TABLE 2. Initial Symptoms

Initial Symptom	TS1	TS2	TS3	TS4	TSX	Total
No symptom	142 (17.3%)	335 (8.8%)	138 (5.4%)	76 (3.7%)	48 (4.1%)	739 (7.1%)
Abdominal pain	206 (25.1%)	1079 (28.3%)	872 (34.4%)	800 (38.6%)	396 (33.9%)	3353 (32.2%)
Body weight loss	22 (2.7%)	154 (4.0%)	108 (4.3%)	82 (4.0%)	63 (5.4%)	429 (4.1%)
Nausea and vomiting	6 (0.7%)	58 (1.5%)	58 (2.3%)	43 (2.1%)	21 (1.8%)	186 (1.8%)
Malnutrition	49 (6.0%)	227 (6.0%)	153 (6.0%)	124 (6.0%)	86 (7.4%)	639 (6.1%)
Jaundice	173 (21.0%)	911 (23.9%)	433 (17.1%)	188 (9.1%)	132 (11.3%)	1837 (17.7%)
General fatigue	38 (4.6%)	187 (4.9%)	88 (3.5%)	57 (2.7%)	49 (4.2%)	419 (4.0%)
Abdominal tumor	0 (0.0%)	18 (0.5%)	21 (0.8%)	40 (1.9%)	12 (1.0%)	91 (0.9%)
Back pain	38 (4.6%)	175 (4.6%)	183 (7.2%)	182 (8.8%)	76 (6.5%)	654 (6.3%)
Exacerbation of diabetes	60 (7.3%)	238 (6.3%)	147 (5.8%)	151 (7.3%)	69 (5.9%)	665 (6.4%)
Unknown	88 (10.7%)	425 (11.2%)	332 (13.1%)	332 (16.0%)	217 (18.6%)	1394 (13.4%)
Total	822 (100.0%)	3807 (100.0%)	2533 (100.0%)	2075 (100.0%)	1169 (100.0%)	10,406 (100.0%)

(PL), and invasion of other organs (OO).[3] The lymph node metastasis (N factor) is divided into 3 groups, and the distant metastasis (M factor) includes the N3 lymph node metastasis.[3] The positive rate of each factor and the resulting stages in patients with TS1 pancreatic cancer are summarized in Table 5. Of 822 patients with TS1 pancreatic cancer, only 216 (26.3%) had T1 tumors because of invasion to adjacent organs. There is no cases of T2 (tumor >2 cm) in patients with TS1 pancreatic cancer by definition. It should be noted that 172 (20.9%) of the patients had invasion to the portal venous system, followed by arterial invasion, plexus invasion, and invasion to the other organs, factors that contribute to classifying the tumor as T4. There were 306 patients (37.2%) who had lymph node metastasis from TS1 pancreatic cancer, of whom 63 patients had distant lymph node metastasis. As a result, 81 patients (9.9%) had distant metastases. The frequency of liver metastasis and the peritoneal dissemination is as low as 1.2% and 1.5%, respectively. Of 822 TS1 pancreatic cancer patients, only 136 (16.5%) had stage I disease.

Tumor Staging and the Survival

Of 822 patients with TS1 pancreatic cancer, 799 (97.2%) underwent pancreatectomy (470 pancreaticoduodenectomy, 132 pylorus-preserving pancreaticoduodenectomy, and 141 distal pancreatectomy). Survival after pancreatectomy, according to the JPS T factor, is shown in Figure 2. When the same patients were classified according to the UICC classification system,[5] survival curves differed, as shown in Figure 3. UICC T4 means that the tumor has invaded the celiac artery or superior mesenteric artery, while JPS T4 does not necessarily mean arterial invasion. Even if the tumor size is ≤2.0 cm, the prognosis for patients with invasion to the adjacent organs is very poor. Figure 4 shows the influence of lymph node metastasis to the survival after pancreatectomy for TS1 pancreatic

TABLE 3. Imaging Modalities to Detect the Tumor for the First Time

Imaging Modality	TS1	TS2	TS3	TS4	TSX	Total
ERCP	144 (17.5%)	361 (9.5%)	202 (8.0%)	165 (8.0%)	117 (10.0%)	989 (9.5%)
Angiography	6 (0.7%)	17 (0.4%)	20 (0.8%)	29 (1.4%)	6 (0.5%)	78 (0.7%)
CT	157 (19.1%)	957 (25.1%)	752 (29.7%)	602 (29.0%)	291 (24.9%)	2759 (26.5%)
US	333 (40.5%)	1634 (42.9%)	1019 (40.2%)	732 (35.3%)	437 (37.4%)	4155 (39.9%)
PTCD	84 (10.2%)	399 (10.5%)	192 (7.6%)	130 (6.3%)	76 (6.5%)	881 (8.5%)
Other	52 (7.5%)	239 (6.4%)	201 (7.9%)	270 (13.0%)	143 (12.3%)	905 (8.8%)
Unknown	36 (4.4%)	200 (5.3%)	147 (5.8%)	147 (7.1%)	99 (8.5%)	629 (6.0%)
Total	822 (100.0%)	3807 (100.0%)	2533 (100.0%)	2075 (100.0%)	1169 (100.0%)	10,406 (100.0%)

ERCP, endoscopic retrograde cholangiopancreatography; CT, computed tomography; US, abdominal ultrasonography; PTCD, percutaneous transhepatic choledochal drainage.

TABLE 4. Sensitivities of the Tumor Markers (From Cases in 2001 and 2002)

Tumor Marker	TS1 (%)	TS2 (%)	TS3 (%)	TS4 (%)	TSX (%)	Total (%)
CA19-9	48.4	72.3	71.6	66.0	69.0	69.0
CEA	19.5	42.4	33.7	53.6	51.7	33.2
DUPAN-2	35.3	61.8	66.1	52.8	66.7	59.7
SPAN-1	47.6	80.2	88.2	65.4	71.4	78.1
Elastase-I	62.2	45.4	47.6	37.1	100.0	48.4

Sensitivities were calculated as the number of positive cases divided by the number of cases in which the tumor markers were precisely evaluated.

cancer. Though the 5-year survival reflects the N factor, the difference between each lymph node group (from N1 to N3) was not as statistically significant as the difference between N0 and N1. UICC N factor and distant metastasis are apparent prognostic factors (data not shown). The resulting JPS stage and survival after pancreatectomy are shown in Figure 5. There is an inversion of prognosis between stage II and III disease. In stage II disease with TS1 tumor, there should be an N1 lymph node metastasis that worsens survival. The same patients were classified by UICC staging system, as shown in Figure 6. There is a significant difference between the survival of patients with UICC stage IIa and those with UICC stage IIb disease. There was no statistical difference between the UICC stage IIb and UICC stage IV disease. These results indicate that TS1 pancreatic cancer is of real favorable prognosis value if the tumor is confined to the pancreas [CH (−), DU (−), S (−), RP (−), PV (−), A (−), PL (−), and OO (−)] with a median survival time of 78.2 months and a 58.1% 5-year survival rate. UICC stage Ia includes cases with intrapancreatic bile duct invasion [CH (+) and all other negative factors], resulting in a less favorable prognosis than that for JPS stage I. Since the majority of distant metastasis in TS1 pancreatic cancer means group 3 lymph node metastasis, as shown in Table 5, most of the patients with JPS stage IVb and UICC stage IV disease underwent pancreatectomy, and some of them survived >5 years. Figure 7 shows the survival rate of patients with stage IVa disease stratified by the tumor size. The survival rate of patients with TS1 pancreatic cancer is better than that of patients with larger tumors, even in the same stage.

DISCUSSION

The concept of "early" pancreatic cancer is still controversial. The biologically low-grade malignancy, intraductal papillary mucinous tumor (IPMT),[6,7] and mucinous cystic

TABLE 5. Extent of Disease in the 822 Patients with TS1 Pancreatic Cancer

T Factor		N Factor		M Factor		Stage	
T1	216 (26.3%)	N0	418 (50.9%)	M0	739 (89.9%)	I	136 (16.5%)
		N1	137 (16.7%)	M1	81 (9.9%)	II	24 (2.9%)
T3	320 (38.9%)	N2	106 (12.9%)	MX	2 (0.2%)	III	250 (30.4%)
T4	215 (26.2%)	N3	63 (7.7%)			IVa	162 (19.7%)
TX	71 (8.6%)	NX	98 (11.9%)			IVb	116 (14.1%)
						Unknown	134 (16.3%)
CH	373 (45.4%)			HEP	10 (1.2%)		
DU	132 (16.1%)			PER	12 (1.5%)		
S	156 (16.1%)			LYM	63 (7.7%)		
RP	177 (21.5%)						
PV	172 (20.9%)						
A	47 (5.7%)						
PL	52 (6.3%)						
OO	33 (4.0%)						

CH, distal bile duct invasion; DU, duodenal invasion; S, serosal invasion; RP, retropancreatic tissue invasion; PV, portal venous system invasion; A, arterial system invasion; PL, extrapancreatic nerve plexus invasion; OO, invasion of other organs; HEP, liver metastasis; PER, peritoneal dissemination; LYM, distant lymph node metastasis including N3.

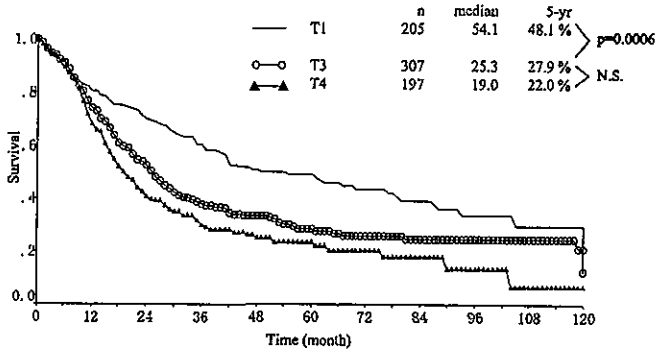

FIGURE 2. JPS T factor and the survival rate after pancreatectomy for TS1 pancreatic cancer. T1, tumor limited to the pancreas, ≤2 cm in greatest dimension; T3, tumor that has extended into any of the following: bile duct, duodenum, peripancreatic tissue; T4, tumor that has extended into any of the following: adjacent large vessels, extrapancreatic nerve plexus, other organs. N.S.: statistically not significant.

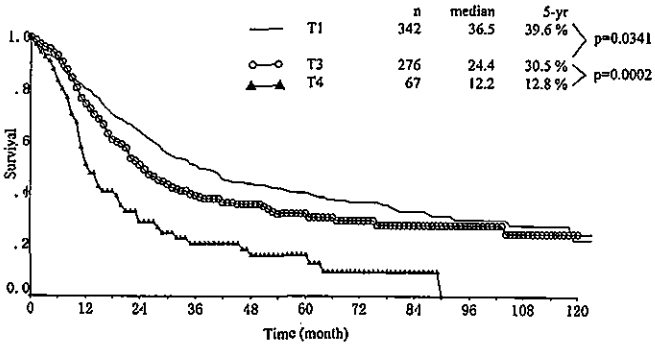

FIGURE 3. UICC T factor and the survival rate after pancreatectomy for TS1 pancreatic cancer. T1, same as JPS but including the tumor invading the bile duct; T3,: tumor extends beyond the pancreas but without involvement of celiac axis or superior mesenteric artery; T4, tumor involves celiac axis or superior mesenteric artery. N.S., statistically not significant.

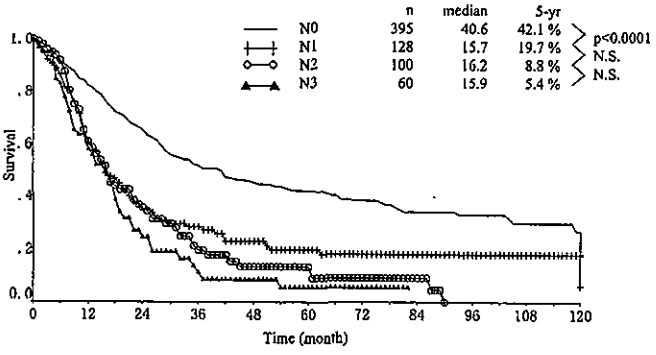

FIGURE 4. JPS N factor and the survival rate after pancreatectomy for TS1 pancreatic cancer. The grouping of the lymph nodes is described elsewhere.[3] N.S., statistically not significant.

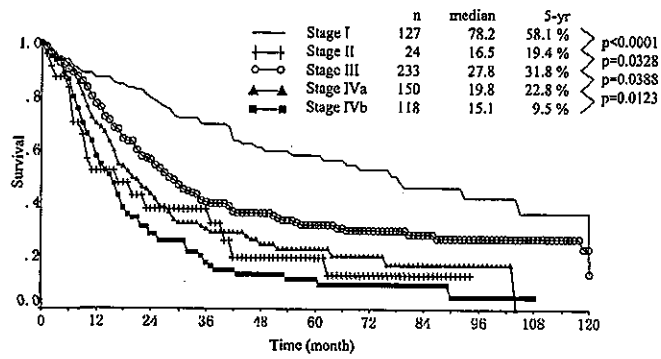

FIGURE 5. JPS stage and the survival rate after pancreatectomy for TS1 pancreatic cancer.

tumors[3] are clinical entities distinct from the common type of pancreatic cancer. Pancreatic intraepithelial neoplasia (PanIN),[8] atypical hyperplasia (PanIN-2), and carcinoma in situ (PanIN-3)[3] may be sequential lesions, but their contiguity to invasive cancer is still unknown. The present study is a retrospective multiinstitutional collection of TS1 pancreatic cancer. Compared with the larger tumor sizes, TS1 pancreatic cancer more frequently includes papillary adenocarcinoma and a well-differentiated type of tubular adenocarcinoma. There might be an important genetic change to make the cancer cells more undifferentiated and an aggressive type during the size progression.

To detect early pancreatic cancer, attention should be paid to the patients presenting with any type of abdominal pain, back pain, exacerbation of diabetes, and jaundice, as these symptoms can indicate pancreatic cancer. Tanaka et al[9] found that a slight dilatation (≥2 mm) of the main pancreatic duct seen by ultrasonography indicates a high risk of pancreatic cancer, with an odds ratio of 32.5 (95% confidence interval, 10.9–107.3). They reported that the slight dilatation observed >4 years before the resection of the pancreatic cancer was detected in 65% of 39 precancer patients, while the fre-

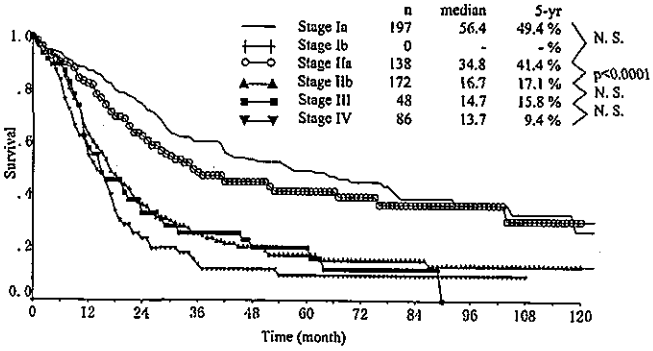

FIGURE 6. UICC stage and the survival rate after pancreatectomy for TS1 pancreatic cancer. N.S., statistically not significant.

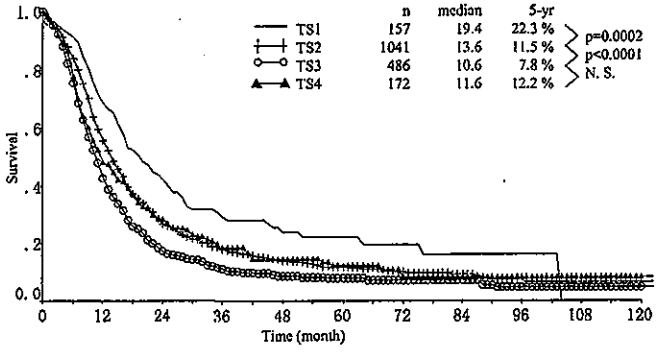

FIGURE 7. Survival rate of patients with stage IVa disease according to the tumor size. N.S., statistically not significant.

quency in the 10,244 age-matched control population was 5.35%. While sensitivities of the relevant tumor markers are not satisfactory, elastase-I may be a sensitive serum marker to detect TS1 pancreatic cancer. Satake et al[10] reported the sensitivity of elastase-I to be 27.8% in TS1 pancreatic cancer, but they measured elastase-I in only 7 cases (2 positive cases). It should be noted that elastase-I has very poor specificity, elevating to 100% in acute pancreatitis and to over one-third of those with chronic pancreatitis.

Surgical resection is the only way to obtain long-term survival,[4] survival being determined by the extent of the tumor. If there is invasion to the adjacent tissue, the survival rate worsens significantly. However, the survival rate of patients with TS1 pancreatic cancer is significantly higher than for those patients with larger tumors of the same stage, as shown in Figure 7. This may be due to the low frequency of liver and peritoneal metastasis and to the postoperative histologic confirmation of distant metastasis, especially N3 node metastasis, from TS1 pancreatic cancer (Table 5). Since the mortality and morbidity from pancreatectomy have been reduced greatly, TS1 pancreatic cancer should be resected other than for massive distant metastasis. Even in TS1 pancreatic cancer, protocols of chemotherapy and radiation therapy should be refined in adjuvant or neoadjuvant settings on a randomized, prospective trial basis. Unconventional modalities such as immunotherapy, gene therapy, and antiangiogenesis therapy, with or without conventional therapies, should be developed.[11]

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The Role of Chromosome 18 Abnormalities in the Progression of Pancreatic Adenocarcinoma

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Abstract: To date, the events that mediate tumor progression in pancreatic cancer are still poorly understood. Cytogenetic, allelotype, and somatic cell hybrid studies in human pancreatic adenocarcinoma have suggested that chromosome 18 may carry tumor suppressor genes (TSGs), including *SMAD4*. We previously identified that LOH of 18q at the *SMAD4* locus, along with LOHs on 17p and 12q, positively associated with poor prognoses of pancreatic cancer patients. However, restoration of the *SMAD4* gene did not suppress in vitro proliferation of pancreatic cancer cells that harbored homozygous deletion of this gene. An intraductal papillary mucinous neoplasm (IPMN) is thought to be one of the premalignant lesions of the pancreas that progresses to carcinoma. Although there were frequent LOH (7/14, 50%) at the *SMAD4* locus in IPMN samples, *SMAD4* protein was observed immunohistochemically in tumor cells, and no mutations of the *SMAD4* gene were observed, suggesting that it is the existence of a TSG in 18q, other than *SMAD4*, that suppresses cell growth. To functionally assess the activity of chromosome 18 in pancreatic cancer, we transferred a normal copy of the chromosome into pancreatic ductal carcinoma cells with and without completely inactivated *SMAD4*. In this study, in vitro growth of the hybrid cells was significantly suppressed compared with the parental cells, regardless of the initial *SMAD4* status. To estimate the metastatic ability of the hybrids, we used a lung colonization model. At the end of the experiment, there was significant suppression of the number of surface metastases developing in mice injected with hybrids in comparison with those injected with parental cells. To identify and characterize genes that are involved in the progression of pancreatic cancer, we used microarray expression analysis employing a 20k oligo-array system. It was revealed that there was increased expression of 4 genes relating to apoptosis in the 18 chromosome hybrids cells compared with the parental cells. We are now analyzing the function of these genes.

Key Words: pancreatic cancer, chromosome 18, SMAD4, chromosome transfer, microarray

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In Japan, there are about 20,000 newly diagnosed cases of pancreatic adenocarcinoma and an approximately equal number of deaths per year from pancreatic adenocarcinoma. It is the fifth leading cause of cancer-related death. Recent progress in medicine has identified cancers as diseases involving an accumulation of genetic alterations of oncogenes and tumor suppressor genes (TSGs). At the preliminary clinical detection of cancer, tumor cells have already accumulated several genetic alterations. In the case of pancreatic cancer, K-*ras*,[1] *TP53*,[2] *MTS1*,[3] and *SMAD4*[4] have been considered to be molecules that play key roles in tumorigenesis. To develop an effective therapeutic intervention for patients with pancreatic cancer, it is necessary to understand which mutated gene(s) is(are) important in terms of clinical features and prognosis. Ductal adenocarcinoma is the most common form of pancreatic cancer. Early detection of small, resectable lesions may improve the outcome of pancreatic cancer,[5] but the optimal approach for early detection of this deadly disease is unknown and still under investigation. Acquisition of efficient approaches and markers that accurately detect the earliest genetic stages of pancreatic cancer should be a priority. Despite the continuous progress in molecular biology, the genetic events involved in the initiation and progression of ductal adenocarcinoma of the pancreas remain largely unclear. SMAD proteins are distributed in the cytoplasm and nucleus. The *SMAD4* gene, located on chromosome 18q21, which is also a putative location for other TSGs,[6] is a recent addition to the group of known TSGs. *SMAD4* is inactivated in one-half of pancreatic carcinomas,[7] which we have previously found to be associated with poor patient prognosis and tumor progression.[8] Cytogenetic, allelotype, and somatic cell hybrid studies in human cancers have suggested that 18q may carry a TSG including

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SMAD4 that plays a role in the carcinogeneses of colorectal or prostate cancer.[9,10] This evidence has encouraged us to pay particular attention to this area of pancreatic cancer. This paper aims to summarize the results that we obtained from studying the implications of chromosome 18 abnormalities in the progression of pancreatic carcinoma.

ALLELOTYPE STUDIES

The molecular pathology of pancreatic carcinogenesis may be characterized by a broad spectrum of distinct gene mutations and chromosomal alterations, of which some seem to be nonrandom. Oncogene K-*ras* and tumor suppressor genes such as *TP53*, *MTS1*, and *SMAD4* have been observed abnormal in many pancreatic cancers.[11,12] Loss of heterozygosity (LOH) is one of the most important mechanisms for inactivation of TSGs. We have analyzed LOH in pancreatic cancer and observed frequent LOH in a number of chromosomal loci.[13] LOH of ≥30% was observed on chromosome arms 18q (43%) and additionally for 17p(47%), 12q (34%), and 6q (30%). Furthermore, this relatively high frequency of LOH for 18q is clearly associated with a poor prognosis for patients with pancreatic cancer.[8] It is important to clarify the mechanism of how 18q abnormalities are involved in the progression of pancreatic cancer to develop a novel therapeutic strategy. There may be 2 or more genes that are integral in pancreatic carcinogenesis: one that is involved in the initial step of carcinogenesis and others that have roles in progression. Several genes, such as *DCC*, *SMAD2*, and *SMAD4*, map closely on 18q21.[11,14] Homozygous deletion was also found in 18q22, which suggests the existence of TSGs other than *SMAD4*. In our previous study, loss of 18q in the vicinity of *SMAD4* was observed in mucin-producing pancreatic tumors and cystadenomas, both putative pancreatic premalignant lesions, at a frequency similar to that observed in pancreatic cancer.[15] Furthermore, an intraductal papillary mucinous neoplasm (IPMN) is thought to correspond with one of the premalignant neoplasms of the pancreas: IPMNs often harbor sequential dysplastic epithelial lesions, from mild dysplasia to severe dysplasia/carcinoma in situ as well as invasive carcinoma.[16,17] We detected frequent LOH (7/14, 50%) at the *SMAD4* locus in IPMN[15]; however, no mutations of *SMAD4* were observed in any of the tumors, and the level of expression of the SMAD4 protein was not reduced in IPMNs. These results strongly suggested that: (1) the loss of 18q is an early event in pancreatic carcinogenesis; (2) an inactivating mutation of the *SMAD4* gene is a rather late genetic change during pancreatic carcinogenesis; and (3) the loss of 18q with 12q and 17p is the prognosis-regulating factor.

SMAD4 RESTORATION IN PANCREATIC CANCER

SMAD proteins are distributed in the cytoplasm and nucleus. The *SMAD4* gene is located on chromosome 18q21, a putative location for other TSGs.[18] The gene is inactivated in half of pancreatic carcinomas,[19] and as we reported previously, *SMAD4* inactivation is associated with poor patient prognosis and tumor progression.[8] SMAD4 interacts with specific SMAD proteins to interfere with the cell cycle, and it plays a pivotal role in embryonic development via distinct transforming growth factor-β (TGF-β)–signaling pathways.[20] More specifically, these pathways function by SMAD4 heterodimerization with activated SMAD2 or its isoform, SMAD3, on TGF-β receptor type II via TGF-β_1) or activin phosphorylation, whereas SMAD1/5/8 act downstream of the bone morphogenic proteins (BMPs) and associate with SMAD4. The resulting complexes translocate into the nucleus, where they regulate transcription of certain genes.[20] In adenocarcinoma, malignant transformation of epithelial cells is the result of a sequence of oncogene and TSG mutations.[21] An interesting finding constitutes the fact that the highly frequent activating mutation of K-*ras* occurs early in gastrointestinal cancer and is reported to jointly disrupt the TGF-β antiproliferative responses incurred by SMAD4 silencing, a later event,[22] through the inhibition of the SMAD2/3 function.[19] The concomitant presence of these 2 mutations may explain why *SMAD4* restoration in some tumor cells did not rescue the TGF-β antiproliferative and gene responses.[19] The simultaneous presence of 2 distinct TGF-β inhibitory hits also suggested that *SMAD4* has pleiotropic effects, which result in its alternative role in vivo and accounts for the suppression of tumor growth. One such effect was recently reported, implicating *SMAD4* for the first time as an inhibitor of pancreatic tumor angiogenesis and identifying thrombospondin-1 and vascular endothelial growth factor (VEGF) as relevant tumor targets.[23]

In our recent study, we investigated both the physiology and the molecular mechanism associated with *SMAD4* inactivation in variety of pancreatic tumor cell lines to better understand the role of *SMAD4* in the progression of this disease.[24] The *SMAD4* gene was restored by adenovirus vector into pancreatic cancer cell lines. It was shown that *SMAD4* did not affect proliferation of cells in vitro, nor did it change the specific gene activation in the pancreatic adenocarcinoma cell lines (Fig. 1). Since the stimulation of TGF-β is mediated by phosphorylation of the type I receptor, activin receptor-like kinase (ALK) 5, the adenovirus vector encoding the active form of ALK5 was infected into pancreatic cancer cells to exclude the possibility of abnormal function of the TGF-β receptor. The in vitro growth curve was not changed after ALK5 induction, when compared with the control curve. It is suggested that *SMAD4* induces G1 cell cycle arrest and apoptosis in SMAD4-null breast and colon cancer cells[25,26]; this pathway is not functional in certain pancreatic tumor cells. On the contrary, a human xenograft tumor model in immunodeficient (SCID) mice showed significant inhibition of in vivo tumor growth by SMAD4. We assessed the growth and the angiogenic response of the human pancreatic tumor in SCID mice, as well as the expression of various factors associated with invasion and lo-

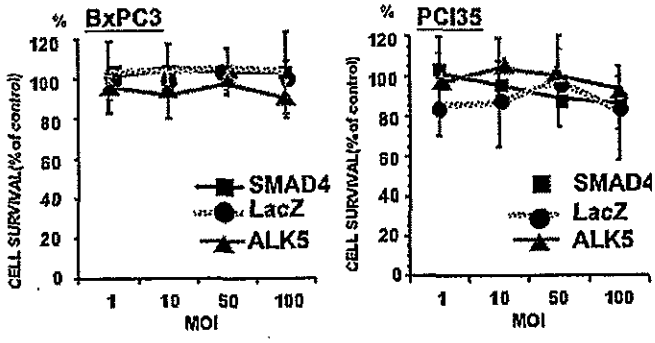

FIGURE 1. SMAD4 or activin receptor-like kinase (ALK) 5 was induced by adenovirus vector into pancreatic cancer cell lines (BxPC3 and PCI35). The cell survival ratios were shown at different multiplicity of infection (MOI) of adenovirus vector. *SMAD4* restoration through efficient adenoviral transfection failed to restore the TGF-β–induced growth inhibition of pancreatic adenocarcinoma cells, as no significant cytotoxic effect was found using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay in comparison with the control (LacZ).

cal angiogenesis balance in these tumors and in primary human tumors. It was demonstrated that adenoviral transfer of *SMAD4* to *SMAD4*-null cells restored its expression and function and correlated with the suppression of angiogenesis (Fig. 2) and invasion in a human xenograft tumor model in SCID mice. Matrix metalloproteinases, the gelatinases in particular, have been found to be involved in tumor growth and invasion.

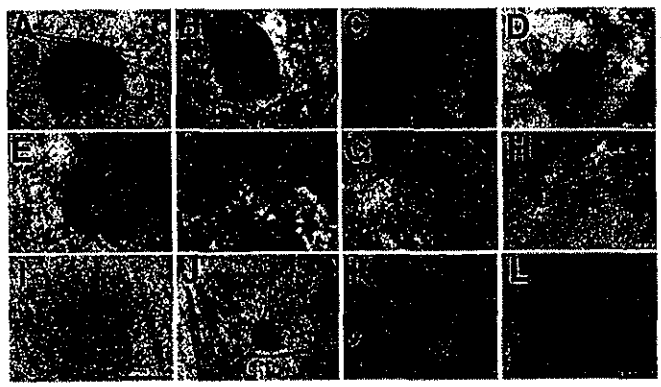

FIGURE 2. Illustration of angiogenesis inhibition in the dorsal transparent chamber of PK-1/SMAD4 (upper panel) cells, in contrast to the PK-1/wild-type and PK-1/LacZ cells (lower panel). The xenograft implantation on day 0 (A, E, and I, respectively) on the striated muscle in the dorsal skinfold chamber was monitored in terms of growth and angiogenesis. Images captured at days 7 (B, F, and J, respectively), 14 (C, G, and K, respectively), and 21 (D, H, and L, respectively) showed the initiation of angiogenesis and the neovasculature network organization surrounding the pellet of wild-type PK-1 cells and LacZ-transfected PK-1 cells, but not SMAD4-expressing PK-1 cells [bar = 1 mm (I)].

In addition, recent evidence suggests that the ETS-1 transcription factor is a key regulator of vasculogenesis and angiogenesis. ETS-1 is expressed in adult lymphocytes as well as in mesoderm lineage including endothelial cells during embryogenesis. Moreover, it is reported that its induction is a mutual phenomenon in response to proangiogenic factors, regulating the expression of VEGF receptors Flt-1[27] and Flk-1 (through interaction with HIF-2α[28] as well as the local proteolytic balance and migration of cells).[29,30] Over the past several years, ETS-1 expression has been documented in a variety of carcinoma cells, including pancreatic, ovarian, and colorectal,[30–32] and it has consistently correlated with tumor invasion and poor prognosis. The silencing of *SMAD4*, a late event in pancreatic carcinoma, can thus be associated with the "switching" on of an invasive and angiogenic phenotype that results in tumor growth, spreading, and metastasis. A molecular understanding of the complex involvement of the frequent *SMAD4* deletion in pancreatic malignancies is crucial because the poor prognosis of patients with these diseases emphasizes the need for new diagnostic and therapeutic approaches.

CHROMOSOME 18 TRANSFER

Chromosome 18 Aberration in Pancreatic Adenocarcinoma Cells

Data from previous studies[13,33,34] clearly indicate that the majority of pancreatic cancer cell lines harbor chromosomal abnormalities, including those of chromosome 18. Hence, we performed dual-color fluorescence in situ hybridization (FISH) analysis on metaphase chromosomes[35] to monitor the status of chromosomes 18. Furthermore, it was necessary to confirm whether these cells could be used as chromosome 18–defective recipients in our functional study.[18] Two different regional probes were used on 18q12 and 18q22, in addition to the chromosome 18–specific centromere probe *L1.84*. In concurrence with previous work, FISH indicated a loss at the locus corresponding to RMC18P012 on 18q22 (which maps closely to *SMAD4*) in >90% of the nuclei counted. Moreover, these cells featured different hyperploid patterns but nevertheless may be considered a useful model of chromosome 18–defective cells.

It has been proposed that the K-*ras* mutation detected in pure pancreatic juice[36] or in stools[37] may be considered a tool for early detection of pancreatic cancer. Since such mutations are also detected in chronic pancreatitis, however, it would be difficult to use this test in population screening. Therefore, we used FISH to analyze the status of 18q in pancreatic juice samples collected from 32 patients with various pancreatic disorders. The cells were isolated from pancreatic juice and subjected to dual-color FISH analysis[35] using 18q-specific probes. The sequence of chromosome 18 was apparently intact in 13 specimens from individuals who had no neoplastic lesions. Subsequently, 12 specimens (63%) from the remaining 19 pa-

tients with neoplastic lesions showed a loss of 18q. We detected chromosomal loss at 18q, although routine cytologic examination and endoscopic retrograde cholangiopancreatography (ERCP) failed to identify any abnormalities in the patients. FISH results and high CA19-9 values prompted us to perform endoscopic ultrasonography (EUS), which revealed a small serous cystadenoma. Thus, the loss of 18q, as detected by FISH, appears to be (1) a useful diagnostic tool for detecting pancreatic tumors, particularly in patients who are not well diagnosed by conventional methods and (2) one of most striking early genetic alterations in pancreatic ductal carcinogenesis.

Suppression of Tumorigenesis by Chromosome 18 Transfer

Several somatic cell hybrid studies of human cancers have suggested that 18q may carry a TSG(s) that plays a role in the carcinogenesis of colorectal or prostate cancer.[9,10] Employing various molecular techniques in several studies, we had consistently found that the loss of 18q is an early genetic event in pancreatic carcinogenesis: LOH of 18q was observed in >90% of pancreatic carcinomas.[38] Therefore, functional evidence involving hybrids containing normal copies of chromosome 18 was desirable. We conducted the following experiments using the microcell hybridization technique to transfer a single chromosome from a normal somatic cell into a human cancer cell line [microcell-mediated chromosome transfer (MMCT)]. To functionally assess the activity of chromosome 18 in pancreatic cancer, we introduced it as a normal copy into pancreatic ductal carcinoma cells with and without completely inactivated *SMAD4*. This method has been proven useful in providing functional evidence of the chromosome location of the TSGs in a variety of cancers, including melanoma,[39] Nijmegen breakage syndrome,[40] and prostate cancer.[10] We first performed MMCT and then confirmed the efficiency of the chromosome transfer using microsatellite and FISH analyses.[18] In our previous study, adenovirus-mediated transfer of *SMAD4* did not suppress in vitro growth of pancreatic cancer cells. On the contrary, in vitro growth of the hybrid cells was significantly suppressed compared with the parental cells, regardless of the initial *SMAD4* status. The latter finding suggests that a product of the gene on chromosome 18 other than *SMAD4* plays a role in this behavior. Additionally, the hybrid cells exhibited a significant decrease in terms of colony formation and invasiveness through the matrigel-reconstituted basement membrane, as compared with parent cells. Since the ability of cells to grow in the soft agar suspension very closely correlated with their tumorigenic potential in vivo,[41] these features strongly suggest that chromosome 18 transfer is associated with major changes in tumor cell behavior. We then assessed the in vivo tumorigenic abilities of each hybrid containing an intact copy of chromosome 18. Despite the inherent interclonal variation, the hybrids showed a significant reduction in tumor volume and a longer latency, in comparison with the parent cells. Our present results provide strong functional evidence of the existence of additional TSG(s) located on chromosome 18 that are distinct from *SMAD4*.

Chromosome 18 Encodes Significant Antimetastatic Activity

Because chromosome 18 has been proven to harbor a cluster of candidate tumor and metastasis suppressor genes, ie, *SMAD2*, *SMAD4*, *DCC*, *maspin*, and *PAI-2*, and because poor prognosis is significantly associated with 18q LOH,[8] it is with great interest that we examined whether genes on chromosome 18 play a role in metastatic processes. To estimate the metastatic ability of the hybrids, we used a lung colonization model.[42] At the end of experiment, there was significant suppression of the number of surface metastases developing in mice injected with hybrids compared with those injected with parent cells. Furthermore, microscopic examination revealed that animals injected with hybrid cells developed a significantly lower number of micrometastases and only very rarely developed macrometastases, in contrast to the mice injected with parent cells. This demonstrates that 18q encodes an important metastasis-suppressor activity and confirms our previous study that clearly correlated LOH of 18q with the poor prognosis of patients with pancreatic cancer. Chromosome 18 transfer induces an inhibitory effect that prevents micrometastases from forming macrometastases. It is speculated that this dormant status is induced not only by the decreased proliferation and increased apoptosis in cancer cells but also by the inhibitory suppression of angiogenesis and invasion in a tumor microenvironment.

Somatic hybrid segregants that underwent nonrandom chromosomal losses were shown to reexpress high tumorigenic and metastatic ability,[43] demonstrating that there are certain gene(s) that when expressed can suppress the metastatic ability of prostatic cancer cells. In this study, spontaneous deletion of portions of human chromosome 11 in some of the clones delineated the minimal portion of human chromosome 11 capable of suppressing prostatic cancer phenotypes. Although the various clones were kept culturing, we could not find such revertant hybrids that show the same tumorigenesis as parent cells. However, we can speculate that either our hybrids are more stable or losses of 18q are required in the last steps of pancreatic tumorigenesis. Although this study clearly implicates the important role of gene(s) on chromosome 18, the precise subchromosomal localization of the metastasis suppressor gene(s) remains in question. In the absence of spontaneous revertant hybrids, the precise localization and identification of a putative metastasis suppressor gene will require further efforts.

Oligonucleotide Microarray Analysis

DNA microarrays provide a powerful means with which to query the relative transcript abundance of many genes in

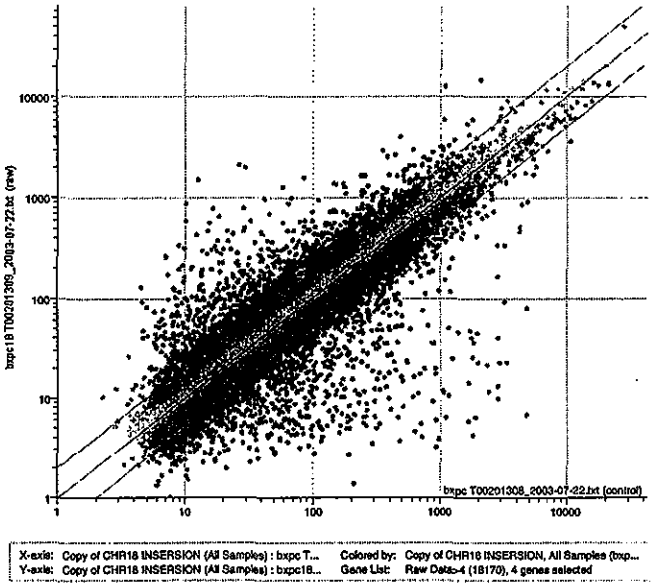

FIGURE 3. A scatter graph showing the differences between a chromosome 18 transfer cell and parent cell, BxPC3, in the expression of 20,000 genes. Four (in black) of 400 genes relating to apoptosis showed more than twofold higher expression after chromosome 18 transfer.

parallel. Array fabrication can be accomplished using in situ synthesis or deposition methods, and nucleic acids on the array may be either oligonucleotides or amplified cDNAs. Coupled with informatics tools, the microarray approach provides valuable insights into the areas of target discovery, mechanisms of drug action, genes and pathways involved in various cellular responses and pathophysiologies, exon mapping, and tumor classification. Because it has been reported that the differential expression ratios obtained with CodeLink expression platform were validated against those obtained with quantitative reverse transcription PCR assays for 54 genes,[44] we employed an oligonucleotides microarray system of CodeLink UniSet Human 20K I Bioarray (Amersham Biosciences, UK) in this experiment. There were approximately 20,000 probes of genes hybridized on one slides. We used total RNA to prepare cRNA and conducted oligonucleotide microarray analysis using CodeLink according to the manufacturer's instructions. Processed slides were scanned using an Axon GenePix 4000 Scanner and GenePix Pro Software (Axon Instruments, Foster City, CA). These data were analyzed by GeneSpring 6 software (Silicon Genetics, Redwood City, CA). The differences in mRNA expression profiles between the hybrids and parent cells were compared in 2 pancreatic cancer cell lines. We confirmed elevated expression of *SMAD4* in BxPC3 H(18) (chromosome 18–transferred cell line) in comparison with BxPC3, which has a homozygous deletion of *SMAD4*. The 400 genes involved in the mechanism of apoptosis were included in this system. The expression of 4 of these 400 genes was elevated in BxPC3 H(18) and in MIAPaCa2 H(18), more than twofold higher than in the parent cells. These 4 genes are colored in black on the scatter graph comparing BxPC3 and BxPC3 H(18) (Fig. 3).

CONCLUSION

The complexity of the processes associated with malignant tumor development and metastasis is a major hurdle in attempting to treat and cure patients with pancreatic cancer. Overall, the results of our study suggest that (1) loss of 18q is an early event in pancreatic carcinogenesis, (2) inactivating the *SMAD4* gene is a rather late genetic change in pancreatic carcinogenesis, (3) losses of 12q, 17p, and 18q are the prognosis-regulating factors, and (4) introduction of chromosome 18 induces significant suppression of both the tumorigenic and metastatic phenotypes. The precise localization and identification of a putative metastasis suppressor gene on chromosome 18 will require further efforts. These results will increase the possibility of improving a genetic diagnosis that provides additional information on prognosis and leads to better clinical management of patients with pancreatic cancer.

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Excerpta Medica

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Functional Analysis of Chromosome 18 in Pancreatic Cancer: Strong Evidence for New Tumour Suppressor Genes

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Original Article

Functional Analysis of Chromosome 18 in Pancreatic Cancer: Strong Evidence for New Tumour Suppressor Genes

Liviu P. Lefter,[1,2] Makoto Sunamura,[1] Toru Furukawa,[2] Toshimasa Yastsuoka,[1] Hisashi Abe,[1] Hiroko Inoue,[1] Tadayoshi Abe,[1] Shinichi Egawa,[1] Koh Miura,[1] Rina Morita,[1] Akira Horii[2] and Seiki Matsuno,[1] Departments of [1]Gastrointestinal Surgery and [2]Molecular Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan.

BACKGROUND: In a previous work, we demonstrated that loss of heterozygosity of 18q is a frequent event significantly associated with poor prognosis in pancreatic cancer. We hypothesized that restoration of heterozygosity of chromosome 18 in pancreatic cancer cells would reduce their tumorigenicity. This study was intended to provide functional evidence for the existence of new tumour suppressor gene(s) located on chromosome 18.

METHOD: Restoration of heterozygosity was achieved by introducing a normal copy of chromosome 18 into pancreatic ductal carcinoma using a microcell-mediated chromosome transfer technique. The tumorigenicity and metastatic ability of both the parental cells and resulting hybrids were assessed *in vitro* and *in vivo*.

RESULTS: *In vitro* growth of hybrid clones was significantly delayed compared to parental cells. This was paralleled by a significantly lower rate of promoting invasive carcinoma in nude mice and a longer latency with hybrid cells compared with parental tumour cells. Hybrid clones showed significant suppression in the number of surface lung metastases when compared with parental cells.

CONCLUSION: These data represent strong functional evidence that chromosome 18q encodes strong tumour and metastasis suppressor activity that is able to switch human pancreatic cancer cells to a dormant phenotype. [*Asian J Surg* 2004;27(2):85-92]

Introduction

Tumour suppressor genes are attractive candidates as markers for early genetic diagnosis because, by definition, their loss should be followed by switching to a tumorigenic phenotype. The molecular pathology of pancreatic carcinogenesis is characterized by a broad spectrum of distinct gene mutations and chromosomal alterations, some of which seem to be non-random. Recent advances in molecular biology have increased our understanding of the pathophysiology of, and the frequent genetic alterations in, this disease.[1]

Many studies employing different molecular techniques have consistently outlined the loss of chromosome 18q as an early event in pancreatic carcinogenesis. Chromosome 18 harbours a cluster of either tumour or metastasis suppressor genes such as *SMAD2*, *SMAD4*, *DCC*, *maspin* and *PAI-2*. Loss of heterozygosity (LOH) of 18q is a common event in more than 90% of pancreatic carcinomas, while only 50% are characterized by bi-allelic inactivation of the *SMAD4* genes.[2,3] This high frequency of losses affecting 18q caused special interest in this region. We reported the loss of 18q in 92% of pancreatic juice samples collected from patients with clinically early pancre-

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atic neoplasia and emphasized this approach as a useful tool in early detection of this deadly disease.[4] In a recent study, we found that LOH of 18q is significantly associated with a poor prognosis in pancreatic cancer.[5] Loss of *SMAD4* expression occurs biologically late in neoplastic progression; therefore, even when clinically early infiltrating pancreatic cancers are detected, they could, in fact, be considered genetically late.[6,7] On the other hand, adenovirus-mediated transfection with *SMAD4* inhibits mouse tumorigenesis by halting angiogenesis but fails to inhibit *in vitro* growth of pancreatic ductal adenocarcinoma cells with completely inactivated *SMAD4*.[8,9]

To date, there is no strong evidence that implicates *SMAD2* or *MADH2*, another candidate tumour suppressor gene residing on chromosome 18q, in colon and other cancers; they are inactivated only in a small fraction, accounting for 5% of colon and other cancers.[10] Loss of *DCC* expression was initially found to be involved in colon cancer,[11] where it could play a role in tumour progression. However, only one recent study reported inactivation of *DCC* by homozygous deletions in a subset of pancreatic and biliary cancers, as an isolated event or in addition to *SMAD4* alterations.[12]

Maspin is initially expressed in normal human mammary and prostate epithelial cells, but is down-regulated during cancer progression. *Maspin* inhibits cell motility, invasion and metastasis in breast and prostate cancers.[13] *Maspin* expression is up-regulated in pancreatic cancer, in contrast to its absence in normal pancreatic tissue.[14,15] However, mutations in these genes alone cannot explain the whole process of pancreatic carcinogenesis; there may be some other genes that play important roles. We have focused on tumour suppressor genes in pancreatic cancer to find effective methods for genetic diagnosis and/or treatment. Given that a few different known and probably unknown tumour and metastasis suppressor genes are clustered on 18q, we presumed that correction of these defects could restore quasi-normal growth status to pancreatic cells.

This study was undertaken to provide functional evidence for the existence of new tumour suppressor genes located on chromosome 18 that play a role in pancreatic tumorigenesis.

Materials and methods

Cell lines

Two-pancreatic cancer cell lines, PK-1 (established in our department from liver metastasis of pancreatic cancer)[16] and Panc-1 (American Type Culture Collection, Rockville, MD, USA), were cultured according to the protocols of the suppliers. The cell lines are well characterized.[17]

Briefly, PK-1 is homozygous for deletion of *SMAD4* (*SMAD4*$^{-/-}$), whereas Panc-1 expresses normal *SMAD4* (*SMAD4*^{wt}) (Table). For each cell line, three stable hybrids containing a normal copy of chromosome 18 (detected by fluorescence *in situ* hybridization, FISH, using mouse DNA as probes) were established: PK-1H(18)-1 through -3 and Panc-1H(18)-1 through -3, respectively. These hybrids were generated by the microcell-mediated chromosome transfer (MMCT) technique and expanded in medium containing 400 μg/mL of G418, as previously described.[18-20] The A9H(18) mouse fibroblast cell line, which carries a single copy of human chromosome 18 and an integrated neomycin resistance gene, was maintained in the same selective medium. All cells were routinely monitored for mycoplasma as well as for mouse hepatitis, Sendai and pneumonia viruses, and were consistently negative.

Table. Oncogenic properties of stable hybrid cells

	PK-1	PK-1H(18)	Panc-1	Panc-1H(18)
Colony formation (number/3 cm dish)*†	228 ± 19.36	49 ± 3.61	196 ± 18.21	34 ± 2.89
Colony size (μm)*†	392 ± 39.22	106.4 ± 19.61	293 ± 32.93	32 ± 29.66
Apoptosis index (%)†‡	2.1 ± 0.5	3.9 ± 0.7	1.9 ± 0.3	4.1 ± 0.5
Tumour size (mm^3 at day 45)†	1945 ± 245	366 ± 98	756 ± 168	142 ± 39
Latency period (d)†§	8 ± 2	14 ± 3	9 ± 3	18 ± 3
Lung metastatic lesion (number/lung)†‖	51 ± 11.8	5.2 ± 2.6	29 ± 9	6 ± 3.4
Proliferation index (PCNA-positive cells/x 40 field)†	57 ± 11.6	16 ± 5.1	42 ± 8.3	9 ± 3.4
SMAD4 status of parental cells	*SMAD4*$^{-/-}$		*SMAD4*$^{+/+}$	

*Colony number and size were measured and averaged from three randomly chosen photographs from each plate; †averaged results ± standard deviation from three independent experiments; ‡apoptotic cells were detected by annexin V/EGFP staining; §latency period is defined as time course when tumours became palpable (reached 4–5 mm in diameter); ‖surface lung metastatic tumours counted and microscopically confirmed on day 30. PCNA = proliferating cell nuclear antigen.

Microsatellite analysis

Genomic DNA from cells and nude mouse tumours was extracted using the Nucleon BACC3/ST kit (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England) and analysed using highly polymorphic microsatellite markers located on 18q, as described previously.[20,21]

Briefly, seven microsatellite markers, D18S1104, D18S463, D18S72, D18S35, D18S1144, D18S483 and D18S58 (http://gdbwww.gdb.org), are spaced at approximately 10 cM intervals (mean, 9.96 cM) along the long arm of chromosome 18. Microsatellite primers were designed to amplify approximately 100 bp to 150 bp products. The forward primers of each pair were end-labelled with ^{32}P-g-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). Polymerase chain reaction (PCR) amplification consisted of initial denaturation at 95°C for 2 minutes, 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 60–62°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. The PCR products were separated in 8 M urea-polyacrylamide gel and autoradiographed. For each marker, PCR amplification was carried out at least twice.

In vitro proliferation

Anchorage-dependent proliferation was monitored using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for 5 days, in the absence of G418, and a daily proliferation index (PI) was calculated for each parental and corresponding hybrid cell line.[22] Conversion of MTT to formazan dye was measured using absorbance at 590 nm in a multi-well plate immunoreader system. Data from three independent experiments were pooled, averaged and analysed.

Colony formation assay

Ten thousand cells were plated in 1 mL medium containing 0.3% Bacto-agar (Becton Dickinson, Sparks, MD, USA) with 10% fetal bovine serum as an upper layer into 30 mm dishes. Another 1 mL medium with 0.7% Bacto-agar was used for the bottom layer. After 2 weeks, viable colonies were stained red by incubation for 3 hours with 0.3 mL of 1 mg/mL 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride (INT; Dojindo Laboratories, Kumamoto, Japan). Red colonies were photographed using a Zeiss microscope (Carl Zeiss, Jena, Germany) with ×5 objective. Both the number and size of colonies were measured and averaged on three randomly chosen photographs from each plate using National Institutes of Health (NIH) 1.62 software. Independent anchorage growth was assessed in triplicate from two independent experiments.

Determination of apoptosis

Apoptotic cells were detected using annexin V/enhanced green fluorescent protein (EGFP) staining with an ApoAlert Annexin V-EGFP kit (Clontech, Palo Alto, CA, USA). Stained cells were quantified using a Becton Dickinson FACScan, and data were analysed using CellQuest software (version 3.1, Becton Dickinson).

Animal experiments

Eight-week-old male athymic nude (BALB/c-nu/nu) mice (Clea Japan Inc, Tokyo, Japan) were maintained under pathogen-free conditions and used in accordance with NIH and Tohoku University Medical School institutional guidelines. Logarithmically growing cells trypsinized from subconfluent monolayers were suspended in medium containing 10% Matrigel Growth Factor Reduced (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 10^7 cells/mL. Then, 3×10^6 cells in 300 μL suspension were injected subcutaneously into the hind flanks of nude mice.

Tumour volume was estimated after biweekly measurements using the formula $V = D \times d^2 \times 0.4$, where V = tumour volume, D = largest dimension and d = smallest dimension. Mice were sacrificed at week 8, when tumours from parental cells reached approximately 2,000 mm^3. Tumours were resected, weighed and bisected; half of the tissue was snap-frozen in liquid nitrogen for molecular investigation and the other half was fixed in neutral buffered formalin for further investigation.

To estimate the metastatic ability of hybrids, we used a lung colonization model.[23] Briefly, parental and hybrid cells were prepared as single-cell suspensions in sterile phosphate-buffered saline at a concentration of 5×10^6/mL, then 250 μL (1.25×10^6 cells, viability 95% as determined by trypan blue exclusion) was injected intravenously via the tail vein of the mice. Animals were sacrificed on day 30, when most control mice became moribund. Surface lung metastatic tumours were counted and microscopically confirmed. Each hybrid clone was assessed using two mice, and data from three independent experiments were pooled for statistical analysis. No spontaneous mortality or dropouts due to incomplete tumour growth were recorded.

Immunohistochemical analysis

Sections (5 μm) were prepared from formalin-fixed, paraffin-embedded specimens. Immunohistochemical reactions were

performed as described previously,[1] using mouse anti-proliferating cell nuclear antigen antibody (anti-PCNA, clone PC10, Dako Corporation, Copenhagen, Denmark), and developed using an Immunomouse kit (Zymed Laboratories Inc, South San Francisco, CA, USA). Proliferating cells were quantitated by counting PCNA-positive cells as well as total cells in 10 arbitrarily selected fields at × 40 magnification in a double-blinded manner. The percentage of PCNA-positive cells per 10 × 40 fields was determined from the number of PCNA-positive cells × 100/total number of cells. Negative control slides were prepared by omitting the primary antibody.

Statistical analysis

A two-tailed Student's *t* test calculated with GraphPad Prism 3.0 software (GraphPad Software Inc, San Diego, CA, USA) was used to determine the statistical significance of measured differences. The level of significance was established at p less than 0.05.

Results

Molecular characterization of parental and hybrid cells

The efficiency of chromosome transfer into hybrids was explored using microsatellite analysis; typical examples are shown in Figure 1. A complete copy, or at least a great majority, of human chromosome 18 was transferred and maintained in each hybrid cell; the band originating from A9H(18) was seen in the DNA of each hybrid cell line. To assess whether the transferred chromosome was maintained indefinitely, we repeated this analysis at the end of the experiment using sample DNA from both the hybrids (after 10–15 passages) and subcutaneous tumours generated in nude mice (Figure 1). The results confirmed that the additional copy of chromosome 18 was efficiently transferred and maintained indefinitely.

Phenotypic assessments

In vitro growth of hybrid cells was significantly suppressed compared with parental cells, regardless of initial *SMAD4* status (Figure 2). No morphological changes were apparent throughout the hybrid clones when compared with either parental or control cells (Figures 2B and C). This suggests that a product of a chromosome 18 gene other than *SMAD4* plays a role in this behaviour of hybrids cells.

To study proliferation and morphology under anchorage-independent conditions, we transferred both parental and hybrid cells from a mono- to a bi-layered Bacto-agar suspension. The size and number of colonies observed among hybrids were significantly smaller than among parental cells (mean, 49 ± 3.6 vs 228 ± 19.36 colonies/wheel and 106 ± 19.61 vs 392 ± 37.22 μm for PK-1H(18) and PK-1, respectively). In addition, parental cells formed tight, densely packed, multicellular spheroids (MCS) easily detected by the naked eye, while hybrids formed smaller, loose spheroids (Figures 2D and E). Because the ability of cells to grow in soft agar suspension very closely correlates with their tumorigenic potential *in vivo*,[24] these features strongly suggest that chromosome 18 transfer is associated with major changes in hybrid cell behaviour.

The percentage of annexin V-positive parental cells (2.1 ± 0.5%) was very low (Table); the percentage increased slightly among hybrid clones (3.9 ± 0.7%). Thus, apoptosis is unlikely to participate in the chromosome 18-mediated growth suppression observed in hybrid cells.

Despite inherent interclonal variation, the hybrids showed a significant reduction in tumour volume and a longer latency compared to parental cells (Figures 3A–D). We found that 16% of tumour cells stained positive for PCNA in hybrid tumours compared with 57% in parental tumours (Figures 3E and F). Thus, proliferation was decreased in hybrid tumours compared with parental tumours (Table), indicating that retardation of tumour growth is caused by diminished proliferation of hybrid cells.

The number of surface metastases in mice lungs injected with hybrids was significantly decreased compared with that in mice injected with parental cells (Table). Furthermore,

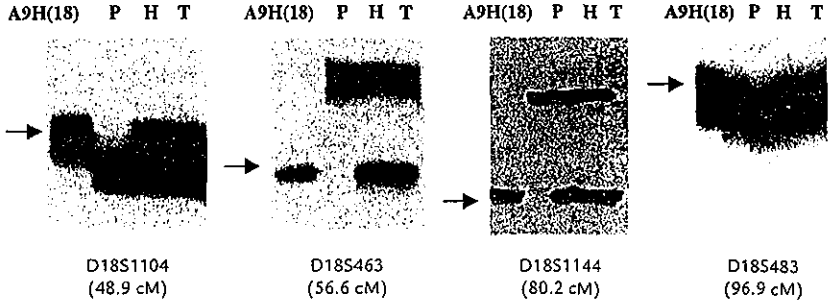

Figure 1. Microsatellite analysis. Representative scanned autoradiographs showing restoration of heterozygosity for the different 18q-microsatellite markers after chromosome 18 transfer into PK-1 cells. Arrows indicate new bands of the same size as those obtained from A9H(18)H used as donor cells. The generic names and cytogenetic position (Genome Database, http://gdbwww.gdb.org) along 18q are indicated below every panel. P = parental cells; H = hybrids; T = nude mouse tumours.

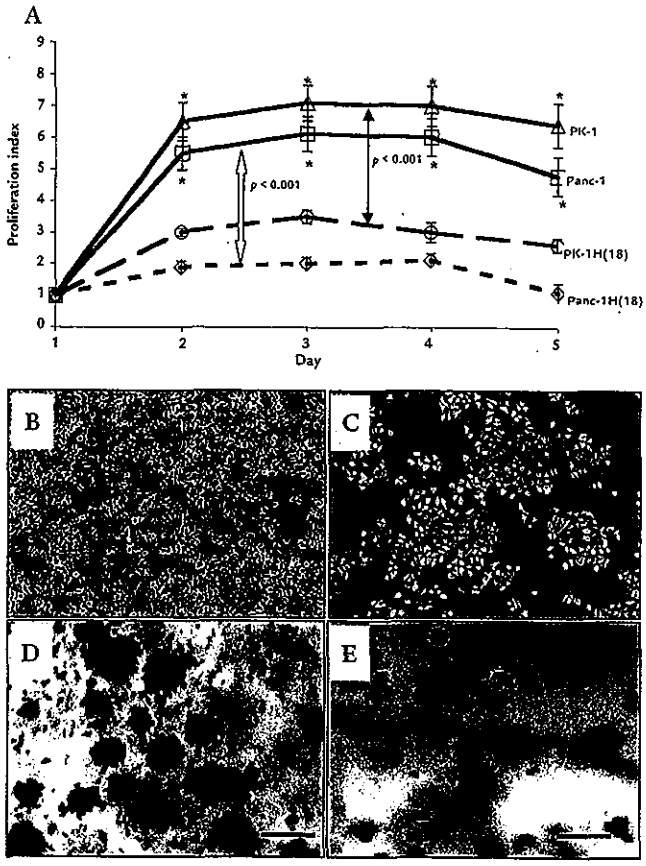

Figure 2. *In vitro* proliferation. A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays indicated that hybrids (dashed lines) derived from *SMAD4*^{wt} cells demonstrated the ability to suppress *in vitro* growth to a greater extent than hybrids derived from *SMAD4*$^{-/-}$ recipients (*$p < 0.05$). Note the same trend for parental cells. The calculated standard deviations (bars) were too low to be resolved in some curves. Up-to-down arrows reflect mean differences recorded between hybrid and parental cell tumours. B, C) PK-1 parental cells and their hybrids at 96 hours under a phase-contrast microscope at original magnification × 20. D, E) Anchorage-independent growth at day 21 shows that hybrids have an increased ability to suppress growth in soft agar (D, PK-1; E, PK-1H(18)). Bar = 500 μm.

lungs showing no metastases at surface examination had few detectable micro-metastases; some of them appeared to be dormant. These results demonstrate that the introduced chromosome 18 provides an important factor that reduces metastatic activity.

Discussion

Pancreatic cancer, like other cancers, is a genetic disease arising from an accumulation of mutations that promote clonal selection of cells with increasingly aggressive behaviour.[25] At the time of the initial detection of clinical cancer, several genetic alterations have already accumulated in tumour cells. In pancreatic cancer, *KRAS*, *TP53*, *p16* and *SMAD4* are thought to play key roles in tumorigenesis.[26] Several lines of accumulated evidence have clearly shown that loss of 18q is one of the more consistent findings among chromosomal abnormalities identified in a variety of cancers, and that this event is associated with a poor clinical outcome.[5] However, functional evidence implicating chromosome 18 in pancreatic tumorigenesis is desirable.

In this study, we used MMCT to introduce a normal copy of human chromosome 18 individually into two pancreatic cancer cell lines. MMCT is a useful tool that has provided functional evidence of the location of tumour suppressor genes in a variety of cancers including melanoma[27] and prostate cancer.[28] We first performed MMCT and then checked the efficiency of chromosome transfer by microsatellite analyses. Although corresponding normal tissues were not available for each cell line, we demonstrated the introduced chromosome 18 by observing the additional band in hybrid cells. Because these cell lines do not have high microsatellite instability,[29] additional bands for each marker are likely to be derived from the introduced 18q copy. Although some microdeletions cannot be totally excluded, it is reasonable to assume that the great majority of the 18q arm was maintained in our hybrid cells. However, in this system, based on a semi-quantitative PCR method, it is impossible to estimate the percentage of cells retaining the transferred chromosome copy.[28]

In vitro growth of the hybrid clones was significantly delayed compared to that of parental cells, apparently regardless of initial *SMAD4* status. The latter fact is not surprising since over-expression of *SMAD4* itself does not affect the *in vitro* proliferative rate of pancreatic cancer cells.[8] The *in vitro* culture of cells in suspension is believed to more closely mimic *in vivo* conditions than the culture of cells in a two-dimensional monolayer.[24] The size and number of colonies were significantly smaller among hybrids than among parental cells. In addition, parental cells formed tight, densely packed MCS easily detected by the naked eye, while hybrids formed smaller, loose spheroids. This is in keeping with changes in the adhesion properties of hybrids, but should be examined in the light of further specific investigation. The ability of cells to grow in soft agar suspension very closely correlates with their tumorigenic potential *in vivo*, so the results of this experiment showed that chromosome 18 transfer is associated with major changes in tumour cell behaviour.

Cells undergoing apoptosis were detected using an annexin V assay, which measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event

typically associated with apoptosis. In comparison with traditional tests, this assay is sensitive and offers the possibility of detecting early phases of apoptosis before the loss of cell-membrane integrity, and permits measurement of the kinetics of apoptotic death in relation to the cell cycle.[30] The percentages of annexin V-positive cells were very low in parental cells and slightly increased among the hybrid clones (Table). Thus, apoptosis is unlikely to be involved in chromosome 18-mediated growth suppression observed throughout the hybrid clones.

Tumorigenesis in nude mice is one of the most stringent tests and yields highly valuable information about cancer-cell behaviour. In order to shorten tumour latency and enhance tumour growth, we mixed cells in a suspension containing Matrigel extract. Despite inherent interclonal variation, hybrids showed a significant reduction in tumour volume and a longer latency compared to parental cells. Remarkably, there was a significant difference in tumour volumes related to *SMAD4* status. Specifically, *SMAD4*$^{+/+}$ cells generated significantly bigger tumours with a shorter latency than *SMAD4*$^{-/-}$ cells. This has recently been explained by the fact that *SMAD4* inhibits angiogenesis via putative down-regulation of vascular endothelial growth factor.[8] We found that 16% of tumour cells stained positive for PCNA in hybrid tumours compared with 57% in parental tumours. Thus, proliferation was decreased in hybrid tumours compared with parental tumours (Table), which indicated that retardation of tumour growth is caused mainly by slower proliferation.

Metastasis-regulatory genes can be broadly categorized as either metastasis-promoting or metastasis-suppressing. Analogous to the role of oncogenes in tumorigenesis, metastasis promoters drive conversion from non-metastatic to metastatic cells.[31] As expected, tumour suppressors inhibit both phenotypes because tumorigenicity is a prerequisite for metas-

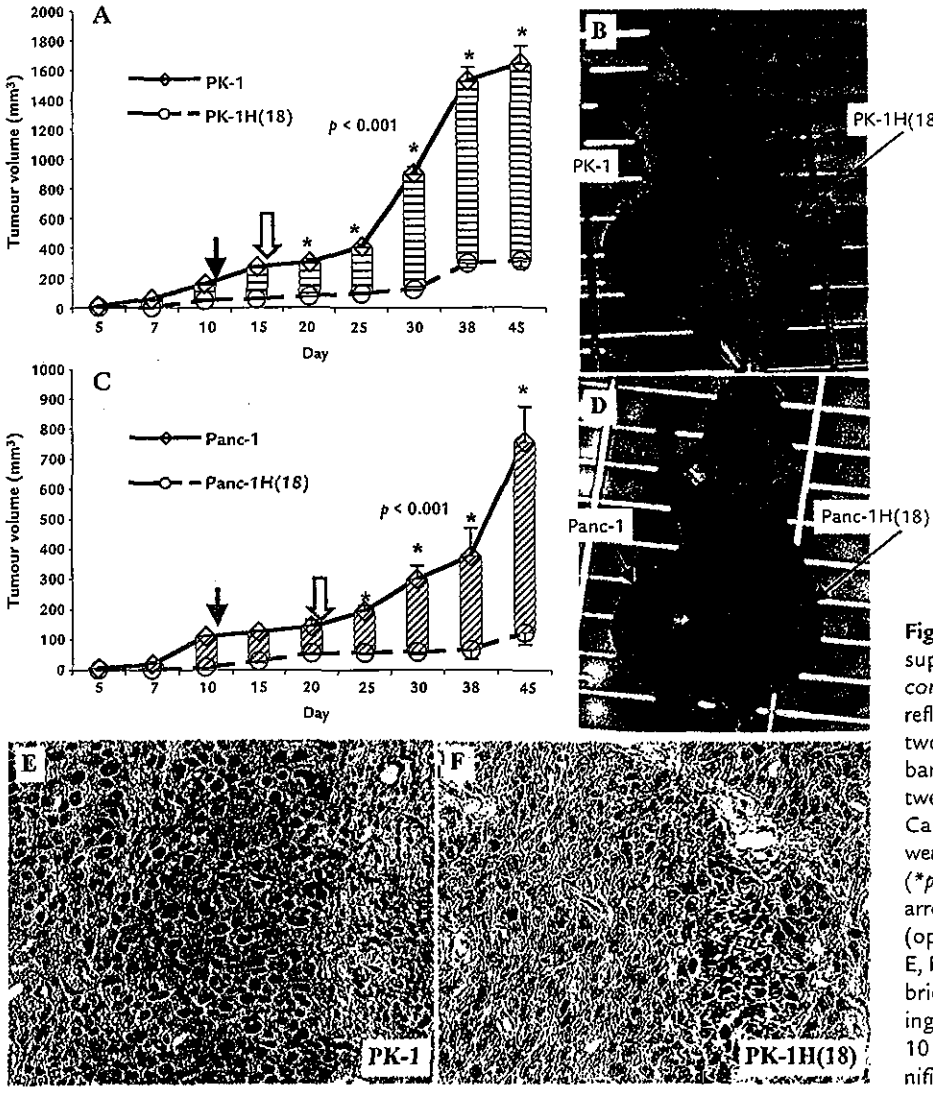

Figure 3. *In vivo* growth. A–D) Significant suppression of tumorigenesis in hybrids compared to parental cells. Each point reflects the average of three tumours from two different experiments. Up-to-down bars reflect mean differences recorded between hybrid and parental cell tumours. Calculated standard deviations (bars) were too low to be resolved in some curves (*$p < 0.001$). In A and C, the up-to-down arrows reflect the latency period for hybrid (open arrows) and parental cell tumours. E, F) Decreasing proliferation index in hybrid xenografts. Percentage of proliferating cell nuclear antigen-positive cells per 10 arbitrarily selected fields at × 40 magnification (E, PK-1; F, PK-1H(18)).

tasis.[32] As chromosome 18 harbours a cluster of candidate tumour and metastasis suppressor genes, i.e. *SMAD2*, *SMAD4*, *DCC*, *maspin* and *PAI-2*, and because poor prognosis is significantly associated with 18q-LOH,[5] it is of great interest to examine whether genes on chromosome 18 play roles in metastatic processes. To estimate the metastatic ability of hybrids, we used a lung colonization model. We recorded a significant suppression in the number of surface metastases in mice injected with hybrids compared to mice injected with parental cells (mean, 5 vs 51 for PK-1(18) and PK-1, respectively) (Table). This demonstrated that 18q encodes an important metastasis suppressor factor and confirmed our previous study that clearly correlated LOH of 18q with poor prognosis in patients with pancreatic cancer.[5]

The MMCT technique means that hybrid cells usually do not contain mouse chromosomes,[19] but there is nevertheless a small possibility that the observed growth suppression could be attributed to the effects of remaining mouse chromosomes. However, we obtained three independent hybrid clones for each parental cell line, and the possibility that these individual clones would harbour the same particular undetected mouse chromosome or chromosome fragments is unlikely. Hence, it is likely that the tumour suppression phenotype is caused by the introduction of normal human chromosome 18 into pancreatic cancer cells.

Nevertheless, whole chromosome transfer raises at least two issues: the direct effects of known or unknown genes located on chromosome 18, and indirect effects through possible interactions among the transferred chromosome and other genes. Although in this setting, these effects could not be clearly delineated, it is conceivable that introduction of an extra copy of chromosome 18 confers a less aggressive tumour phenotype on pancreatic cancer cells. In other words, the metastatic inhibition encoded by 18q could explain the dormant status of the hybrids and, in turn, the presence of micro-metastases unable to form macro-metastases. Although this study clearly implicates the important role of gene(s) on chromosome 18, the precise sub-chromosomal localization of the metastasis suppressor gene(s) remains an open question. In the absence of spontaneous revertant hybrids, the precise localization and identification of a putative metastasis suppressor gene will require further effort. In an attempt to define new presumable interactions between genes on chromosome 18 and others, a microarray analysis is currently under way in our department.

Briefly, we achieved significant suppression of both *in vitro* and *in vivo* growth of pancreatic cancer cells by transfer of chromosome 18. The suppression was observed regardless of *SMAD4* status, and induction and restoration of *SMAD4* could not prevent *in vitro* growth, regardless of *SMAD4* mutational background.[8] These functional data bring into sharp relief the implication of chromosome 18 in pancreatic carcinogenesis, but new research will be able to locate tumour suppressor gene(s) in this region.

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Phase III Trial of Radiosensitizer PR-350 Combined With Intraoperative Radiotherapy for the Treatment of Locally Advanced Pancreatic Cancer

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Abstract: A randomized, controlled trial was conducted to clarify the effect of novel radiosensitizer, PR-350, accompanied by intraoperative radiology (IOR) on locally advanced pancreatic cancer. Between July 1999 and March 2002, 48 patients were enrolled in this clinical trial and received either PR-350 or placebo. Any differences between the PR-350 group (n = 22) and control group (n = 25) were not statically significant. All patients were evaluated, and none of them showed toxicity, with the exception of 1 patient from the control group, and the PR-350 compound was considered to be safe. The efficacy of IOR with PR-350 was evaluated using CT examination. The committee responsible for evaluating efficacy reported that 47.4% of the PR-350 group showed the effective response, compared with 21.7% of the control group ($P = 0.1067$, Fisher analysis). At 6 months following treatment, the tumor mass reduction rate in the PR-350 group was significantly improved ($P = 0.0274$). By the time of the last follow-up in July 2003, 17 PR-350 patients and 24 control patients group had died of the disease. The median survival period of the PR-350 group was thus 318.5 days and that of the control group is 303.0 days. One-year survival rates of the PR-350 group and control group were 36.4% and 32.0%, respectively. Although the PR-350 group did not demonstrate significantly better survival than the control group, 4 of 22 PR-350 patients were still living more than 2 years after the end of the trial, compared with only 1 of 25 patients from the control group. The mechanism of this increased therapeutic response to radiotherapy using PR-350 must be clarified to establish more effective strategy for pancreatic cancer treatment.

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Ever since Abe and Arakawa[1] first reported that intraoperative electron beam radiation therapy (IOR) minimizes radiation damage to normal structures, the use of IOR in Japan has spread and been adopted to treat pancreatic adenocarcinoma.[2] The advantages of IOR are that it (1) minimizes damage to normal tissues, which can be shifted from the field of treatment and (2) uses high-energy electrons that can deliver high doses of radiation to induce tumoricidal effects on the cancer. Goldson et al[3] demonstrated these tumoricidal effects in pathologic specimens that showed massive tumor necrosis and fibrous replacement following IOR. However, Goldson et al also recommended additional external beam radiotherapy (EBRT) because viable cancer was noted in all cases.

IOR typically employs single high doses of radiation, ie, 15–30 Gy. It is expected that such high doses destroy almost all oxygenated cells, leaving only hypoxic cells to survive. Recent studies have demonstrated that hypoxic cells exist in many human malignant tumors, including pancreatic cancer. Since it is probable that hypoxic cells within solid tumors limit tumor curability by radiation therapy and certain chemotherapeutic agents, patients with hypoxic cell tumors have poor prognoses in comparison to patients with oxygenated tumors. Therefore, it seems quite reasonable to employ hypoxic cell sensitizers, along with IOR, in the treatment of unresectable or macroscopically residual tumors.

Misonidazole in combination with IOR was tested in Japan in the 1980s, but no definite conclusions on its efficacy were drawn. Misonidazole[4,5] and etanidazole,[6,7] of the 2-nitroimidazole family of radiosensitizers, were considered to be 2 of the most applicable and effective compounds in pancreatic cancer treatment. However, these hypoxic cell radiosensitizers induce inevitable side effects in humans that are predomi-