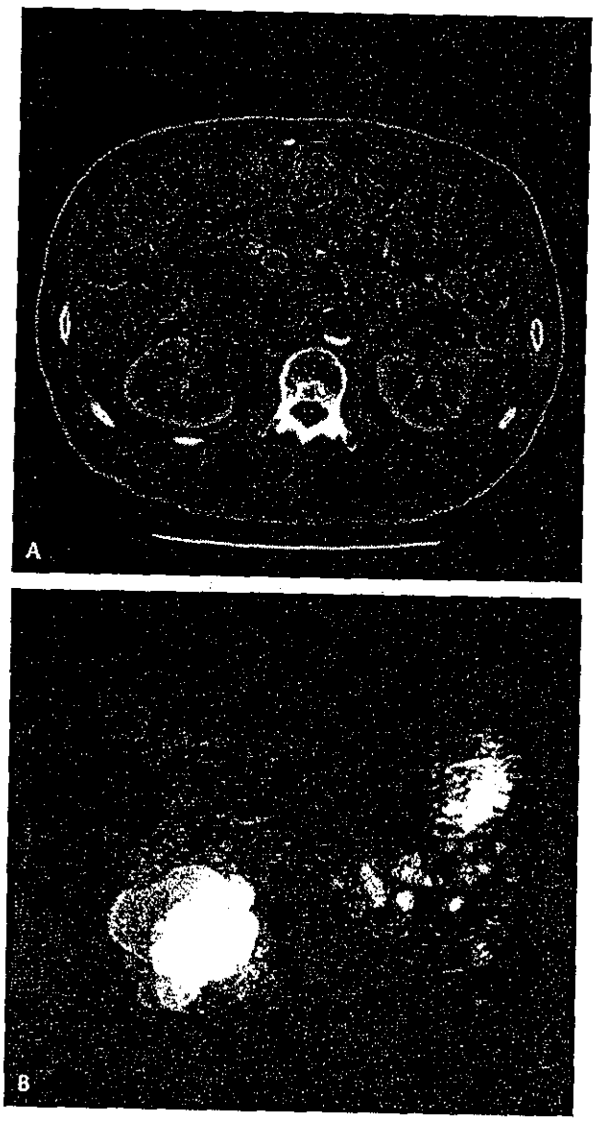

FIGURE 3. Multiple branch-type IPMNs in the head, body, and tail of the pancreas, as shown by CT (A) and magnetic resonance pancreatography (B).

FIGURE 4. Endoscopic retrograde balloon pancreatogram demonstrating a main duct–type IPMN. Mural nodules subsequently proved to be carcinoma (arrow).

When main duct–type IPMN is associated with invasive carcinoma, the gross appearance may not demonstrate the presence of the IPMN. Only on rare occasions does the pancreatic parenchyma around the main duct appear gelatinous.

Mixed Type

Any combination of the above 2 types of IPMN is designated as mixed-type IPMN. In mixed-type IPMN, in addition to the presence of branch-type IPMN, the main pancreatic duct contains papillary growth of columnar epithelia of various degrees of dysplasia that produce excessive mucin (Fig. 1). Mere dilatation of the main pancreatic duct due to excessive mucin production by the branch-type IPMN should not be designated mixed-type IPMN. Mixed-type IPMN is an advanced form of the branch type, in which the IPMN has spread to the main pancreatic duct, or an ultimate form of the main duct type, in which the IPMN has involved the branch ducts as well.

DIAGNOSIS

When the IPMN produces large enough amounts of mucin, the ampulla of Vater becomes enlarged and the orifice wide open (Fig. 5). It is this endoscopic finding that initially brought this disease to our attention: the characteristic, excessive secretion of mucin indicated the presence of IPMN.[21,22,34] This unique widening of the orifice is sometimes observed at the minor papilla and/or the major papilla. A growing awareness of this indication of IPMN has resulted in an increasing number of patients diagnosed during routine upper gastrointestinal endoscopy in Japan.

Serum levels of tumor markers are typically within the normal limits, but serum CEA and CA19-9 may be elevated in

FIGURE 5. A wide-open orifice of the ampulla of Vater in a patient with a branch-type IPMN.

the presence of IPMN. Ultrasonography, endosonography, and computed tomography (CT)[31,35] may reveal one or more cystic dilatations in the pancreas (branch type) or diffuse or segmental dilatation of the main pancreatic duct (main duct type) with or without polypoid lesions. Magnetic resonance pancreatography is the preferred method of use to visualize the entire outline of either main duct- or branch-type IPMN. Endoscopic retrograde pancreatography reveals any dilatation of the main pancreatic duct or branches with filling defects due to the presence of either mural nodules or mucin. Communication between branch-type IPMN and the main pancreatic duct is usually evident and was seen in 45 (85%) of 53 patients in our series.[8]

The diameter of the main pancreatic duct is generally larger in malignant cases of IPMN than in benign cases. Main pancreatic duct aspirate at ERCP may be positive for malignant cells by cytology and/or shows high levels of CEA and CA19-9.[17] The rate of positive transpapillary biopsy and cytology is <50%.[10,13] In a main duct IPMN, peroral pancreatoscopy may demonstrate the fish-egg appearance of a papillary neoplasm, granular or polypoid mucosa, or rough mucosa. In a branch-type IPMN, ERCP and/or peroral pancreatoscopy may reveal mucin in the main pancreatic duct. Full visualization of the ductal system and either the main duct or branch IPMN by ERCP is frequently difficult due to the presence of viscid mucin. Angiography and enhanced CT are helpful in determining extrapancreatic invasion and resectability of an invasive IPMN.

In the preoperative diagnosis of malignancy grade, the presence of a main duct IPMN, marked dilatation (>1 cm) of the main pancreatic duct, and the presence of a mural nodule(s) (>1 cm) are highly suggestive of malignancy.[30] The presence or absence of mural nodules or solid components may be preoperatively confirmed by endosonography, which is superior to any other diagnostic modality in this regard. Although it is still in the investigational phase, intraductal probe ultrasonography appears to be quite useful in the demonstration of mural nodules.[36] However, differentiation between mucin plugs and mural nodules remains difficult at times.

Differential Diagnosis

The IPMN is typically easily distinguishable from other tumors, such as nonneoplastic cysts, serous cystadenomas, solid-pseudopapillary tumors, acinar cell cystadenocarcinomas, and cystic endocrine tumors. However, differentiation between the IPMN and the mucinous cystic neoplasm still presents some complications. Distinction of IPMN from chronic pancreatitis is also of clinical significance, especially in patients with main duct-type IPMN.

Tumor cells of the mucinous cystic neoplasm have the same cytologic features as those of the IPMN. It is impossible, therefore, to distinguish the two by pancreatic juice cytology or even histology. The mucinous cystic neoplasm is predominantly found in middle-aged women in the tail of the pancreas. The patient may complain of nonspecific abdominal discomfort not akin to the symptoms of pancreatitis. Typically, the tumor appears as a spherical multilocular cyst with a considerably thick, smooth wall and does not show communication with the pancreatic duct. It has been proposed that ovarian-type stroma is a characteristic histologic feature of the IPMN tumor.[30,37,39]

A nonneoplastic mucosal change known as intraductal papillary hyperplasia possesses similar cytologic and histologic features as an IPMN. Intraductal papillary hyperplasia is a small lesion that usually affects the secondary branches and may produce similar dilatation of those branches. This hyperplasia may possibly be a precursor to an IPMN adenoma and should therefore be considered a component of an IPMN.

Association With Other Malignancy

Another unique feature of an IPMN is its association with malignancy in other organs. Not infrequently, patients with an IPMN have synchronous or metachronous malignancy in various organs. The rate of association of IPMNs with malignant neoplasms in extrapancreatic organs has been reported to range from 23.6% to 32%.[8,40,41] Yamaguchi and Tanaka[8] described nonpancreatic malignancies in 18 of 56 patients (32%) with resected IPMNs. Sugiyama and Atomi[40] reported that 15 of 42 patients (32%) with benign and malignant IPMNs had also nonpancreatic neoplasms including colorectal, gastric, and bile duct cancer before, at, or after surgery for an

IPMN. Osanai et al[41] reported 35 of 148 patients (23.6%) with malignancies of the colon (n = 11), stomach (n = 8), and lung (n = 5) before (n = 13), at (n = 12), or after (n = 10) diagnosis of an IPMN.

We previously reported a high prevalence of pancreatic cancer in approximately 10% of patients, independent of IPMN in those with branch-type IPMN.[42] Seven of 76 patients (9.2%) with resected IPMN had synchronous (n = 5), metachronous (n = 1), or both synchronous and metachronous (n = 1) occurrence of ordinary-type pancreatic cancer, independent of the IPMN. The presence of IPMN led to a diagnosis of concomitant ductal cancer in 4 of these 7 patients. The 7 patients included 2 with in situ carcinoma,[43] 1 with minimally invasive carcinoma, and 4 with invasive carcinoma. All 7 IPMNs associated with pancreatic cancer were branch-type adenomas with mild dysplasia and located in the head of the pancreas in 3 patients, in the pancreatic body in 2, and in the pancreatic tail in the remaining 2 male patients. Nakaizumi et al[44] also recently described the same combination of branch-type IPMNs and common-type pancreatic carcinoma in 5 male patients.

These phenomena may suggest that patients with IPMN may be genetically predisposed to the development of malignant tumors in a variety of organs, including the stomach, colon, breast, pancreas, and others. This may be biased, however, in part by the fact that some IPMNs are detected during follow-up of other, previously treated malignant tumors. Nonetheless, we should be well aware of the possibility that an IPMN may be an indicator of pancreatic cancer.[43] Increased knowledge of an IPMN and its relationship with pancreatic cancer may lead to an earlier diagnosis of pancreatic cancer and improved patient prognosis.

TREATMENT

The prognosis for patients with benign IPMNs and malignant but noninvasive IPMNs is excellent after complete resection.[15,45,47] Fortunately, only ~40% of all malignant IPMNs are invasive,[7] and the 5-year survival rate is ~80% even for individuals with malignant IPMN.[38] Nevertheless, inadequate and/or incomplete resection may lead to recurrence. The prognosis of an invasive malignant IPMN depends on its histologic type and the extent of invasion and metastasis.[1] In our series, the 3-year survival rate for 10 patients with malignant IPMNs was 48% after potentially curative resection.[8]

It has been reported that the malignant IPMN acquires aggressive behavior similar to that of common-type pancreatic carcinoma once it has invaded the pancreatic parenchyma.[48] In such an instance, adequate pancreatic resection should be performed with D1 plus alpha or preferably D2 lymph node dissection. However, D1 lymph node dissection is sufficient in most patients who have a malignant IPMN without massive parenchymal invasion.[37,49] Accurate preoperative or intraoperative determination of the degree of parenchymal invasion is a topic of current interest.

Branch Type

When the branch-type IPMN causes acute pancreatitis or other symptoms or when it possesses mural nodules that suggest malignancy, complete surgical resection should be performed. If the IPMN appears benign, as in the case of acute pancreatitis, a variety of limited pancreatic resections may be attempted.[50,57] A branch-type IPMN that is asymptomatic and without mural nodules may be followed up without resection. However, on rare occasions, even IPMN patients who do not show mural nodules have in situ carcinoma or minimally invasive carcinoma. In this regard, Maguchi et al[33] have recommended that a branch-type IPMN be excised when it is ≥25 mm in size and has mural nodules ≥6 mm in diameter and/or the main pancreatic duct is dilated to ≥7 mm. The same authors stated that the branch-type IPMN with mural nodules ≥6 mm in diameter is most likely carcinoma and that the IPMN showing branch dilatation ≥25 mm or main duct dilatation of ≥7 mm is frequently adenoma.[58] They emphasize the importance of the initial diagnosis in determining indications for surgery. When the branch-type IPMN is to be followed up without resection, they recommend meticulous patient screenings by CT or ultrasonography at 3, 6, and 12 months to prevent any potential rapid changes and yearly MRCP thereafter.

Main Duct Type

The main duct–type IPMN is frequently malignant and requires careful evaluation in regard to the presence of mural nodules, potential extraductal and extrapancreatic invasion, indications for surgery, and, finally, the appropriate area and extent of resection. Intraoperative sonography[59] and pancreatoscopy may be helpful in determining the site of resection. However, there may exist a discrepancy between the dilated ductal segment and the actual localization of IPMN cells,[2] and the IPMN may still affect the stump of resection. Intraoperative, frozen-section diagnosis must be adequately used, although diagnosis by frozen histology is not always accurate and definite. Some patients may necessitate total pancreatectomy to achieve complete resection; indications for this, however, must be examined very carefully because total pancreatectomy causes severe and permanent endocrine and exocrine pancreatic insufficiency. Because most IPMNs are slow growing and affect elderly people and the prognosis may be favorable even when the IPMN is malignant,[15,46] the benefits of such an aggressive treatment must be balanced against operative and postoperative risks.

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