Predictors of Malignancy for Branch Duct Type IPMN

In the univariate analysis, we found 7 significant factors predicting the malignancy of branch duct type IPMNs: the presence of jaundice ($P < 0.001$), a tumor occupying the pancreatic head ($P = 0.006$), a MPD size larger than 5 mm ($P < 0.001$), mural nodule size larger than 5 mm ($P < 0.001$), elevated serum CA19–9 ($P = 0.002$), positive cytology (class IV or V) in the pancreatic juice ($P = 0.023$), and a CEA level in the pancreatic juice >30 ng/mL ($P < 0.001$) (Table 3). Furthermore, a mural nodule size larger than 5 mm ($P = 0.003$; odds ratio = 12.9) and a CEA level in the pancreatic juice higher than 30 ng/mL ($P < 0.001$; odds ratio = 299) were independent malignant predictors in the subsequent multivariate analysis (Table 4).

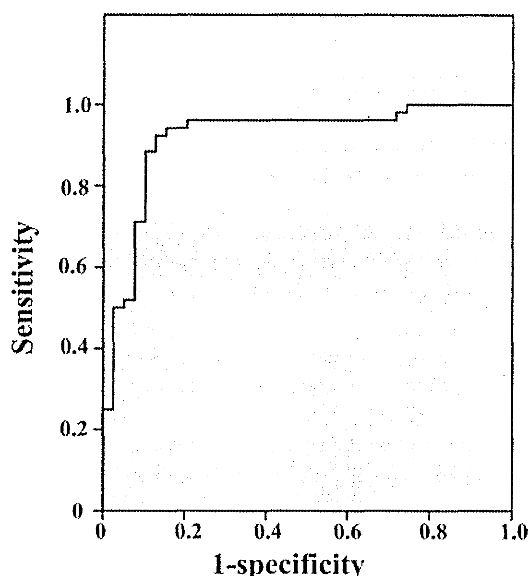

FIGURE 1. The receiver operating characteristic curve used to determine the optimal CEA cutoff levels in the pancreatic juice obtained by preoperative endoscopic retrograde pancreatography for the prediction of the malignancy of branch duct type IPMN. The area under the curve for the CEA levels in the pancreatic juice was 0.92, and the determined cutoff level for differentiation between benign and malignant IPMNs was 30 ng/mL.

TABLE 2. The Diagnostic Cutoff Levels of the Tumor Size, Main Duct Size, Mural Nodule Size, and CEA Levels in the Pancreatic Juice for Differentiating Between Benign and Malignant IPMN Based on the Receiver Operating Characteristic Curves

	Area Under Curve	Cutoff Value
Tumor size	0.612	30 mm
Main pancreatic duct size	0.711	5 mm
Mural nodule size	0.819	5 mm
CEA levels in the pancreatic juice*	0.920	30 ng/mL

*The CEA in the pancreatic juice could be measured in 91 patients who received preoperative ERP.

With regard to the mural nodule size, the sensitivity, specificity, and accuracy of the cutoff (5 mm) for differentiating between benign and malignant IPMN were 74.4%, 80.4%, and 76.9%, respectively and those for the CEA cutoff level in the pancreatic juice (30 ng/mL) were 94.2%, 84.6%, and 90.1%, respectively.

We found that mural nodule size determined by EUS and CEA levels in the pancreatic juice were significantly correlated ($P < 0.001$; Table 5). There were 5 patients with just 5 mm of mural nodule with a CEA level in the pancreatic juice higher than 30 ng/mL, and they included 4 patients with adenoma and 1 patient with CIS.

Combination Analysis of the Mural Nodule Size and CEA Level in the Pancreatic Juice

If the analyses of the mural nodule size and CEA level in the pancreatic juice were combined, all patients with a mural nodule size larger than 5 mm who also had a CEA level in the pancreatic juice higher than 30 ng/mL had malignancy, indicating that the positive predictive value of both a mural nodule larger than 5 mm and a CEA level in the pancreatic juice higher than 30 ng/mL was 100%. Moreover, 26 patients of the 27 patients with a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice higher than 30 ng/mL had benign IPMN, indicating that the negative predictive value of both a mural nodule size larger than 5 mm or a CEA level in the pancreatic juice higher than 30 ng/mL was 96.3% (Fig. 2).

DISCUSSION

Surgical resection offers the best chance for a cure for the patients with IPMN; however, observation may be a better management strategy for the patients with a low risk of malignancy, because a pancreatectomy is an invasive procedure, especially in elderly patients.[16] Therefore, many investigators have performed studies to identify factors that can be used to predict the likelihood of malignancy in the patients with IPMNs. Most clinicians have agreed that the main duct type IPMN has high malignant potential, and surgical resection is recommended for all patients with main duct type IPMN.[4–6,10] However, there is still no definite management consensus, including the surgical indications, for patients with branch duct type IPMN, because the malignant potential of branch duct type IPMN is relatively low.[10–14] Thus, it is necessary to identify more accurate factors that can predict the malignancy and determine the indications for surgical resection for the patients with the branch duct type IPMN.

The International Consensus Guidelines have put forward an algorithm for the surgical management of branch duct type IPMN, which is based on the tumor size, patient symptoms, and "high risk stigmata" (mural nodule and positive cytology in the pancreatic juice).[17] Several recent studies reported that the size of mural nodules was a more significant malignant factor than the tumor size for predicting the malignancy of branch duct type IPMN.[6,10,12,14] In addition, the MPD size, positive EUS fine-needle aspiration (FNA) cytology, and high CEA or carbohydrate antigen (CA)72.4 levels in the cystic fluid have been reported to be factors that can be used to predict the malignancy of branch duct type IPMN.[5,18–21] However, the accuracies of these factors were not high enough to distinguish between benign and malignant IPMNs. Moreover, the cytology of the pancreatic juice should be a criterion standard for the preoperative pathological diagnosis. However, the sensitivity of preoperative cytology was only 11.1% in this series. Therefore, we tried to identify more accurate predictors of the malignant potential of branch duct IPMN to determine an optimal management consensus. If CIS could be determined for the patients with branch duct type IPMN by several parameters, that would be powerful. However, in this study, the number of CIS was small; only 41 patients. Therefore, we could not analyze separately in each group. In the future, we would like to try

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TABLE 3. The Results of the Univariate Analysis of the Malignant Predictive Factors for Branch Duct Type IPMN

	Benign (n = 56), n (%)	Malignant (n = 78), n (%)	*P*
Age, >70 yr	25/56 (44.6%)	44/78 (56.4%)	0.179
Sex, male	30/56 (53.6%)	44/78 (56.4%)	0.745
Symptom	27/56 (48.2%)	46/78 (59%)	0.217
Jaundice	0 (0%)	14 (18%)	<0.001
Body weight loss	5 (8.9%)	11 (14.1%)	0.362
Abdominal pain	14 (25%)	23 (29.5%)	0.567
Back pain	9 (16.1%)	9 (11.5%)	0.448
Onset or worsening of diabetes mellitus	13/56 (23.2%)	26/78 (33.3%)	0.203
Tumor occupied location, head	35/56 (62.5%)	65/78 (83.3%)	0.006
Tumor size, >30 mm	20/56 (35.7%)	37/78 (47.4%)	0.176
Main pancreatic duct size, >5 mm	16/56 (28.6%)	52/78 (66.7%)	<0.001
Mural nodule size, >5 mm	11/56 (19.6%)	57/78 (73.1%)	<0.001
Serum CEA, elevated	3/56 (5.4%)	9/78 (11.5%)	0.217
Serum CA19–9, elevated	6/56 (10.7%)	27/78 (34.6%)	0.002
Cytology in the pancreatic juice,* class IV or V	0/44 (0%)	6/54 (11.1%)	0.023
CEA levels in the pancreatic juice,† >30 ng/m	6/39 (15.4%)	49/52 (94.2%)	<0.001

*Cytological examination of the pancreatic juice was possible in 98 patients.
†The CEA in the pancreatic juice could be measured in 91 patients.

TABLE 4. The Results of the Multivariate Analysis of the Malignant Predictive Factors for Branch Duct Type IPMN

	P	Odds Ratio	95% Confidence Interval
Jaundice	0.989		
Tumor occupied location, head	0.136		
Main pancreatic duct size, >5 mm	0.082		
Mural nodule size, >5 mm	0.003	12.9	2.38–70.3
Serum CA19–9, elevated	0.803		
Cytology in the pancreatic, class IV or V	0.983		
CEA levels in the pancreatic juice, >30 ng/mL	<0.001	299	17.7–5067

TABLE 5. Correlation Between Findings of EUS and CEA in the Pancreatic Juice Obtained by ERP for the Patients With Branch Duct Type IPMN

EUS Findings (Mural Nodule Size)	CEA Levels in the Pancreatic Juice Obtained by ERP		
	≤30 ng/mL (n = 36)	>30 ng/mL (n = 55)	*P*
≤5 mm (n = 45)	27	18	<0.001
>5 mm (n = 46)	9	37	

to analyze by clustering the patients with branch duct type IPMN into 3 groups—adenoma, CIS, and invasive IPMC.

In this study, we defined branch duct type IPMN as that with cyst formation caused by BPD dilation with or without MPD dilation. The reasons that we included mixed type IPMN into the branch duct type IPMN are as follows:

1. It is difficult to distinguish branch duct type IPMN with MPD dilatation caused by outflow of copious mucin from a side branch cyst from the mixed type IPMN with MPD dilatation resulting from the production of mucin in the MPD,[6] by preoperative radiological imaging.
2. Branch duct type IPMN sometimes has microscopic involvement into the MPD without radiological evidence of dilatation of the MPD; however, the preoperative prediction of this phenomenon is impossible.
3. The malignant potential of mixed type IPMN was reported to be lower than that of the main duct type,[6] although there have been a limited number of reports about the potential of mixed type IPMN.

Indeed, in this study, we found 22 patients with branch duct IPMN with MPD dilation, ie, mixed type on imaging findings (the pathological diagnosis in branch pancreatic duct is 5 adenoma, 10 CIS, and 7 invasive IPMC). In 5 mixed type IPMN patients with adenoma, only 1 patient had an adenoma in MPD. Remaining 4 patients had no atypia in MPD, which had the secondary dilation of MPD because of inflow of mucinous fluid from cystic branch duct. Thus, it is very difficult to distinguish branch duct type IPMN from mixed type IPMN by radiological imaging, because we could not predict microscopic involvement into MPD in mixed type IPMN patients.

For this study, we reanalyzed the cutoff values for the tumor size, MPD size, mural nodule size, and CEA level in the pancreatic juice to distinguish between benign and malignant IPMN exclusively in patients with branch duct type IPMN, using ROC curves. Our results suggested that a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice obtained by preoperative ERP higher than 30 ng/mL were independent significant predictors of malignancy in a multivariate analysis. In several significant malignant predictors for branch duct type IPMN on univariate analysis, but not significant on multivariate analysis, we found jaundice, MPD dilation, and elevated serum CA19–9 as malignant predictive factors for IPMN.[10–14] All 8 branch duct type IPMN patients with jaundice, MPD dilation, and elevated serum CA19–9 had malignancy, whereas in 32 patients without jaundice, MPD dilation or elevated serum CA19–9, 10 patients had malignancy with a high CEA in the pancreatic juice. Therefore, the branch duct type IPMN patients with jaundice, MPD dilation, and elevated serum CA19–9 may not require ERP, because the positive predictive values for malignancy of combination of these 3 factors are high, whereas ERP may be useful procedure to distinguish between

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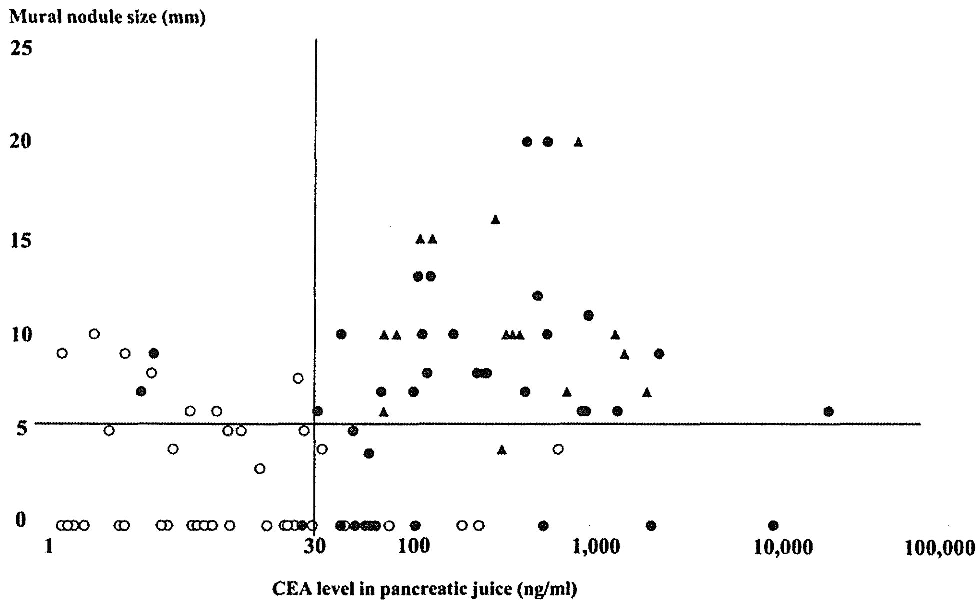

FIGURE 2. The distribution of the mural nodule size and CEA levels in the pancreatic juice in patients with branch duct type IPMN. IPMNs classified as adenoma and borderline neoplasm are indicated by white circles (○), carcinoma in situ by black circles (•), and invasive IPMC by black triangles (▲). All patients (100%) with a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice higher than 30 ng/mL had malignant IPMN, whereas only 1 patient among 27 with a mural nodule size $\leq$5 mm and a CEA level in the pancreatic juice 30 ng/mL or lower had malignant IPMN.

benign and malignant IPMNs for patients without jaundice, MPD dilation, nor elevated serum CA19–9.

We previously reported that the cutoff value for the CEA level in the pancreatic juice to distinguish malignant from benign IPMN was 110 ng/mL for the patients with all types IPMN,[2] whereas this study suggested that the cutoff value was 30 ng/mL for the patients with branch duct type IPMN. It is considered that the CEA levels in main duct type IPMN are higher than those of other types of IPMN, because the CEA levels of the pancreatic juice obtained from the MPD reflect the direct secretion of CEA for the main duct type, whereas the CEA levels for the branch duct type IPMN reflect the outflow into the MPD from cystic side branch secretions.

Recently, measurements of CEA or/and CA72.4 in the cystic fluid obtained by EUS-FNA were reported to be useful for the differentiation of malignant from benign IPMN.[20] In our institute, we have measured the CEA levels in the pancreatic juice obtained from the MPD by preoperative ERP in the patients with all types IPMN, but not in the cyst fluid obtained by EUS-FNA, because (1) obtaining pancreatic juice by ERP is not associated with any risk of peritoneal seeding, and actually, peritoneal seeding was not found in all patients, whose pancreatic juice obtained by preoperative ERP, and (2) the branch duct type IPMNs are often consisted of multilocular cysts, and it is unknown which cyst has the most severe atypia, whereas the pancreatic juice in the MPD obtained by ERP includes secreted CEA from all of the pancreatic ducts. However, further studies of the evaluation of the correlations between the CEA levels in the pancreatic juice in the MPD and the CEA levels in the cyst fluid should be performed.

In this study, there were 29 patients with a mural nodule size 5 mm or smaller and a tumor size 30 mm or larger, who were predicted to have benign IPMN by findings of EUS and CT. Among 29 patients, we had 8 malignant IPMN patients with a CEA level in the pancreatic juice obtained by ERP more than 30 ng/mL. Furthermore, in 23 patients with branch duct type IPMN without a mural nodule and a tumor size 30 mm or smaller, 6 patients had malignancy with a CEA level in the pancreatic juice higher than 30 ng/mL. These results suggest that ERP may be a useful procedure for some branch duct type IPMN patients with a mural nodule size 5 mm or smaller and a tumor size 30 mm or smaller, whereas we cannot clarify which types of patients would be benefited from this additional invasive procedure.

When the combination of a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice higher than 30 ng/mL is used, the predictive value is excellent (100%), indicating that branch duct type IPMN with a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice higher than 30 ng/mL would be recommended for resection. Only 1 patient (1 of 27 patients) with a mural nodule size 5 mm or smaller and a CEA level in the pancreatic juice 30 ng/mL or lower had malignant IPMN, which suggests that the patients with branch duct type IPMN in this group might be better treated by strict observation.

In conclusion, we identified 2 useful predictive factors for malignancy in branch duct type IPMN; a mural nodule size larger than 5 mm and a CEA level in the pancreatic juice obtained by preoperative ERP more than 30 ng/mL. Additional studies in other populations will be needed to confirm the validity of our findings.

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Review Article

Epithelial–mesenchymal transition in cancer development and its clinical significance

Masaaki Iwatsuki,[1,2] Koshi Mimori,[1] Takehiko Yokobori,[1] Hideshi Ishi,[1,3] Toru Beppu,[2] Shoji Nakamori,[4] Hideo Baba[2] and Masaki Mori[1,3,5]

[1]Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu; [2]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto; [3]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka; [4]Department of Surgery, Osaka National Hospital, Osaka, Japan

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The epithelial–mesenchymal transition (EMT) plays a critical role in embryonic development. EMT is also involved in cancer progression and metastasis and it is probable that a common molecular mechanism is shared by these processes. Cancer cells undergoing EMT can acquire invasive properties and enter the surrounding stroma, resulting in the creation of a favorable microenvironment for cancer progression and metastasis. Furthermore, the acquisition of EMT features has been associated with chemoresistance which could give rise to recurrence and metastasis after standard chemotherapeutic treatment. Thus, EMT could be closely involved in carcinogenesis, invasion, metastasis, recurrence, and chemoresistance. Research into EMT and its role in cancer pathogenesis has progressed rapidly and it is now hypothesized that novel concepts such as cancer stem cells and microRNA could be involved in EMT. However, the involvement of EMT varies greatly among cancer types, and much remains to be learned. In this review, we present recent findings regarding the involvement of EMT in cancer progression and metastasis and provide a perspective from clinical and translational viewpoints. (*Cancer Sci* 2010; 101: 293–299)

Development of distant metastases is the final stage of solid cancer progression and is responsible for the majority of cancer-related deaths.[1] Distant metastasis alone or with concurrent locoregional recurrence accounts for nearly 80% of all first relapses in women with breast cancer.[2] While clinically of great importance, the biology of metastasis remains unsolved. The process of tumor metastasis consists of multiple steps, all of which are required to achieve tumor spreading.[3,4] First, cancer cells escape from the primary tumor site. Next, cancer cells invade the tumor stroma and enter the blood circulation directly or the lymphatic system via intravasation. Most circulating cancer cells undergo apoptosis due to anoikis conditions.[5] If cancer cells survive in circulation they may reach more suitable sites by attaching to endothelial cells and extravasating from the circulation into the surrounding tissues. Finally, distal colonization requires that cancer cells invade and grow in the new environment.

Recently, the concept of the epithelial–mesenchymal transition (EMT), as developed in the field of embryology, has been extended to cancer progression and metastasis.[6,7] *In vitro* and experimental animal model data now support the role of EMT in metastasis, concepts supported by analyses of clinical samples. Indeed, the biology of EMT has been clarified in tumor samples through use of EMT-associated markers, such as mesenchymal-specific markers (i.e. vimentin and fibronectin),[8,9] epithelial specific markers (i.e. E-cadherin and cytokeratin),[10,11] and transcription factors (i.e. SNAIL and SLUG).[12]

Most recently, several intriguing studies have described the novel mechanism underlying EMT activation. In the current study, we will discuss the role of small non-coding RNA (microRNA) in regulating EMT-related genes.[13–15] Furthermore, Mani *et al.* disclosed that EMT could generate breast cancer cells with stem cell-like characteristics.[16] Here, we update and discuss recent progress in studies of EMT. These new data improve our understanding of the mechanisms of cancer progression and metastasis as well as therapy resistance. This new information may lead to development of novel clinical targets and improve the clinical management of cancer patients.

Involvement of EMT in Cancer Progression

In the 1980s, Greenburg and Hey first analyzed EMT-associated changes in cell phenotype and mesenchymal states in adult and embryonic epithelia.[17] EMT and the inverse process of mesenchymal–epithelial transition (MET) are major embryological mechanisms for tissue remodeling, as in gastrulation and segment formation.[18] The process of EMT consists of multiple steps.[19,20] First, cell–cell adhesion disintegrates with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin. Next, there is a loss of baso-apical polarization and the acquisition of front-rear polarization. Then, the cytoskeleton undergoes remodeling, with changes in cortical actin and actin stress fibers. Finally, cell-matrix adhesion is altered, with activation of proteolytic enzymes such as matrix metalloproteases. Note that the process of metastasis in epithelial cancer also consists of multiple steps.[3,4] That is, cells detach from the primary tumor and invade the surrounding tumor stroma. They subsequently enter into the circulation and reach new metastatic sites. Therefore, the process of EMT during cancer progression and metastasis closely resembles that observed in embryologic development. Accordingly, molecular analyses based on EMT in embryology have been applied to cancer progression.

In the 1990s, accumulating evidence indicated that EMT was associated with cancer progression.[7] Indeed, these transformations may be associated with EMT-related signal pathways during development.[7,21] However, Boyer *et al.* stated that EMT during development depends on additional activities of distinct and specific signaling molecules which are highly controlled spatially and temporally, and which do not occur under normal circumstances. On the other hand, EMT in cancer progression could be due to autonomous oncogenic activation of signaling molecules without additional stimulation.[22] Therefore,

[5]To whom correspondence should be addressed.
E-mail: mmori@gesurg.med.osaka-u.ac.jp

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comparisons of EMT signaling pathways in embryological development and cancer progression may make it possible to identify novel pathways specific to cancer progression and to suggest new therapeutic strategies in cancer therapy.[23]

The Molecular Mechanism of EMT in Cancer Progression

Multiple complex signaling systems are required for induction of EMT because epithelial cells undergoing EMT must undergo both functional and morphologic changes. Studies of the crosstalk among the intracellular signal networks could help us to understand the mechanisms regulating EMT. Here, we discuss the regulation of representative molecules, E-cadherin, a major EMT inducer, transforming growth factor-β (TGF-β) signal pathways, and microRNA regulation reported in recent studies (Fig. 1).

E-cadherin regulation. One of the characteristic findings in EMT is the loss of cell–cell adhesion with diminished expression of E-cadherin. E-cadherin, a calcium-dependent transmembrane glycoprotein expressed in most epithelial tissues, constructs a tight junction which connects adjacent cells. The loss of E-cadherin can lead to tumor progression, metastasis, and poorer prognosis in various human carcinomas.[10,11,24,25] Genetic or epigenetic alterations cause a functional loss of E-cadherin. For instance, mutations in E-cadherin are found in diffuse gastric cancer[26] and lobular breast carcinoma.[27] In addition, hypermethylation of the E-cadherin promoter region is found in various human carcinomas, resulting in frequent loss of E-cadherin expression.[28,29] Interestingly, Graff *et al.* proposed that the degree of methylation of the E-cadherin promoter region during metastatic progression is unstable and heterogeneous.[28] This finding suggests that the loss of E-cadherin by methylation in a primary lesion may drive metastatic progression, indicating that EMT is involved in cancer metastasis. Besides genetic or epigenetic control, E-cadherin is regulated by various signal networks, such as TGF-β signaling and transcription factors as discussed in more detail below.

TGF-β signaling. Miettinen *et al.* first revealed that TGF-β induced EMT in normal mammary epithelial cells.[30] In fact, TGF-β is an important inducer of EMT in cancer progression. However, TGF-β is well known to induce multiple responses in cancer progression.[31] For example, loss of the TGF-β signaling pathway results in the progression of cancer because TGF-β is a strong growth inhibitor.[32] Indeed, Hahn *et al.* reported that mutations in TGF-β and Smad4 give rise to pancreatic cancer[33] and colorectal cancer.[34] On the other hand, TGF-β can protect against apoptosis, and promote angiogenesis and immune suppression.[35] TGF-β induces EMT through multiple signal pathways, including direct phosphorylation of Smad 2 and Smad 3. As shown in Figure 1, TGF-β also activates other EMT-related signal pathways, including integrin, Notch, and Wnt signal pathways, all of which trigger EMT programs.

Transcription factors. Transcriptional repressors of E-cadherin such as zinc finger proteins (ZEB1, ZEB2), bHLH protein (Twist), and the snail family of zinc finger proteins (Snail, Slug) are associated with EMT.[36–40] As shown in Figure 1, various signal pathways such as TGF-β,[20] the Wnt cascade, and PI3K/AKT (phosphatidylinositol 3′ kinase–serine/threonine kinase) axis are connected with these transcriptional repressors of E-cadherin.[41] Recent studies have demonstrated that transcriptional repressors of E-cadherin are regulated by microRNAs as described below. Several transcriptional factors such as Snail, Slug, and Twist are useful markers to predict prognosis in various human carcinomas (Table 1). Peinado *et al.* proposed that E-cadherin repressors might participate in the process of EMT as follows. First, Snail and ZEB2 would initiate down-regulation of E-cadherin. Then, Slug and ZEB1 would maintain repression of E-cadherin.[42] However, the effect of E-cadherin repressors on mesenchymal markers such as vimentin and N-cadherin remains unsolved.

Regulation of EMT by microRNA. Recent studies of small non-coding RNAs are shedding light on the regulation of gene expression and proteins in metastasis. It was shown that miR-10b overexpression is associated with invasiveness and metastatic potential.[43] miR-10b is overexpressed in metastatic breast cancer, and up-regulated by EMT transcription factor Twist. Recent independent studies revealed that the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 play critical roles in regulating EMT, targeting the E-cadherin repressors ZEB1 and ZEB2.[13,15] Gibbons *et al.* found that metastasis-prone tumor cells established from

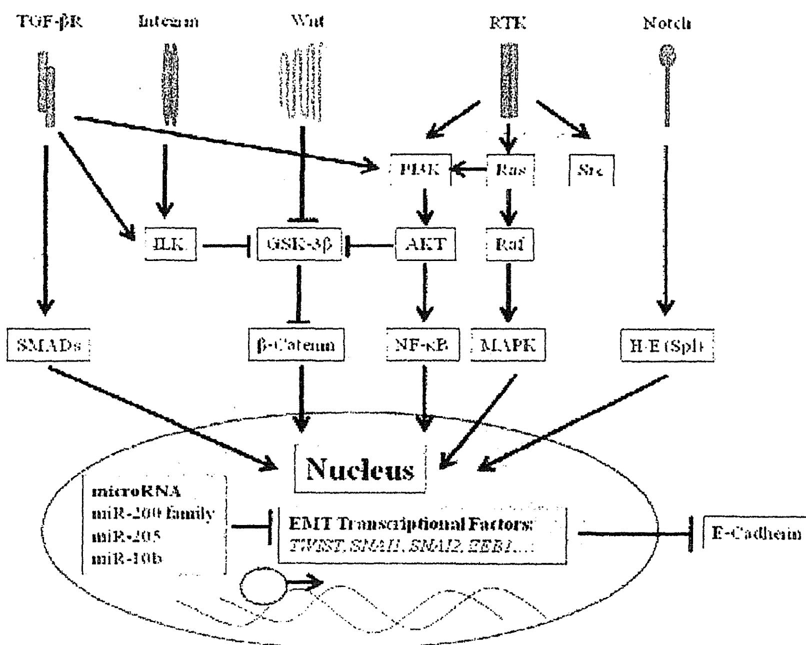

Fig. 1. Depiction of signal pathways regulating the epithelial–mesenchymal transition (EMT). Selected signal pathways regulating E-cadherin are schematized. Transforming growth factor (TGF)-β signals toward the SMAD pathway or the PI3K/AKT axis. Wnt ligands block β-catenin degradation. Excess β-catenin enters the nucleus and upregulates *SLUG* and *SNAIL* transcription. In integrin signaling, *overexpression of ILK leads to nuclear translocation* of β-catenin. Signals via RTK lead to EMT through the Ras-Raf-MAPK pathway or the PI3K/AKT pathway. AKT, serine/threonine kinase; GSK-3β, glycogen synthase kinase-3β; H/E (Spl), Hairy and enhancer of split; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3′ kinase; RTK, receptor tyrosine kinase; TGF-βR, transforming growth factor-β receptor.

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Table 1. **Epithelial–mesenchymal transition (EMT)-associated markers in clinical samples predict patient prognosis**

EMT-associated gene	Characteristics	Cancer types	Reference (author)
Epithelial marker			
E-cadherin	Type I cell–cell adhesion glycoprotein	Breast cancer	Gould Rothberg and Bracken[25]
		Gastric cancer	Chan *et al.*[24]
		Colorectal cancer	Doridi *et al.*[84]
Claudin-1	Tight junctions restrict lateral diffusion of lipids and membrane proteins	Lung cancer	Chao *et al.*[85]
		Renal cell carcinoma	Fritzsche *et al.*[86]
		Ovarian carcinoma	Kleinberg *et al.*[87]
Mesenchymal marker			
Vimentin	Intermediate filaments represent a third class of cytoskeletal elements	Breast cancer	Thomas *et al.*[88]
		Lung cancer	Al-Saad *et al.*[89]
		Gastric cancer	Utsunomiya *et al.*[90]
N-cadherin	Type I cell–cell adhesion glycoprotein	Esophageal cancer	Yoshinaga *et al.*[91]
		Lung cancer	Nakashima *et al.*[92]
		Urothelial tumor	Lascombe *et al.*[93]
Fibronectin	High-molecular weight extracellular matrix glycoprotein	Bladder tumor	Mutlu *et al.*[94]
		Colorectal cancer	Inufusa *et al.*[95]
		Ovarian carcinoma	Franke *et al.*[96]
Transcription factor			
Snail	Zinc finger transcriptional repressor	Adenocortical carcinoma	Waldmann *et al.*[97]
		Esophageal cancer	Natsugoe *et al.*[98]
		Hepatocellular carcinoma	Miyoshi *et al.*[99]
Slug	Zinc finger transcriptional repressor	Lung cancer	Shih *et al.*[100]
		Colorectal cancer	Shioiri *et al.*[101]
		Esophageal cancer	Uchikado *et al.*[102]
Twist	Basic helix-loop-helix transcription factors	Cervical cancer	Shibata *et al.*[103]
		Ovarian carcinoma	Hosono *et al.*[104]
		Breast cancer	Martin *et al.*[105]

metastatic lung adenocarcinoma (with evidence of mutant K-ras and p53) could transit reversibly between epithelial and mesenchymal states, a property that was regulated by the miR-200 family.[44] Furthermore, two recent independent studies showed that members of the miR-200 family can induce the EMT process and regulate the sensitivity to epidermal growth factor receptor (EGFR) in bladder cancer cells and to gemcitabine in pancreatic cancer cells.[45,46] As for regulating TGF-β, microRNAs related to TGF-β signaling such as miR-155 and miR-29a have been identified in breast cancer tissues.[47,48] It is important to identify microRNAs involved in EMT to elucidate up-stream regulators of various known signal pathways.

Microenvironment and EMT

The tumor microenvironment is composed of the extracellular matrix (ECM), cancer-associated fibroblasts, myofibroblasts, immune cells, and soluble factors required for cancer progression and metastasis. Interaction among cancer cells in the tumor microenvironment can induce EMT by auto- and/or paracrine secretion of mediators such as growth factors, cytokines, and ECM proteins.[21] Media conditioned by cultures of cancer-associated fibroblast induce EMT in breast cancer cells.[49] In a comparison of the central areas of primary colorectal cancer and corresponding metastases, nuclear β-catenin was found in dedifferentiated mesenchyme-like tumor cells at the invasive front and it was localized to the membrane and cytoplasm.[50] This study suggested that the tumor microenvironment may induce or maintain EMT (Fig. 2). For instance, cancer-associated fibroblasts may be supplied from cancer cells undergoing EMT.[51] Similarly, oral squamous cancer cells can directly induce a myofibroblastic phenotype via secretion of TGF-β. TGF-β signaling by stromal myofibroblast can induce secretion of hepatocyte growth factor (HGF) which promotes cancer cell proliferation and invasion.[52]

Drug Resistance and EMT

Cells undergoing EMT become invasive and develop resistance to anticancer agents (Fig. 2). In fact, EMT can be induced by anticancer agents, and stress conditions such as exposure to radiation and hypoxic conditions.[53,54] Up-regulation of *TWIST* was associated with cellular resistance to paclitaxel in human nasopharyngeal, bladder, ovarian, and prostate cancers.[55] In colorectal cancer, stable oxaliplatin-resistant cells established by chronic exposure to oxaliplatin can acquire the ability to migrate and invade with phenotypic changes resembling EMT (spindle-cell shape, loss of polarity, intercellular separation, and pseudopodia formation).[56] In pancreatic and ovarian cancer, stable cell lines resistant to gemcitabine and paclitaxel established by continuous exposure can undergo EMT with increased expression of Snail and Twist, EMT-regulatory transcription factors.[57,58]

Various types of molecularly targeted agents have been developed and used against many carcinomas with or without combination of traditional anticancer agents, leading to improved clinical outcome and survival rate.[59,60] However, EMT reportedly confers resistance to these targeted agents. For example, lung cancer cell lines having undergone EMT, expressing vimentin and/or fibronectin, were insensitive to the growth inhibitory effects of EGFR kinase inhibition (erotinib) *in vitro* and in xenografts[61] as well as other EGFR inhibitors such as gefitinib and cetuximab.[62,63] We have often encountered patients who have suffered relapses after drug treatment, even when the tumors were initially highly sensitive. Thus, EMT can lead to resistance to multiple drugs and permit rapid progression of the tumor. These clinical findings may be attributed to the inherent characteristics of EMT. Clarifying the correlation between EMT and drug resistance may help clinicians select an optimal anticancer drug treatment.

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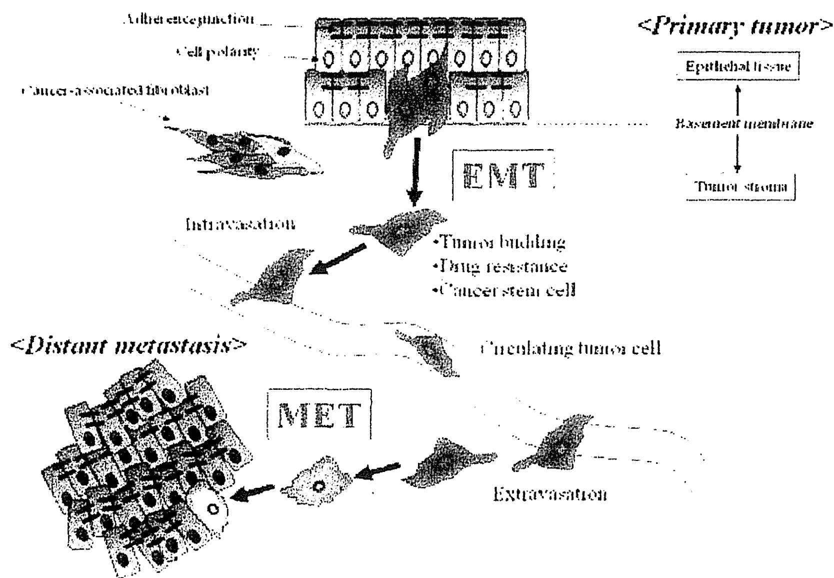

Fig. 2. The epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are involved in cancer metastasis. Cancer cells undergoing EMT in a primary tumor disseminate through the fragmented basement membrane and acquire the characteristics of drug resistance and cancer stem cells. They can be recognized in tumor buds in histological specimens. EMT cells invade into tumor stroma and enter the circulation, allowing transport to distant organs. At metastatic sites, solitary cancer cells form the new metastatic focus through MET.

Cancer Stem Cells and EMT

Cancer researchers have recently found a minor fraction of cells (cancer stem cells [CSC]) with the ability to self-renew and give rise to differentiated tumor cells. CSC have been identified in breast, colon, and pancreatic cancer.(64–66) CSC as well as cells undergoing EMT are considered to be more resistant to toxic injuries and chemoradiation therapy than differentiated daughter cells.(67,68) Furthermore, cancer cells under hypoxic conditions acquire the properties of CSC.(69,70) Even though evidence indicates a relationship between EMT and cancer cells with the traits of stemness,(71) CSC are rare in whole tumor tissues.(68,72) However, it remains controversial among pathologists whether CSC as well as cells undergoing EMT exist in human cancer tissues.(73) Intriguingly, Mani *et al.* initially disclosed that immortalized human mammary epithelial cells (HMLEs) undergoing EMT are CSC-like as characterized by their $CD44^{high}/CD24^{low}$ phenotype.(16) These investigators induced EMT in HMLEs by ectopic expression of Twist or Snail, known inducers of EMT. The cells undergoing EMT acquired a fibroblastoid mesenchymal appearance. Furthermore, Mani *et al.* observed down-regulation of epithelial markers such as E-cadherin and up-regulation of mesenchymal markers such as N-cadherin, vimentin, and fibronectin. They also noted a $CD44^{high}/CD24^{low}$ expression pattern associated with human breast CSCs. Furthermore, they revealed that the cells undergoing EMT had the properties of CSC, including self-renewal and the capacity to form mammospheres. These findings suggest that EMT may play a role in the development of CSC and properties of invasiveness, metastasis, recurrence, and chemoresistance (Fig. 2).

Clinical Significance of EMT

EMT-associated markers in clinical samples and their effects on prognosis are summarized in Table 1. Most EMT-associated markers have been identified in histological specimens. However, the existence of EMT cells in clinical specimens has been challenged.(74) In response, Voulgari *et al.* suggested that the controversy between experimental and clinical studies is due to the 'spatial' and 'temporal' heterogeneity of EMT (Fig. 3).(19) Cells undergoing EMT may gain metastatic potential but may constitute only a small proportion of the total population of tumor cells. Tumor budding is commonly observed in clinical practice, and it consists of a single cancer cell or small cell cluster at the invasive front of tumor tissues. Indeed, cancer cells in tumor buds have down-regulated E-cadherin(75) and have characteristics of CSC.(76) Therefore, identification of cancer cells undergoing EMT in clinical specimens is difficult for pathologists.

The temporal heterogeneity of EMT (and the reverse, MET) is readily explained. MET is observed *in vitro* following addition of bone morphogenetic protein 7 (BMP7), removal of an EMT-inducer such as TGF-β, and establishment of hypoxic conditions.(54,77) A similar process may occur at metastatic sites which require cancer cells to recover the expression of E-cadherin for cell adhesion. The phenotypes of metastatic specimens are often compared with primary specimens to confirm the diagnosis by hematoxylin–eosin staining. The presence of the same cancer cell characteristics or phenotypes in both primary and metastatic lesions can provide the diagnosis of cancer metastasis. Therefore, the occurrence of MET could make it difficult to prove that EMT, a transient phenomenon that involves only a minority of cells, has occurred in human cancer specimens. However, EMT-associated genes obviously are useful as predictive biomarkers (Table 1). Clinical verification of EMT will require advanced techniques such as *in vivo* imaging.

Treatments Targeting EMT

As shown in Figure 1, EMT-related pathways provide targets for therapy. For instance, inhibition of integrin-linked kinase (ILK) increases the sensitivity of mesenchymal cells to EGFR-target therapy in hepatocellular carcinoma.(63) In *in vitro* studies, Src kinase inhibitors effectively inhibit the growth of cells undergoing EMT.(78) Furthermore, the inhibition of hedgehog signaling can prevent pancreatic cancer cells from acquiring tumor-initiating property and undergoing EMT.(79,80)

RNA interference and microRNA are new technologies in drug development. For instance, silencing of Snail by shRNA induced MET and reduced *in vivo* tumor growth.(81) As for microRNA, Krutzfeldt *et al.* disclosed that specific silencers of endogenous miRNAs, antagomirs, are powerful tools to silence specific miRNAs *in vivo*.(82) Therefore, microRNAs associated with EMT such as the miR-10b and miR-200 family could be exploited as therapeutic strategies in the future.

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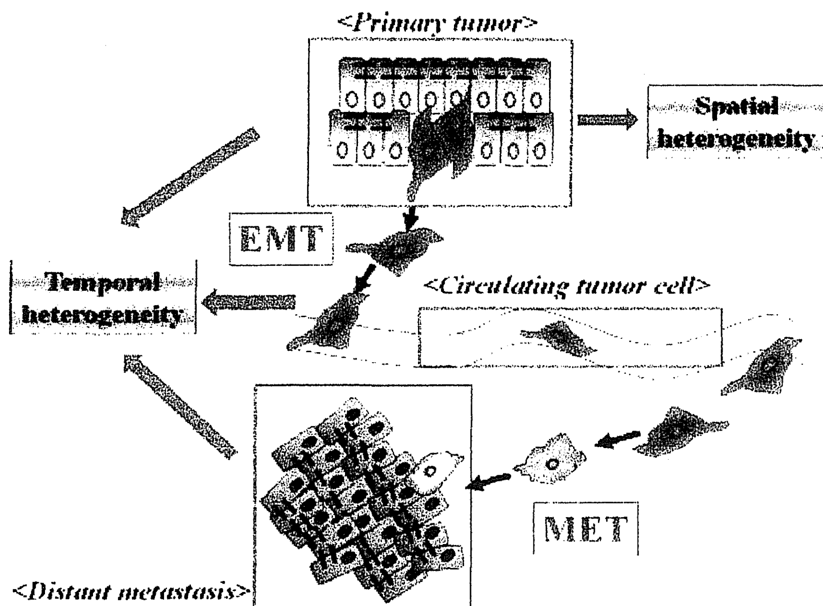

Fig. 3. Spatial and temporal heterogeneity of the epithelial–mesenchymal transition (EMT). Cancer cells undergoing EMT are expected to be only a small proportion of primary tumor tissues. EMT cells transported to metastatic sites are expected to undergo and mesenchymal–epithelial transition (MET). Therefore, the spatial and temporal heterogeneity of EMT/MET severely restricts the ability of pathologists to detect cancer cells undergoing EMT in histological sections.

Furthermore, the tumor microenvironment, which contributes to the maintenance of EMT, could be targeted. A small-interfering RNA targeted at TGF-β reportedly reduces metastasis *in vivo*,[83] and this observation could be applied to TGF-β secreted by tumor stroma. Note that reducing EMT could also lessen the occurrence of anticancer drug resistance and thereby improve the efficacy of conventional therapy. To eradicate cancer cells effectively and cause minimal toxicity to normal cells, further studies are required to define the molecular differences between EMT in embryological development and that in cancer progression.

Perspectives

During the past few decades, an increasing number of studies have shown that EMT is associated with cancer progression, metastasis, and drug resistance. Furthermore, improved understanding of microRNAs and cancer stem cells will clarify the processes underlying EMT. Current understanding of traditional signal pathways coupled with these new concepts could accelerate progress in cancer research. However, the multimodal nature of these complex pathways presents formidable challenges to researchers attempting to inhibit the onset of EMT. Finally, the clinical evidence supporting the role of EMT in cancer progression is still relatively weak. Thus, better methods for EMT detection in patient samples are needed.

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Dermokine Expression in Intraductal Papillary-Mucinous Neoplasm and Invasive Pancreatic Carcinoma

KENJI WATANABE[1*], TOSHIYA OOCHIAI[1*], SHOJIRO KIKUCHI[1,2], TATSUYA KUMANO[1], TAKESHI MATSUI[3], KOJI MORIMOTO[4,5], SATORU YASUKAWA[6], SHOJI NAKAMORI[5], MITSURU SASAKO[2], AKIO YANAGISAWA[6] and EIGO OTSUJI[1]

Department of [1]Surgery and [6]Pathology Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto ,Japan;
[2]Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan;
[3]Institute for Integrated Cell-Material Science (iCeMS), Kyoto University, Sakyo-ku, Kyoto, Japan;
[4]Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan;
[5]Department of Surgery, Osaka National Hospital, Chuo-ku, Osaka, Japan

Abstract. *Background: Serum dermokine (DK) is a novel biomarker for early colorectal cancer. To our knowledge this is the first study of DK expression in intraductal papillary mucinous neoplasm (IPMN) and pancreatic cancer. Materials and Methods: DK expression in human pancreatic cancer cell lines and tissues was assessed. We compared the sensitivities of common diagnostic markers, carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), s-pancreas-1 antigen (SPAN-1), pancreatic cancer associated antigen (DUPAN-2), and Nation Cancer Center-Stomach-439 (NCC-ST-439) in 26 patients with pancreatic neoplasms. Results: DK was expressed in pancreatic cancer cell lines. Immunohistochemical staining revealed that DK was expressed in atypical and cancerous tissues, but not in the normal pancreatic tissue. Serum DK was relatively high in patients with IPMN. The sensitivities of a serum multimarker test including DK was 76.5% (n=13/17) for IPMA/IPMC/invasive carcinoma derived from IPMN, and 100% (n=9/9) for ordinary invasive ductal carcinoma. Conclusion: Serum DK is a potential biomarker in IPMN and invasive ductal carcinoma when used with a combination of conventional biomarkers.*

*These authors contributed equally to this work.

Correspondence to: Shojiro Kikuchi, Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan. Tel: +81 798456372, Fax: +81 798456373, e-mail: skikuchi@hyo-med.ac.jp

Key Words: Serum marker, dermokine, intraductal papillary mucinous neoplasm, pancreatic cancer.

Pancreatic cancer is diagnosed in most patients once it has reached an advanced stage. The survival rate is limited to 5% because 80-85% of patients have unresectable cancer at the time of diagnosis (1, 2). Ohashi *et al.* reported intraductal papillary mucin-producing neoplasms (IPMNs) with dilatation of the pancreatic duct in 1982, which has a relatively good prognosis compared with ordinary pancreatic cancer (3). Novel biomarkers and cell biological analysis are required for (IPMN) because of the diverse grades of malignancy. IPMN is suggested to be a pre-cancerous lesion in the pancreatic duct, which is similar to colonic adenoma. IPMN grows slowly but it can develop into invasive ductal carcinoma. Consequently, early-stage diagnosis is important to improve the prognosis of pancreatic cancer. The standard diagnostic serum markers of pancreatic cancer are carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), s-pancreas-1 antigen (SPAN-1), pancreatic cancer associated antigen (DUPAN-2), and Nation Cancer Center-Stomach-439 (NCC-ST-439). However, these markers are not sufficiently useful to diagnose early pancreatic cancer. We previously reported serum Dermokine (DK) test was beneficial to diagnose early colonic cancer (4). DK was expressed in colorectal cancer at the early stage of carcinogenesis including severe adenoma. The expression of DK in pancreatic cancer and IPMN tissue were still unclear.

In the present study, we aimed to clarify the expression of DK in pancreatic neoplasms, including IPMN, and the utility of a serum DK test.

Materials and Methods

Cell culture. A total of six pancreatic cancer cell lines were purchased from RIKEN BioResource Center (Tsukuba, Japan). All cells were maintained in Dulbecco's modified Eagle's medium

Table I. *Serum marker tests in pancreatic neoplasms. The serum concentration of each marker is indicated. The cuts-off value of each marker is shown at the bottom. The gray panels mean positive cases.*

Case number	DK	CEA	CA19-9	Span-1	DUPAN-2	NCC-ST-43	Pathological diagnosis
1	53.4	5.2	60.1	76	440	2.2	IPMA
2	27.3	2	12	7.8	25	1	IPMA
3	40	3.2	330.1	20	45	1.6	IPMA
4	80.7	2.2	6	4.7	25	1	IPMA
5	22.3	1.5	4.8	3.4	25	1	IPMA
6	28	1.4	6.3	7.1	25	1	IPMA
7	57.4	4	13.1	8.4	25	1	IPMC
8	54.3	1.7	21.3	17	25	2.6	IPMC
9	19.4	2.5	5.9	12	25	56	IPMC
10	47.7	7.6	72	41	25	3.1	IPMC
11	27.2	3.6	914.5	52	25	4	IPMA, IPMC,ordinary invasive ductal
12	28.8	3.7	8.8	12	130	1	IPMA,ordinary invasive ductal carcinoma
13	48.6	3.1	1958.3	260	1200	3.4	IPMA,ordinary invasive ductal carcinoma
14	104.7	4.3	486.1	43	280	1	IPMC,ordinary invasive ductal carcinoma
15	66.4	5.5	4.9	2.1	25	1	IPMC,ordinary invasive ductal carcinoma
16	47.2	3.9	388.8	290	53	11	Invasive carcinoma derived from IPMN
17	25.5	3	3723.7	430	690	2.3	Invasive carcinoma derived from IPMN
18	30.4	2.1	57.1	30	60	1	Ordinary invasive ductal carcinoma
19	24.8	5.1	1002	160	770	2.8	Ordinary invasive ductal carcinoma
20	36	2	205.7	52	230	2.2	Ordinary invasive ductal carcinoma
21	54.5	1.8	40.2	22	25	2	Ordinary invasive ductal carcinoma
22	31.2	2.9	87.6	28	25	1.6	Ordinary invasive ductal carcinoma
23	54.6	1.2	16.4	7.7	25	1.2	Ordinary invasive ductal carcinoma
24	26	7.4	3.9	25	7.5	1	Ordinary invasive ductal carcinoma
25	41.7	4.2	5.1	12	7.5	37	Ordinary invasive ductal carcinoma
26	25.6	2.8	114.1	280	49	1.1	Ordinary invasive ductal carcinoma
Cut-off value	51 U/ml	5 ng/ml	37 U/ml	30 U/ml	150 U/ml	7 U/ml	

(Sigma–Aldrich Japan, Tokyo,Japan) supplemented with 10% fetal calf serum.

Patients and samples. Serum samples and specimens of pancreatic neoplasms were obtained from patients (n=26) under a protocol approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (KPUM) at the KPUM Hospital from 2006 to 2009. The eligibility criteria of patients were: (i) histologically proven primary pancreatic tumor; (ii) no active double cancer (synchronous or metachronous double cancer); and (iii) no prior chemotherapy or radiotherapy for any other malignancy. Serum samples from randomly selected healthy volunteers were also collected from KPUM (n=25). All patients gave written informed consent, and all aspects of these studies were approved by the Ethics Committees of KPUM.

Blood was collected with the Vacutainer blood collection system (Kyokuto Pharmaceutical Industrial Co. Ltd. Tokyo, Japan). All serum was centrifuged (3,000 rpm, 5 min), aliquoted and stored at –80˚C. Pancreatic tissues were fixed in 10% paraformaldehyde, and whole tissue was prepared to make continuous sections along the pancreatic duct. At the Department of Pathology of KPUM, paraffin-embedded sections were subjected to immunohistochemical and hematoxylin and eosin (HE) staining to map the tumor distribution in the whole resected specimen. We analyzed samples intraductal papillary mucin-producing adenoma (IPMA)/ intraductal papillary mucin-producing carcinoma (IPMC)/ordinary invasive ductal carcinoma derived from IPMN (n=12, cases1-10, 16, and 17) and IPMN with ordinary invasive carcinoma (n=5, cases11-15) as share in Table I. Subtypes were gastric type (n=12), intestinal type (n=2) and pancreatobiliary type (n=3), but there was no oncocytic type. One case of very mild dilatation of the pancreatic duct was included as IPMN because of the microscopically aggregated atypical and papillary lesion in the head of the pancreas.

Reverse-transcriptase polymerase chain reaction. For reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of pancreatic cancer cell lines, total RNA was prepared from the pancreatic cancer cell lines using TRIsure (Nippon Genetics Tokyo, Japan). For detection of DK-γ mRNA and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), first-strand cDNA synthesis and RT-PCR were performed in duplicate with PrimeScript™ II 1st strand cDNA Synthesis kit (Takara Bio, Otsu, Japan) with oligo-dT primers. PCR was performed with AmpliTaq Gold®PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and PCR Thermal Cycler Dice (Takara Bio) according to the manufacturer's protocol. Primers for the experiment were as follows: DK-γ forward 5'-ATGCCATAAACAAGGACCAGAGAA-3', reverse 5'-ACACCACC CTCTCATCACTAATCTC-3'; GAPDH forward 5'-ACCTGCC GTCTAGAAAAACCTGC-3', reverse 5'-CTCCTCACAGTTGC CATGTAGACC-3'.

Antibody preparation. Monoclonal antibody (mAb) generation was performed by Kohjin Bio Co. Ltd. (Sakado, Japan) using the antigen hDK- βΔC-SEAP (His)6 as previously described (4). To detect serum DK-β/γ in patients with colorectal cancer, we established a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with anti-DK-β/γ mAbs as previously described (4). We generated anti-DK-β/γ mAb, not anti-DK-γ mAb, because DK-γ specific mAb was difficult to generate.

Immunohistochemistry. Paraffin sections (5-μm-thick) of tumor tissues were subjected to immunohistochemical staining for the DK protein with the tyramide amplification method, which uses fluorescyl-tyramide. Antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (DAKO Japan. Tokyo, Japan), for 40 minutes at 98°C. Endogenous peroxidases were quenched by incubating the sections for 30 minutes in 3% H2O2. After a brief wash with phosphate-buffered saline (PBS) (pH 7.2) and 0.3% polyoxyethylene sorbitan monolaurate (Sigma–Aldrich), the sections were incubated for 45 minutes at room temperature with blocking reagent (Block Ace®; DS Pharma Biomedical Co. Osaka, Japan) to reduce the background signals. Each section was incubated at 4°C overnight with anti-DK mAb. CSAII (Dako) was used for color development according to the manufacturer's protocols. The sections were counterstained with hematoxylin.

Detection of serum DK by ELISA. We developed a DK-specific ELISA that used an mAb against DK-β/γ. DK-β/γ was captured in 96-well plates for ELISA as follows. First, the captured mAb anti-DK-β/γ (IgG1) was added to each well of a 96-well plate and plates were here incubated overnight at 4°C. Wells were then washed with PBS and incubated with PBS containing 1% Block Ace (DS Pharmaceutical) to block non-specific antibody binding. The serum samples (prepared as described above) were added to each well, and the plates were incubated overnight at 4°C. Horseradish peroxidase-conjugated anti-DK-β/γ mAb (IgG2a) was added and the plates were incubated at room temperature for 1 hour. DK was then detected with tetramethylbenzidine Liquid Substrate System for ELISA (Sigma–Aldrich). The standard used in these assays was recombinant DK-β expressed in 293/EBNA-1 cells. Standard curves were prepared for each assay. Limit of detection was estimated 26.3U/ml as mean of 20 control sample (from 10 separate sources) assay results plus 3times the standard deviation of the mean. Limit of quantitation was estimated 36.7U/ml as the mean of same results plus 10 times of the standard deviation. Serum CEA, CA19-9 (Abbott Japan Co., Ltd., Tokyo, Japan) SPAN-1, DUPAN-2, elastase and NCC-ST-439 were quantified at a commercial laboratory (Falco Biosystems Ltd., Kyoto, Japan).

Results

DK mRNA was found to be expressed in human pancreatic cell lines. We performed RT-PCR analysis of DK-γ in six pancreatic cell lines. DK-γ mRNA was expressed in PK-59, NOR-P1, PK-45H, PK-1 and KP4 cells. MIA Paca2 cells did not express DK-γ (Figure 1). The sizes of amplicons were 121 bp for DK-γ and 401 bp for GAPDH.

Immunohistochemistry. Immunohistochemical staining revealed DK expression in IPMN of gastric type (Figure 2).

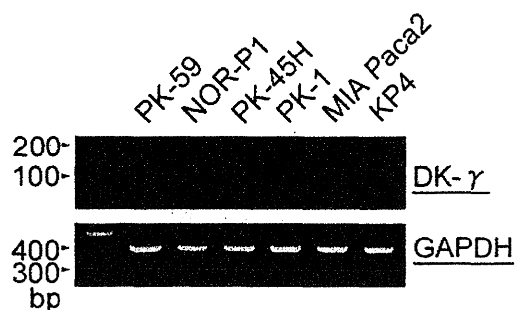

Figure 1. *Dermokine (DK) expression in pancreatic cancer cell lines. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of DK-γ in six pancreatic cell lines. DK-γ mRNAs was expressed in PK-59, NOR-P1, PK-45H, PK-1 and KP4 cells, but not in MIA Paca2 cells. The sizes of amplicons were 121 bp for DK-γ and 401 bp for GAPDH. DNA fragments were separated by 3% agarose gel electrophoresis.*

Multilocular cystic tumor was mainly located in the main pancreatic duct (asterisk) and its branch. The distribution of adenoma is indicated by the blue lines and carcinoma by the red lines (Figure 2a and b). Although normal pancreatic duct (Figure 2g and h) and mucus cell hyperplasia in the main pancreatic duct (Figure 2c and d) did not express DK, areas of carcinoma exhibited DK expression in the cytoplasm (Figure 2e and f). In carcinoma, DK was diffusely localized in the cytoplasm, as demonstrated by loss of cell polarities and normal structures (Figure 2e). We compared DK expression at a different stage of atypia in the epithelium of the pancreatic duct (Figure 3). In papillary epithelium with mild atypia, DK was not expressed (Figure 3a). DK was expressed in moderate atypia (Figure 3c) and severe atypia (Figure 3e); in severe atypia, DK expression was similar to that in carcinoma (Figure 2e).

Detection of serum DK by ELISA. We measured serum DK-β/γ levels in 26 patients with pancreatic neoplasms and compared them with those in 25 randomly selected, healthy volunteers for whom there were full data on their medical condition. To simulate the diagnostic use of this test, we proposed a cut-off value (51 U/ml; derived from the median 36.1 U/ml ± 2 S.D. 14.9 U/ml). The median serum DK-β/γ level was slightly higher in IPMA/IPMC/invasive ductal carcinoma derived from IPMN [median=47.2 U/ml, interquartile range (IQR)=27.3-55.9 U/ml] than in healthy volunteers [median=36.1 U/ml, IQR=30.5-37.7 U/ml] (Control in Figure 4a). The specificity of the serum DK-β/γ test was 92.0% in 25 healthy volunteers.

The data of each marker test for pancreatic neoplasms are shown in Table I. The serum concentration of DK was high in eight of 26 cases (30.8%), six of which were IPMA/IPMC/invasive carcinoma derived from IPMN. CA19-

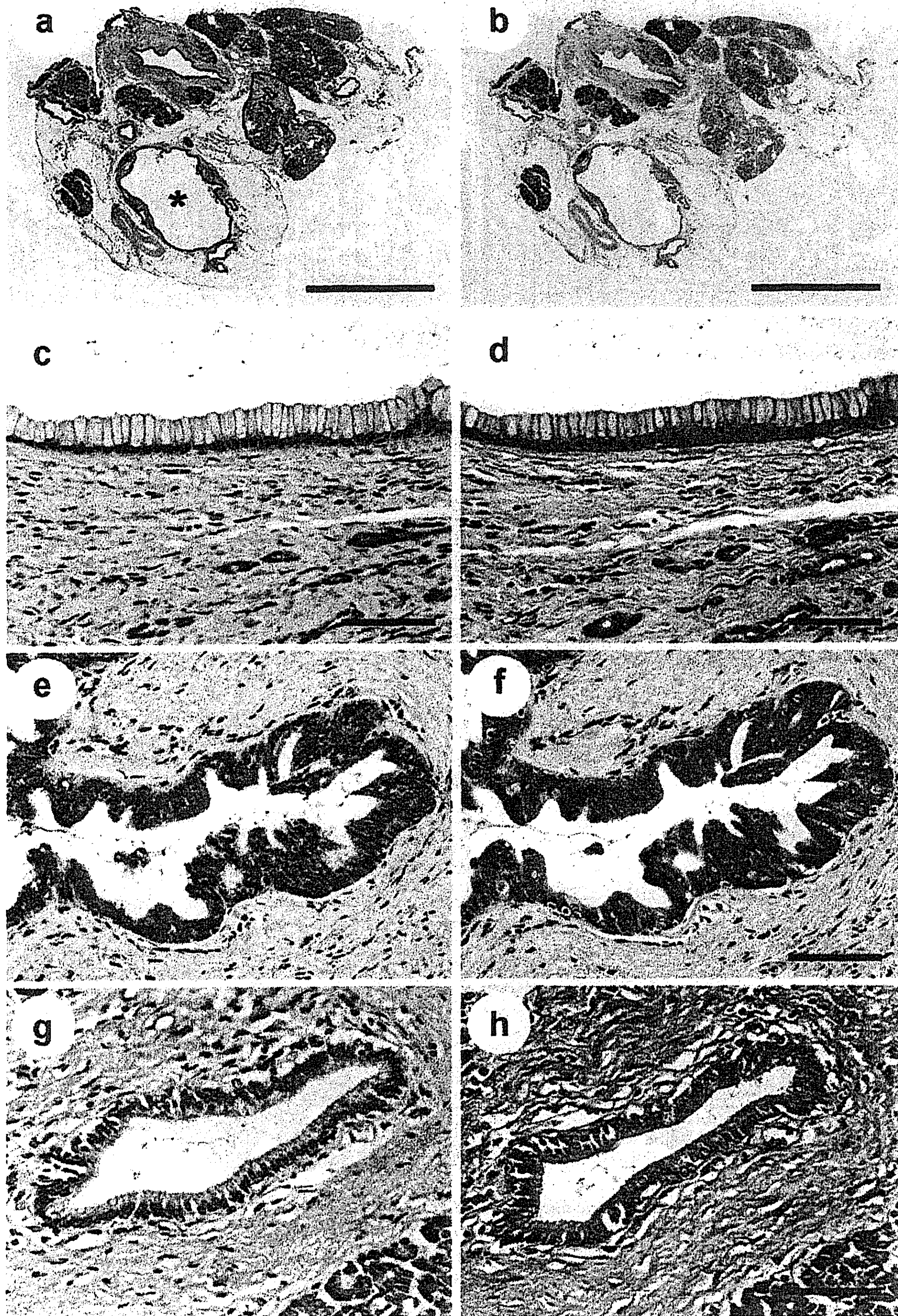

Figure 2. *Dermokine (DK) expression in Intraductal papillary mucinous neoplasm IPMN. a, b; Low-power view of IPMN. b; Adenoma (blue lines) and carcinoma in situ (Tis, red lines). c, d; Mucus cell hyperplasia of the pancreatic duct did not express DK. e, f; Carcinoma expressed DK (brown color). DK was mainly located diffusely in the cytoplasm of the carcinoma cells. g, h; Normal epithelium did not express DK. Asterisk, Main pancreatic duct; a, DK stain; b, HE stain. a, c, e and g: immunohistochemical staining of IPMN and the epithelium of pancreatic duct with anti-DK mAb; b, d, f and h: HE stain. Bar, 10 mm (a, b), 50 μm (c-h). The serum concentration of DK was 26 U/ml (within normal limits) in this patient, but expression of DK was confirmed in the tissue.*

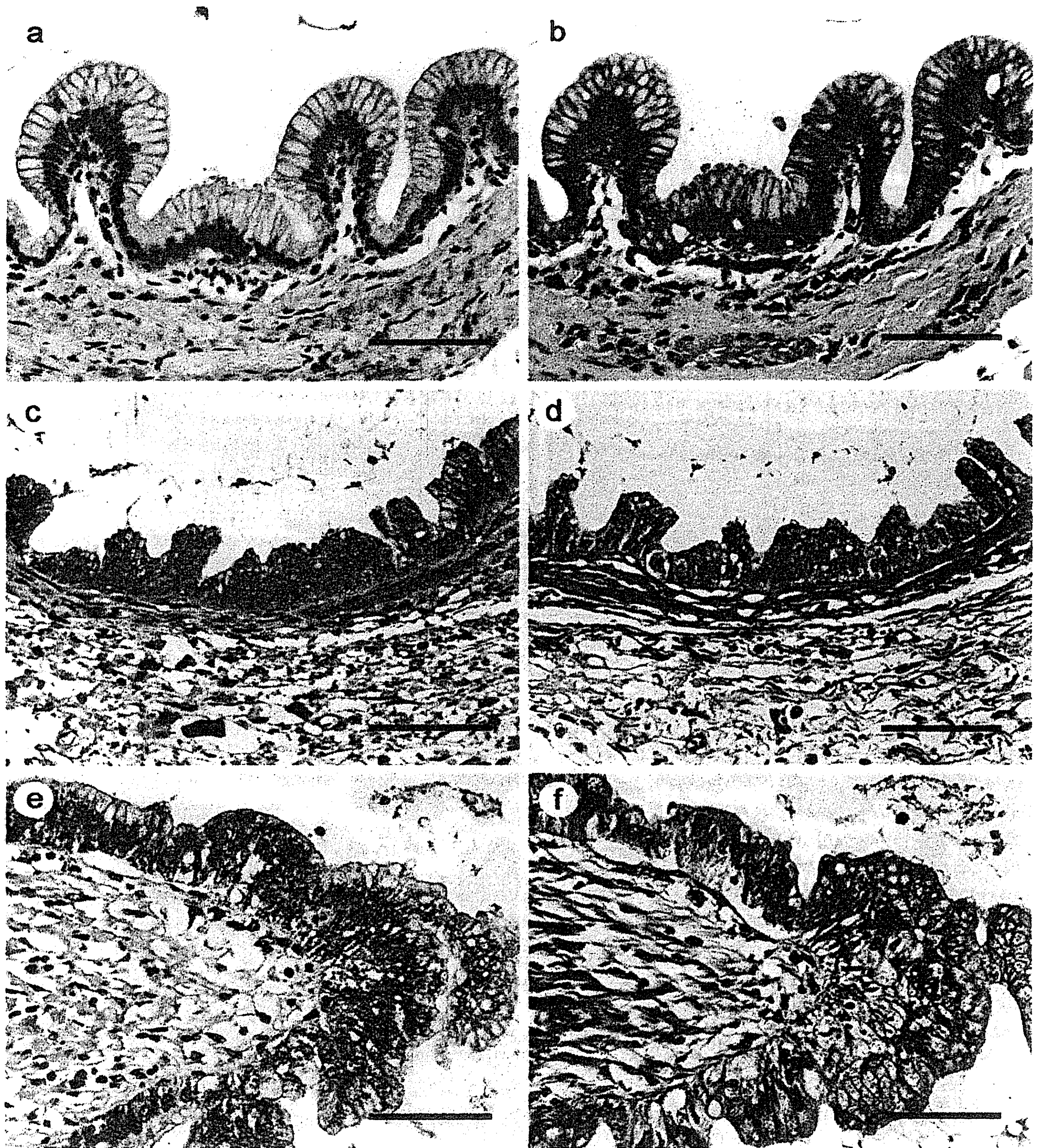

Figure 3. *Dermokine (DK) expression in dysplastic pancreatic duct. (a, c and e) Immunohistochemical staining of the atypia of pancreatic duct with anti-DK mAb. (b, d and f)HE stain. a, b; Mild atypia did not expressed DK. c, d; Moderately atypical cells expressed DK in basolateral areas. e, f; In severe atypia, cells lost polarity and cytoplasmic DK of epithelial cells was diffusely localized in the basolateral and apical areas. The localization of cytoplasmic DK was different in the various dysplastic epitheliums. Bar, 50 μm.*

9 was the most sensitive serum marker in both IPMA/IPMC/invasive carcinoma derived from IPMN and ordinary invasive ductal carcinoma (Table II). To examine the correlation of DK with other serum tumor markers, we analyzed the concentration of CA19-9, CEA, SPAN-1 and DUPAN-2 (Figure 4). In most cases, DK was found to be a unique marker for the detection of IPMA/IPMC/invasive carcinoma derived from IPMN (Figure 4b). Serum CEA-, CA19-9-, SPAN-1 and DUPAN-2-positive cases were often also positive for one of the other markers. In our data, six of

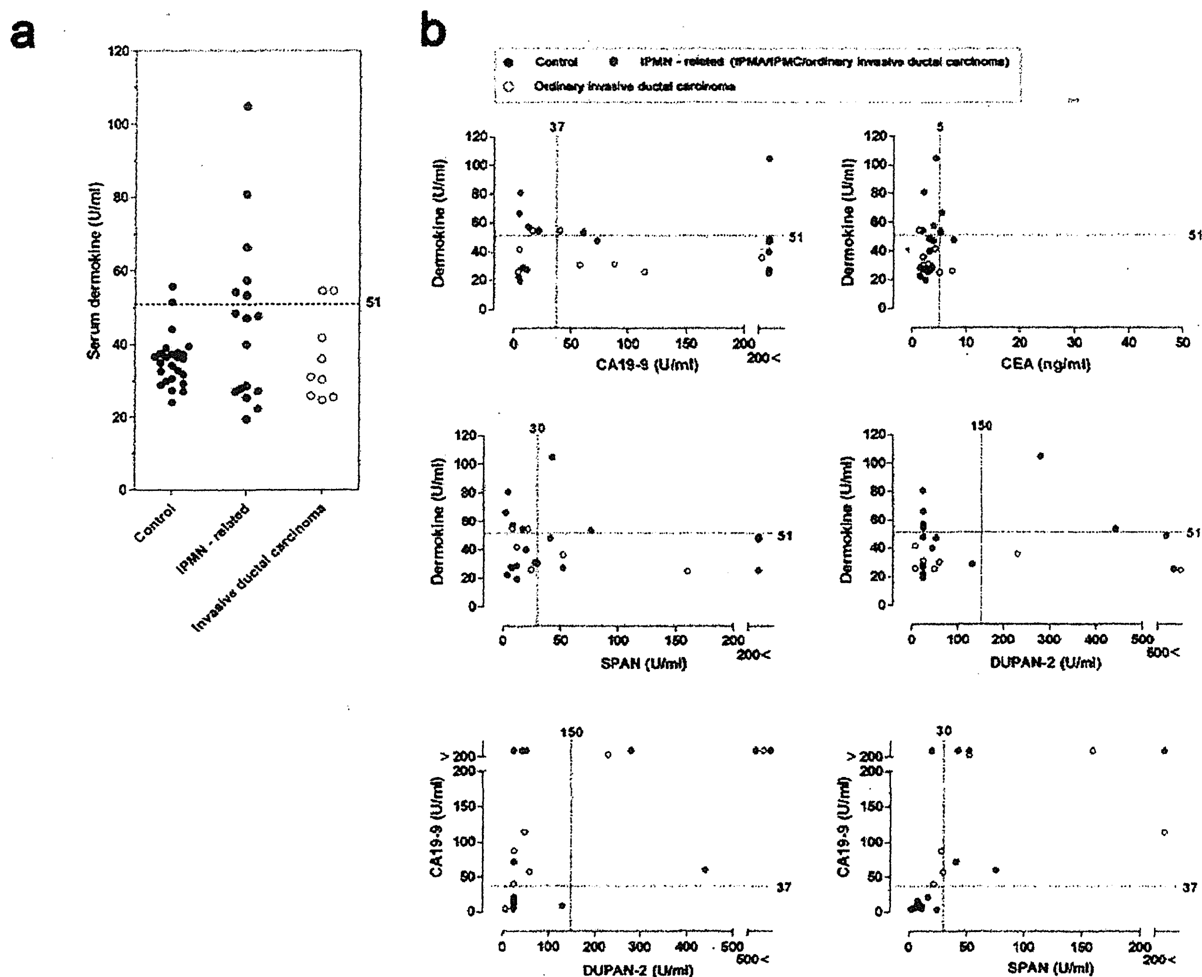

Figure 4. *Dermokine (DK) Enzyme-Linked Immunosorbent Assay (ELISA). a; Serum from 26 patients with pancreatic neoplasms (Intraductal papillary mucin-producing adenoma (IPMA)/ Intraductal papillary mucin-producing carcinoma (IPMC)/invasive carcinoma derived from IPMN, n=17; ordinary invasive ductal carcinoma, n=9) and 25 randomly selected volunteers (controls) was subjected to DK ELISA. The cut-off value was 51 U/ml. a; Six patients with IPMA/IPMC/invasive carcinoma derived from IPMN (n=6/17, 35.3%) had a high level of serum DK. b; Comparison of the sensitivity of the diagnostic markers dermokine, carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), s-pancreas-1 antigen (SPAN-1), and pancreatic cancer associated antigen (DUPAN-2) according to the histopathological diagnosis. The correlations of the sensitivities for the serum DK test with CA19-9, Span-1 and DUPAN-2 are shown in the panels. DK were complementary in IPMA/IPMC/invasive carcinoma derived from IPMN, in CA19-9 (upper left panel), CEA (upper right panel), SPAN-1 (middle left panel) and DUPAN-2 (middle right panel). DUPAN-2 and SPAN-1 positivity coincided with CA19-9 positivity (bottom panels) in most patients. The cuts-off value for each test are indicated by dotted lines.*

26 patients were double positive (Figure 4b, bottom left panel). The diagnostic rate was improved to 76.5% (IPMN, n=13/17) and 100.0% (invasive ductal carcinoma, n=9/9) when using DK in combination with the other five tumor markers (Table II). DUPAN-2 is also known as the carbohydrate antigen complex (Lc4), which is complementary to CA19-9.

Discussion

Dermokine was identified as a gene expressed in the spinous and granular layer of stratified squamous epithelium by high-throughput in situ hybridization, and is related to cell differentiation and multilayer structure (5). We have reported ectopic expression of DK in lesions with high cellularity in colonic adenoma and carcinoma, and its cytoplasmic distribution changes at different stages of carcinogenesis (4). Briefly, the distribution of DK was limited to the apical lesion of adenoma cells, although the location of DK was diffuse in the cytoplasm of carcinoma. Immunohistochemistry revealed that the normal epithelial cells of the pancreatic duct did not express DK (Figure 2). The ectopic expression of DK changed the cytoplasmic distributions, with the loss of

Table II. *Sensitivities of multimarker tests in detecting pancreatic neoplasms. The serodiagnostic positive rates for all six markers dermokine (DK), carbohydrate antigen 19-9(CA19-9), carcinoembryonic antigen (CEA), s-pancreas-1 antigen (SPAN-1), pancreatic cancer associated antigen (DUPAN-2), and Nation Cancer Center-Stomach-439 (NCC-ST-439), and their combinations are showns. Serum DK was high in IPMA/IPMC/invasive carcinoma derived from IPMN. CA19-9 was the most sensitive marker for invasive ductal carcinoma and IPMA/IPMC/invasive carcinoma derived from IPMN. Serum tests for all six markers had 100% sensitivity for ordinary invasive ductal carcinoma (n=9/9), and 76.5% of IPMA/IPMC/invasive carcinoma derived from IPMN (n=13/17). This was because the DK test was complementary to the tests for other markers. DK was the best partner for CA19-9 for a diagnosis of pancreatic neoplasm.*

	IPMA/IPMC/invasive carcinoma derived from IPMN	Ordinary invasive ductal carcinoma
DK	6 (35.3%)	2 (22.2%)
CEA	3 (17.6%)	2 (22.2%)
CA19-9	8 (47.1%)	6 (66.7%)
Span-1	7 (41.2%)	3 (33.3%)
DUPAN-2	4 (23.5%)	2 (22.2%)
NCC-ST-439	2 (11.8%)	1 (11.1%)
DK and CA19-9	12 (70.6%)	8 (88.9%)
5 Markers without DK	10 (58.8%)	8 (88.9%)
6 Markers	13 (76.5%)	9 (100%)
Total cases (%)	17 (100%)	9 (100%)

cellular polarity (Figure 3). Although the molecular function of DK is still unclear, it was interesting that the cytoplasmic distribution was different in atypical tissues of the pancreatic duct, colonic adenoma and colonic carcinoma, as we previously described (4). These findings are suggestive of the different manners of carcinogenesis in colonic cancer and IPMN-induced pancreatic cancer. To our knowledge, this is the first study of DK expression in pancreatic neoplasm.

There are two hypotheses to explain pancreatic carcinogenesis. The first is pancreatic intra-epithelial neoplasia: the PanIN hypothesis. PanIN lesions are associated with somatic alterations in canonical oncogenes and tumor-suppressor genes. Most notably, early PanIN lesions and almost all pancreatic ductal adenocarcinomas involve mutations in the KRAS oncogene. Thus, it is believed that activation of KRAS mutations is crucial for the initiation of pancreatic ductal carcinogenesis (6). The other hypothesis is that of hyperplasia/adenoma/carcinoma in IPMN. The molecular abnormalities in IPMN are still unclear because some genetic changes are similar but others are clearly different to those of PanIN (7). Recently, Izeradjene *et al.* have reported that $Kras^{G12D}$ and *Smad4/Dpc4* haploinsufficiency co-operated to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas in a mouse model (8). However, IPMN is a disease of the ductal epithelium and represents a spectrum of disease, ranging from benign to malignant lesions in humans. The early detection and characterization of IPMN lesions are important because definitive management is surgical resection for malignant lesions and for benign lesions with malignant potential.

IPMN has been reported to account for approximately 7% of clinically diagnosed pancreatic neoplasms and up to 50% of incidentally detected pancreatic cysts (9). The incidence of these mucin-producing epithelial tumors of the exocrine pancreas has been increasing (10). This is probably attributed to improvements in technology and diagnostic imaging, as well as more distinct nomenclature. Although adequate screening tests have not been established to detect malignant IPMN, Nara *et al.* reported that a high level of serum CA19-9 is a poor prognostic factor in patients with invasive ductal carcinoma derived from IPMN (11).

Computed tomography, ultrasonography and CA19-9 blood test are widely performed to investigate pancreatic neoplasms, but these procedures are not adequate in terms of cost and specificity. CA19-9 is a terminal structure in various glycoproteins and glycolipids. It is a carbohydrate antigen complex whose expression is dependent on the activity of fucosyltransferase. This means that in 4-10% of the population (Lewis antigen-negative) in Japan, patients are genetically negative for CA19-9 even if they have a pancreatic neoplasm. In addition, CA19-9 is located in the epithelium of normal pancreatic duct, but its cytoplasmic distribution is limited to apical lesions, similar to CEA in normal colon epithelium. This is one reason for the low CA19-9 concentration in normal structures. Thus, the serum concentration of CA19-9 is high in patients with an inflammatory or cystic disease due to a loss of cell polarity, and the presence of abnormal structures. The conventional biomarkers, CEA, SPAN-1 and DUPAN-2 show no correlation with the aggressive nature of IPMN (12).

As shown in Figure 4 and Table I, DK is a unique marker and it improved the diagnostic sensitivity for IPMN and invasive ductal carcinoma. We suggest that a serum DK test might be the best partner for CA19-9 for a diagnosis of pancreatic neoplasm.

In conclusion, serum DK is a potential biomarker in IPMA/IPMC/invasive carcinoma derived from IPMN and ordinary invasive ductal carcinoma of pancreas when used in combination with other conventional biomarkers.

Conflicts of Interest

The Authors have no potential conflicts of interest to disclosed.

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ORIGINAL ARTICLE

Pancreatic Volumetric Assessment as a Predictor of New-Onset Diabetes Following Distal Pancreatectomy

Sachiyo Shirakawa · Ippei Matsumoto · Hirochika Toyama · Makoto Shinzeki · Tetsuo Ajiki · Takumi Fukumoto · Yonson Ku

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Abstract
Introduction Pancreatogenic diabetes after pancreatectomy is of growing importance due to the increasing life expectancy of pancreatectomized patients. Although reduction of pancreatic volume is thought to affect glucose metabolism, a consistent relationship has yet to be determined. This study aimed to investigate functional consequences of distal pancreatectomy (DP) in preoperatively non-diabetic patients.
Methods This study included 61 non-diabetic patients who underwent DP. Clinical data were obtained, and the percent resected volume (PRV) of each pancreas was determined via multi-detector row computed tomography volumetry.
Results During the follow-up period (median 26 months), 22 patients (36 %) developed new-onset diabetes within a median onset time of 8 months (range 0.5–42 months) postoperatively. The remaining 39 patients also showed impaired glucose metabolism. Multivariate analysis identified preoperative hemoglobin A1c≥5.7 % (odds ratio 15.6, p=0.001) and PRV> 44 % (odds ratio 11.3, p=0.004) as independent risk factors for new-onset diabetes.
Conclusions Key determinants of postoperative glycemic control include preoperative functional reserve of the endocrine pancreas and the volume reduction of pancreatic parenchyma. Our findings enable reliable preoperative evaluation of the risk of postoperative diabetes and appropriate postoperative surveillance, which is helpful for early intervention in high risk patients.

Keywords Pancreas · Volumetry · Pancreatic diabetes

Introduction

Pancreatogenic diabetes, classified as type 3c by the American Diabetes Association,[1] is associated with diseases of the exocrine pancreas including pancreatitis, benign and malignant neoplasm, cystic fibrosis, hemochromatosis, fibrocalculous pancreatopathy, and trauma and pancreatectomy. Among the 8–9 % of the general diabetes population with type 3c diabetes in Western countries, 2–3 % are those who underwent pancreatectomy.[2,3] Pancreatectomized patients are at high risk for type 3c diabetes, as well as type 2, because surgery inevitably results in a deficit in the exocrine and endocrine pancreas, and also can promote the progression of underlying disease. Due to improved diagnostic modalities and a more refined understanding of pancreatic neoplasm pathogenesis, pancreatectomies for benign or low-grade malignant tumors are more frequent, and the life expectancy of patients undergoing pancreatectomy has increased in recent years. As the frequency of pancreatectomy and length of life expectancy increase, so does the importance of the risk of pancreatogenic diabetes associated with pancreatic surgery.

Distal pancreatectomy (DP) is the standard procedure used for removal of lesions in the body and tail of the pancreas. Long-term disturbances in glucose metabolism are a major concern after DP because previous studies have found that postoperative diabetes develops in from 4.8 to 38 % of patients after DP.[4–8] Physiological factors reported to correlate with postoperative pancreatic endocrine function include preoperative fasting plasma glucose (FPG), body mass index

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S. Shirakawa · I. Matsumoto (✉) · H. Toyama · M. Shinzeki · T. Ajiki · T. Fukumoto · Y. Ku
Division of Hepato-Biliary-Pancreatic Surgery, Department of Surgery, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
e-mail: imatsu@med.kobe-u.ac.jp

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