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Immunohistochemical Expression of Receptor-Tyrosine Kinase c-kit Protein and TGF-β1 in Invasive Ductal Carcinoma of the Pancreas

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Abstract. *Background: The receptor tyrosine kinase c-kit is known to play an important role in the progression of gastrointestinal stromal tumors, but its biological significance in other solid malignancies is unclear. Recent publications have suggested a regulatory role for TGF-β1 in c-kit-mediated cell growth. The present study assessed the clinicopathological significance of c-kit protein (KIT) and TGF-β1 expression in resectable invasive ductal carcinomas (IDCs) of the pancreas. Patients and Methods: This study included 91 pancreatic IDC patients who received a pancreatectomy between 1982 and 2003. The expression of KIT and TGF-β1 was analyzed by immunohistochemistry. Results: KIT and TGF-β1 were expressed in 77% (70/91) and 59% (54/91) of the IDC, respectively. The expression of KIT was not correlated with that of TGF-β1. TGF-β1 expression correlated inversely with nodal involvement, but KIT expression did not correlate with any clinicopathological factors. KIT expression had no significant influence on the survival of the patients, whereas the survival rate of TGF-β1 (+) IDC patients was significantly higher than that of TGF-β1 (–) IDC patients. Co-expression analysis of KIT and TGF-β1 indicated that, in patients with KIT (+) IDC, the TGF-β1 (+) group showed a significantly better survival rate than the TGF-β1 (–) group. Neither KIT expression nor TGF-β1 expression had a significant effect on the efficacy of adjuvant chemotherapy (ACT). In multivariate analysis, TGF-β1 expression was one of the significant variables for survival in IDC patients overall, but KIT expression was not. Conclusion: TGF-β1 expression is suggested to have a significant influence on c-kit-mediated cell proliferation in human pancreatic IDCs.*

The c-kit proto-oncogene has recently been identified as a member of the receptor (Rcp)-tyrosine kinase family and, more specifically, as a member of the platelet-derived growth factor Rcp family (1, 2). c-kit encodes a transmembrane Rcp with a molecular weight of 145-160 kDa (1) and has also been shown to be identical with the product of the W locus in mice, and as such is integral to the development of mast cells and hematopoiesis (3, 4). More recently, the ligand of the c-kit Rcp has been identified and characterized, and was shown to be encoded at the murine steel locus (5).

The activation of c-kit by the c-kit ligand, also known as the stem cell factor (SCF), is essential to melanocyte and germ cell development and during the early stages of hematopoiesis (6). The expression of c-kit protein (KIT) was observed in various malignancies such as neuroblastomas (7) and testicular germ cell tumors (8). Recently, c-kit was shown to play an important role in the progression of gastrointestinal stromal tumors (9, 10). In addition, KIT and SCF are coexpressed in some breast and colorectal cancers (11, 12), suggesting that c-kit may serve an autocrine role in normal or malignant epithelial tissues. On the other hand, it has also been suggested that TGF-β1 may play a regulatory role in c-kit-mediated cell growth, and that TGF-β1 inhibited

Abbreviations: Ab, antibody; ACT, adjuvant chemotherapy; Cdk, cyclin-dependent kinase; CPA, cyclophosphamide; 5-FU, 5-fluorouracil; GIST, gastrointestinal stromal tumor; IDC, invasive ductal carcinoma; KIT, c-kit protein; pAb, polyclonal Ab; PBS, phosphate-buffered saline; Rcp, receptor; RT, radiotherapy; SA, surgery alone; SCF, stem cell factor; TGF-β1, transforming growth factor-beta 1

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Key Words: c-kit, TGF-β1, pancreatic cancer, immunohistochemistry, tyrosine kinase.

Table I. *KIT and TGF-β1 expression and clinicopathological characteristics.*

Feature		KIT(+)	Correlation coefficient*	TGF-β (+)	Correlation coefficient*
Overall (n=91)		70(77%)		54(59%)	
TGF-β1	(+) (n=54)	43(80%)	0.078 (p=0.4657)		
	(–) (n=37)	27(73%)			
Grade**:	1 (n=38)	30(79%)	–0.091 (p=0.3903)	20(53%)	0.087 (p=0.4126)
	2 (n=46)	36(78%)		30(65%)	
	3 (n=7)	4(57%)		4(57%)	
Stage:	I (n=11)	7(64%)	0.069 (p=0.5146)	8(73%)	–0.125 (p=0.2371)
	II (n=5)	4(80%)		4(80%)	
	III (n=46)	37(80%)		26(57%)	
	IV (n=29)	22(76%)		16(55%)	
pT***:	1 (n=7)	6(86%)	0.004 (p=0.9676)	5(71%)	–0.158 (p=0.1354)
	2 (n=31)	23(74%)		21(68%)	
	3 (n=29)	22(76%)		16(55%)	
	4 (n=24)	19(79%)		12(50%)	
N:	(–) (n=10)	6(60%)	0.141 (p=0.1826)	9(90%)	–0.219 (p=0.0365)
	(+) (n=81)	64(79%)		45(56%)	

*numbers indicate r-value

**histological grade: 1, well-differentiated; 2, moderately-diffferentiated; 3, poorly-differentiated

***1, limited to pancreas, <2.0 cm; 2, limited to pancreas, >2.0 cm; 3, extended to peripancreatic structures including the duodenum, bile duct, mesentery, mesocolon, omentum and peritoneum; 4, extended to adjacent structures including the stomach, spleen, colon, portal vein, celiac artery and the superior mesenteric and common hepatic arteries and veins.

the growth stimulation *via* c-kit in colorectal carcinoma cells (11). Accordingly, c-kit may play an important role in the regulation of cell growth in various malignancies.

Invasive ductal carcinoma (IDC) of the pancreas is one of the most common causes of cancer death in developed countries (13) and has been a challenge to clinical oncologists. Although recent progress in surgical treatment and other combination therapies for pancreatic IDC has brought about an improvement in the overall results, it is still an undeniable fact that the prognosis of patients with pancreatic IDC is extremely poor, and IDC is highly resistant to various cancer therapies (14). Many studies, including ours, have indicated that pancreatic cancer is associated with alterations in various oncogenes and the overexpression of growth factors. Assaying these oncogenes and growth factors yields prognostic information independent of standard clinicopathological factors such as clinical stage and histological grade. However, the epidemiology and oncogenetic background of pancreatic IDCs are still unclear.

The present study assessed the clinicopathological significance of KIT and TGF-β1 expression in resectable IDCs of the pancreas.

Patients and Methods

Patients. Ninety-one patients (46 females and 45 males; 35-80 years old; mean 65.8 years) with pancreatic IDC underwent pancreatectomies between 1982 and 2003 at the Department of Cardiovascular and Digestive Surgery, Shimane University School of Medicine, Shimane, Japan. The present study does not include mucinous cystic adenocarcinomas or intraductal papillary mucinous neoplasms with adenocarcinoma, because they have a better prognosis than IDC. A standard or pylorus-preserving pancreatoduodenectomy was performed in 49 patients, a distal pancreatectomy in 29 and a total pancreatectomy in 13 patients. The tumors were staged according to the UICC classification (TNM classification) (15). Histopathologically, all specimens were verified as IDCs of the pancreas. The IDC profiles are summarized in Table I. None of the patients received any type of treatment prior to their surgical procedures. After surgery, some patients were treated with adjuvant chemotherapy (ACT) and/or radiotherapy (RT), and were followed-up. All patients were followed-up in our department, and the survival of the patients was surveyed on October 1, 2004. The postoperative survival was defined as the time that elapsed from the surgery to a cancer-related death.

Adjuvant chemotherapy (ACT). Of the 91 patients, 30 received surgery alone (SA), 53 received ACT and 8 received both ACT and RT. In Japan, under the universal health insurance system, the Japanese Ministry of Health, Labor and Welfare strictly regulates the use of anticancer agents. Accordingly, the ACT typically involves only approved agents.

In our department, we have no standard regimen for ACT against pancreatic IDC, because there is no evidence supporting the survival benefits of adjuvant therapy for pancreatic IDC at present. Accordingly, the use of ACT was decided on by the respective doctors with the informed consent of the patients and/or their family. Sixty-one patients received ACT after their surgery, and most patients were given 5-fluorouracil (5-FU) or its derivative

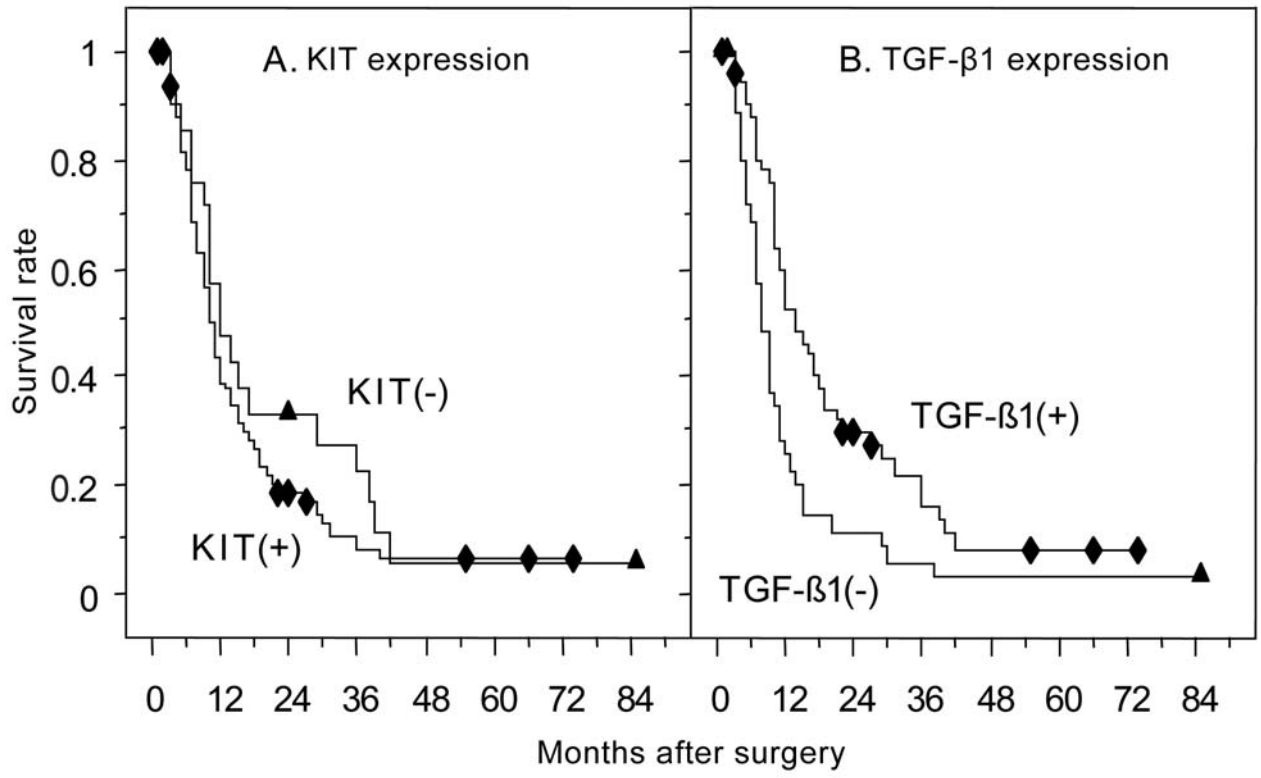

Figure 1. *Effect of KIT and TGF-β1 expression on patient survival. A. KIT expression and survival curves. KIT (+), n=70; KIT (–), n=21. KIT (+) vs. KIT (–), p=0.2996. B. TGF-β1 expression and survival curves. TGF-β1 (+), n=54; TGF-β1 (–), n=37. TGF-β1 (+) vs. TGF-β1 (–), p=0.0007.*

UFT alone or with cyclophosphamide (CPA), and some received intensive regimens including gemcitabine, adriamycin and cisplatin. Eight patients in the ACT group also received adjuvant RT using LINAC (ML-15MDX, 10MVX, Mitsubishi Electric Co. Ltd., Tokyo, Japan) at 50 Gy (2Gy x 25 times) after surgery.

Antibodies. The anti-TGF-β1 antibody (Ab) (sc-146) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was an affinity-purified rabbit polyclonal Ab (pAb) raised against a peptide corresponding to the sequence mapping at the carboxy-terminal 328-353 amino acids of human TGF-β1, and was specific for TGF-β1 but not cross-reactive with other isoforms. It was used at a dilution of 1:100 (16).

The anti-KIT rabbit Ab (AB-1, Oncogene Science, Uniondale, NY, USA) was a purified rabbit pAb raised against a peptide corresponding to a sequence found at the carboxy-terminal 961-976 amino acids of the human c-kit protein. It was diluted at 5 μg/ml for use (17).

Immunohistochemistry. The specimens were immuno-stained primarily according to the labelled polymer method using DAKO EnVision+™, Peroxidase, Rabbit kit (DAKO Corp., Carpenteria, CA, USA), which is a goat anti-rabbit immunoglobulin conjugated to a peroxidase labelled-dextran polymer. Formalin-fixed, paraffin-embedded specimens were cut into 4-μm sections. The sections were deparaffinized in xylene for 5 min 3 times, hydrated in 100%, 95%, and 45% ethanol and finally in phosphate-buffered saline (PBS). The immuno-staining procedures for KIT and TGF-β1 were different in the procedure for antigen retrieval.

Immuno-staining was performed according to instructions from the manufacturer (DAKO) as follows:

Step 1: for KIT staining, slides were pretreated in 6 M urea at 95˚C for 10 min (17), whereas for TGF-β1 staining, slides were placed in 10 mM sodium citrate buffer (pH 6.0) at 95˚C for 40 min in a water bath (Yamato BM400, Tokyo, Japan) and cooled for 20 min at room temperature for antigen retrieval. Slides were then immersed in PBS.

Step 2: endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 10 min.

Step 3: Specimens were incubated with the primary Ab for 2 h at room temperature, and then rinsed twice in PBS.

Step 4: Specimens were incubated with EnVision+, Peroxidase, Rabbit at room temperature for 30 min, and then rinsed twice in PBS.

Final step: Specimens were treated with a 0.05% 3,3'-diaminobenzidine solution for 5 min at room temperature. After washing in distilled water, specimens were counter-stained with hematoxylin, and then mounted in Entellan-new with a coverslip.

Evaluation of immuno-staining. Immuno-staining was considered positive for both KIT and TGF-β1 only when the cytoplasmic immunoreactivity was greater than 30% of the tumor cells (16). Those cases with only faint immuno-staining were regarded as negative.

Statistical analysis. A Chi-square test and Student's *t*-test were used for comparisons of the patients' clinicopathological backgrounds. The correlations between the clinicopathological factors and the

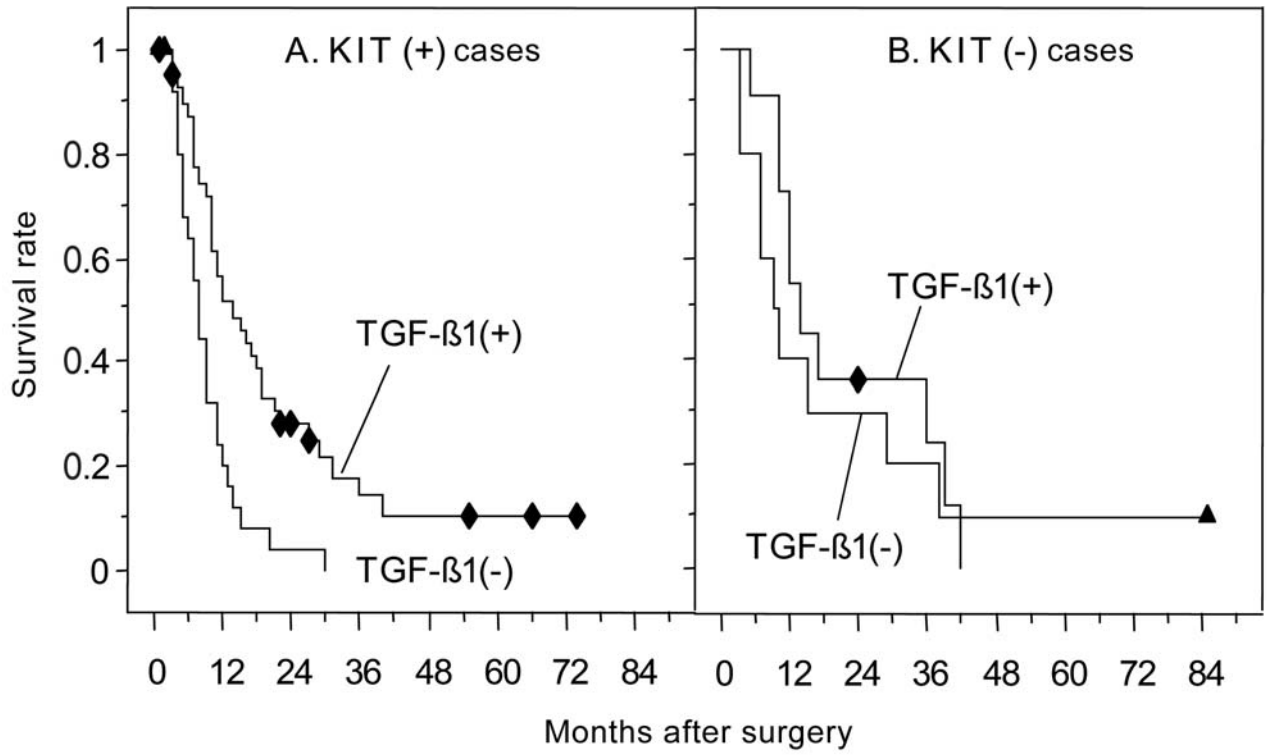

Figure 2. *Effect of KIT and TGF-β1 co-expression on patient survival. A. KIT (+) IDC patients. TGF-β1 (+), n=43; TGF-β1 (–), n=27. TGF-β1 (+) vs. TGF-β1 (–), p=0.0012. B. KIT (–) IDC patients. TGF-β1 (+), n=11; TGF-β1 (–), n=10. TGF-β1 (+) vs. TGF-β1 (–), p=0.2088.*

expressions of TGF-β1 and KIT were examined using Pearson's correlation analysis. The cumulative survival rates were calculated according to the Kaplan-Meier method, and were compared by the Cox-Mantel test. A multivariate analysis of Cox's proportional hazard risk model was used to obtain the conditional risk of death due to IDC of the pancreas. Statistically significant differences were defined at $p<0.05$.

Results

KIT and TGF-β1 were expressed in 77% (70/91) and 59% (54/91) of the patients, respectively, and the correlations between their expression and clinicopathological factors are shown in Table I. KIT expression was not correlated with that of TGF-β1. In addition, KIT expression did not correlate with other clinicopathological factors, but TGF-β1 correlated inversely with the nodal involvement ($p=0.0365$).

Median survival was 15.8±1.3 months for all patients. KIT expression was not significantly associated with survival, because there was no difference in the survival rate between the KIT (–) group and the KIT (+) one ($p=0.2996$) (Figure 1A). By contrast, the survival rate of the TGF-β1 (+) group was significantly higher than that of the TGF-β1 (–) group ($p=0.0007$) (Figure 1B). The effect of the co-expression of KIT and TGF-β1 is shown in Figure 2A and 2B. In patients with KIT (+) IDCs, the TGF-β1 (+) group had a significantly better survival rate than the TGF-β1 (–) group ($p=0.0012$) (Figure 2A), but there was no difference in survival rates between the TGF-β1 (+) and (–) groups in the KIT (–) IDCs ($p=0.2088$) (Figure 2B).

The survival rate of the ACT group was significantly higher than that of the surgery alone group ($p<0.0001$) (Figure 3). In both KIT (+) IDC and KIT (–) patients, the survival rate of the ACT (+) group was higher than that of the ACT (–) group. Furthermore, in both TGF-β1 (+) and TGF-β1 (–) IDCs, the survival rate of the ACT (+) group was higher than that of the ACT (–) group (data not shown). These results indicated that neither KIT expression nor TGF-β1 expression had any significant effect on the efficacy of the ACT.

In the multivariate analysis (Table II), the stage, grade, ACT and TGF-β1 expression were all significant variables for survival in the IDC patients overall. In KIT (+) IDCs, TGF-β1 expression was one of the significant variables for survival along with stage, grade and ACT, whereas in the KIT (–) IDCs, TGF-β1 expression was not a significant variable, but ACT and stage were.

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

	Variables	Risk ratio (95% confidence)	*p*-value
Overall	Adjuvant chemotherapy	0.333(0.188 - 0.590)	0.0002
	pTNM	1.618(1.197 - 2.187)	0.0018
	TGF-β1expression	0.485(0.300 - 0.785)	0.0033
	Histological grade	1.762(1.154 - 2.690)	0.0087
	KIT expression	1.380(0.788 - 2.416)	0.2603
KIT (+)	TGF-β1expression	0.391(0.219 - 0.699)	0.0015
	Adjuvant chemotherapy	0.3561(0.187 - 0.677)	0.0016
	Histological grade	2.174(1.088 - 3.233)	0.0033
	pTNM	1.400(1.004 - 1.953)	0.0473
KIT (–)	Adjuvant chemotherapy	0.154(0.031 - 0.750)	0.0206
	pTNM	2.240(1.066–4.705)	0.0332
	Histological grade	1.516(0.587 - 3.914)	0.3902
	TGF-β1expression	0.892(0.301 - 2.640)	0.8364

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

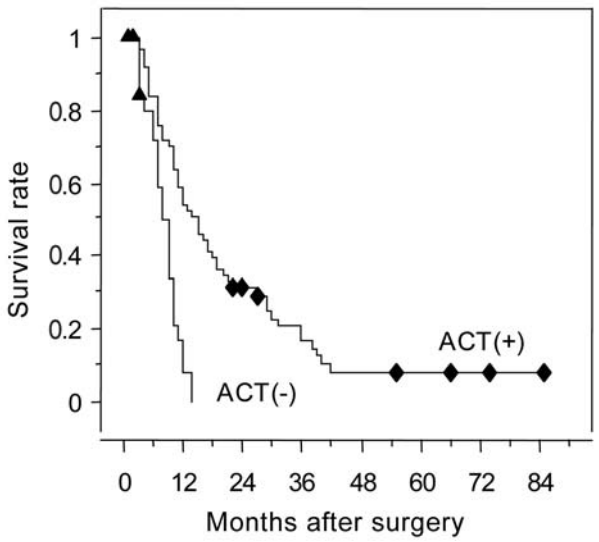

Figure 3. *Effect of the adjuvant chemotherapy on patient survival. ACT (+), n=61; ACT (–), n=30. ACT (+) vs. ACT (–), p<0.0001.*

Discussion

It has been reported that protein or mRNA for c-kit are expressed in various human solid cancers and their cell lines, such as breast cancer (18, 19), lung cancer (20) and colon cancer (21). To our knowledge, only one study has reported KIT expression in human pancreatic cancer, in which 3 cases of human pancreatic cancer did not express KIT in an immunohistochemical study (20). However, in the present study, KIT was expressed in about 80% of human pancreatic IDCs. These different results may be due to the different methods for antigen retrieval, since the paraffin sections in the present study were pretreated with 6M urea at 95˚C for 10 min according to the manufacturer's instructions, whereas the previous authors did not use an antigen retrieval method. KIT expression was studied in gastrointestinal stromal tumors (GISTs) by applying a new c-kit inhibitor, STI571 (Glivec) for the treatment of KIT (+) GISTs. In our experience, when antigen retrieval was not performed, the immuno-staining of KIT was very weak. One GIST case, which was known to express c-kit mRNA by RT-PCR and responded to STI571 treatment, was evaluated as KIT (–) in an immunohistochemical study. Furthermore, in the present study, a new labelled-polymer method was used for the immunohistochemical staining in order to avoid non-specific staining of the endogenous biotin in the tissue. Accordingly, while we are confident of these results, KIT expression in pancreatic IDC should be further replicated.

In the present study, KIT expression did not correlate with any clinicopathological factors, but TGF-β1 correlated significantly with nodal involvement. Although KIT expression had no significant influence on the survival of these patients, the survival rate of the TGF-β1 (+) IDC patients was significantly higher than that of the TGF-β1 (–) IDC patients. These results suggest that TGF-β1 expression has more influence on the progression of pancreatic IDC than KIT expression. However, the co-expression analysis of KIT and TGF-β1 indicated that, in patients with KIT (+) IDCs, the TGF-β1 (+) group had a significantly higher survival rate than the TGF-β1 (–) group, but there was no difference in survival rate between the TGF-β1 (+) and (–) groups in the KIT (–) IDCs. These results suggest that TGF-β1 may exert an inhibitory effect on the progression of c-kit dominant IDCs, and may inhibit the growth stimulation of c-kit in human pancreatic IDCs. This is supported by the results from the present multivariate analysis that in the KIT (+) IDCs, TGF-β1 expression was a significant variable for survival, whereas in the KIT (–) IDCs, TGF-β1 expression was not a significant variable (Table II).

It has recently been reported that TGF-β1 inhibits the growth stimulation of colorectal carcinoma cells *via* the c-kit receptor (21), and the potential of TGF-β1 to up-regulate the expression of Bcl-2 and p27 could be counteracted by the c-kit ligand SCF (22). The present study focused on the coordination of TGF-β1 and c-kit in the progression of human pancreatic IDCs. Here, KIT was expressed in 77% (70/91) of pancreatic IDCs, indicating that

c-kit may play an important role in the progression of pancreatic IDCs. On the other hand, TGF-β1 was expressed in 59% (54/91) of the pancreatic IDCs. However, the expression of TGF-β1 was not correlated with that of KIT. Accordingly, it is still unclear whether TGF-β1 and c-kit may affect each other.

TGF-β1 is a multifunctional cytokine involved in the regulation of cell growth and differentiation, extracellular matrix deposition, cellular adhesion properties, angiogenesis and immune functions (23, 24). Growth inhibition by TGF-β1 is attributed to the induction of WAF1/p21 (25), and the expression of Bcl-2 is also up-regulated by TGF-β1 (26). Furthermore, TGF-β1 induces cell cycle arrest at G1, and this regulatory effect of TGF-β1 is ascribed to the activities of G1 cyclins and cyclin-dependent kinases (Cdks) (26-28). TGF-β1 is also induced by other Cdk inhibitors, such as p15 or p27 (29-31). Although it has been unclear what mechanisms are responsible for the inhibitory effects of TGF-β1 on cell growth induced by c-kit, several reports have indicated that TGF-β1 inhibits the expression of the gene products for steel factor and its receptor, c-kit (32), and that TGF-β1 interferes with the proliferation-inducing activity of stem cell factor in myelogenous leukemia blasts through a functional down-regulation of the c-kit proto-oncogene product (33). Furthermore, it has also been reported that TGF-β1 regulates c-kit message stability and cell-surface protein expression in hematopoietic progenitors (34). These reports suggest that TGF-β1 plays an important role in the regulation of the c-kit-stimulated growth of malignant or blast cells, and this may be compatible with the present results that in KIT (+) IDCs, TGF-β1 expression is a significant prognostic factor (Table II). However, in KIT (–) IDCs, TGF-β1 had no significant influence on patient survival, suggesting that the progression of KIT (–) IDCs may not be regulated by TGF-β1.

In the multivariate analysis, ACT was one of the significant variables for the prognosis of KIT (+) IDCs, whereas in the KIT (–) IDCs, ACT was not a significant variable. These results suggest that ACT may be beneficial against KIT (+) IDCs. Although there are no reports on the involvement of KIT expression in the response to chemotherapy, it has been reported that the leukemia cell line MO7e, which was transduced by a mutant c-kit cDNA, acquired a growth advantage and resistance to apoptosis in response to chemotherapeutic agents and ionizing radiation (35). This report supports a possible implication for KIT expression in the response of these cells to the chemotherapy. Recently, a new c-kit inhibitor, STI571 was used for the treatment of KIT (+) GISTs, and resulted in a marked improvement in response rates. However, to our knowledge, there have been no reports on the relationship between KIT expression and the efficacy of chemotherapy, except for STI571. In the present study, most patients from the ACT group received 5-FU or its derivative UFT and CPA, and these chemotherapeutic agents may be effective against KIT (+) IDCs. Furthermore, KIT was expressed in about 80% of human IDCs, suggesting that STI571 may be a beneficial agent for chemotherapy against human pancreatic IDCs.

In conclusion, TGF-β1 expression is indicated to have a significant influence on the c-kit-mediated cell proliferation system in human pancreatic IDCs.

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