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.

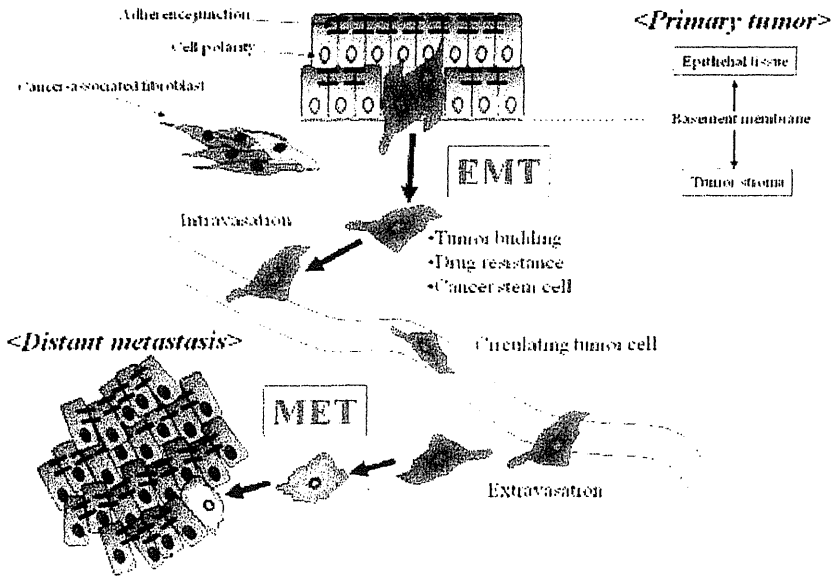

Fig. 2. The epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are involved in cancer metastasis. Cancer cells undergoing EMT in a primary tumor disseminate through the fragmented basement membrane and acquire the characteristics of drug resistance and cancer stem cells. 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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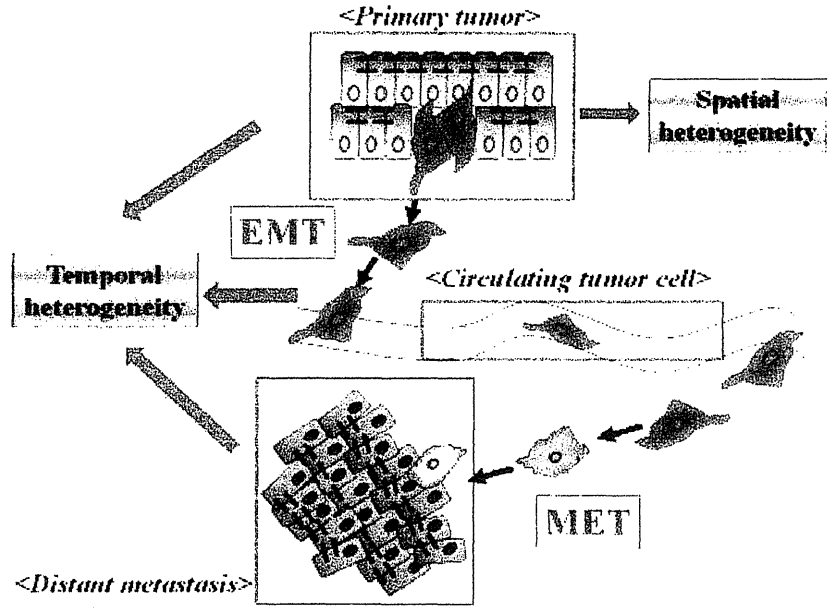

Fig. 3. Spatial and temporal heterogeneity of the epithelial–mesenchymal transition (EMT). Cancer cells undergoing EMT are expected to be only a small proportion of primary tumor tissues. EMT cells transported to metastatic sites are expected to undergo and mesenchymal–epithelial transition (MET). Therefore, 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

KEŃJI 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).

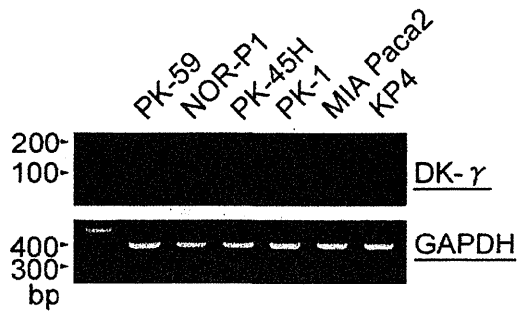

Figure 1. *Dermokine (DK) expression in pancreatic cancer cell lines. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of DK-γ in six pancreatic cell lines. DK-γ mRNAs was expressed in PK-59, NOR-P1, PK-45H, PK-1 and KP4 cells, but not in MIA Paca2 cells. The sizes of amplicons were 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-

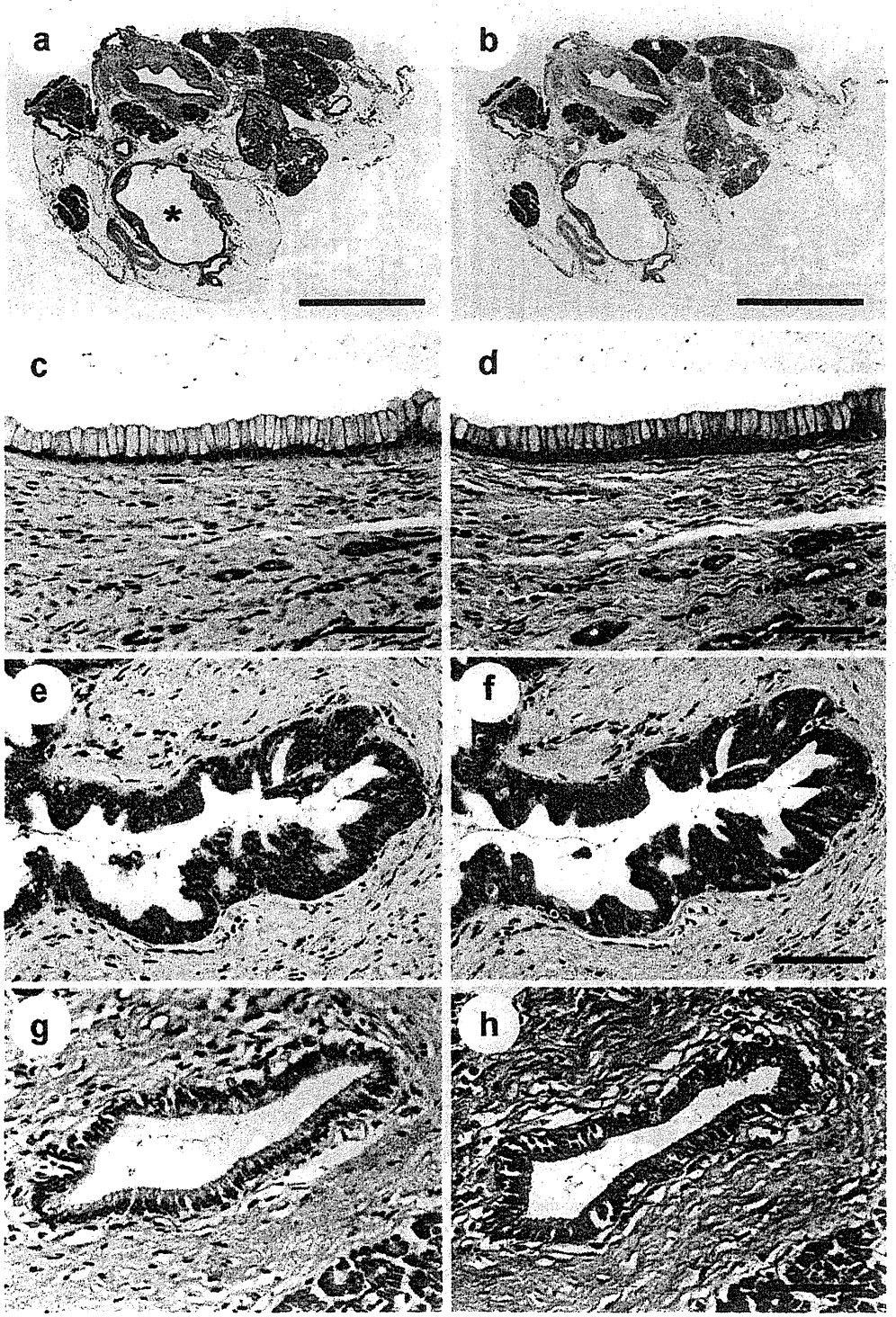

Figure 2. *Dermokine (DK) expression in Intraductal papillary mucinous neoplasm IPMN. a, b; Low-power view of IPMN. b; Adenoma (blue lines) and carcinoma in situ (Tis, red lines). c, d; Mucus cell hyperplasia of the pancreatic duct did not express DK. e, f; Carcinoma expressed DK (brown color). DK was 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.*

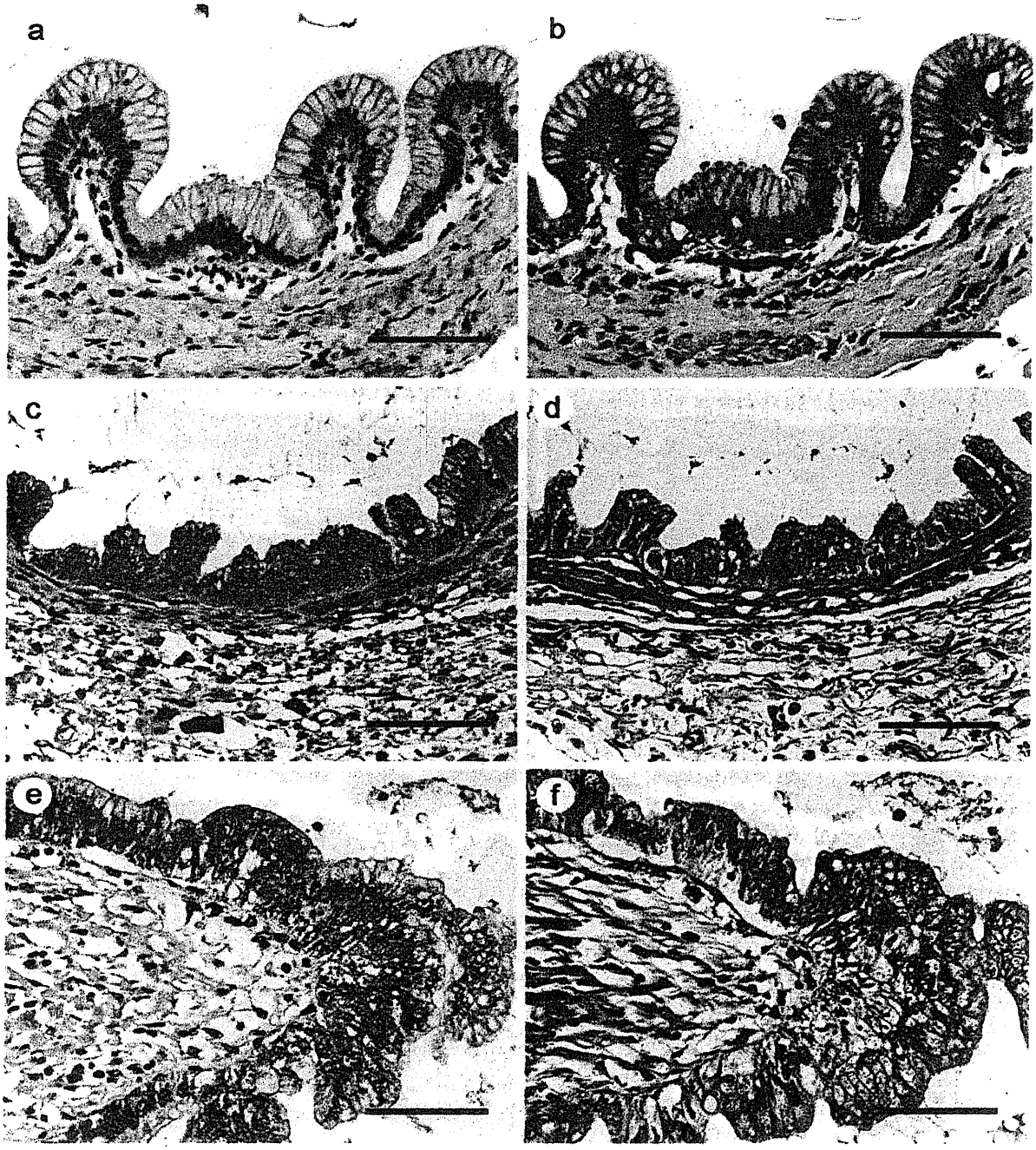

Figure 3. *Dermokine (DK) expression in dysplastic pancreatic duct. (a, c and e) Immunohistochemical staining of the atypia of pancreatic duct with anti-DK mAb. (b, d and f)HE stain. a, b; Mild atypia did not expressed DK. c, d; Moderately atypical cells expressed DK in basolateral areas. e, f; In severe 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

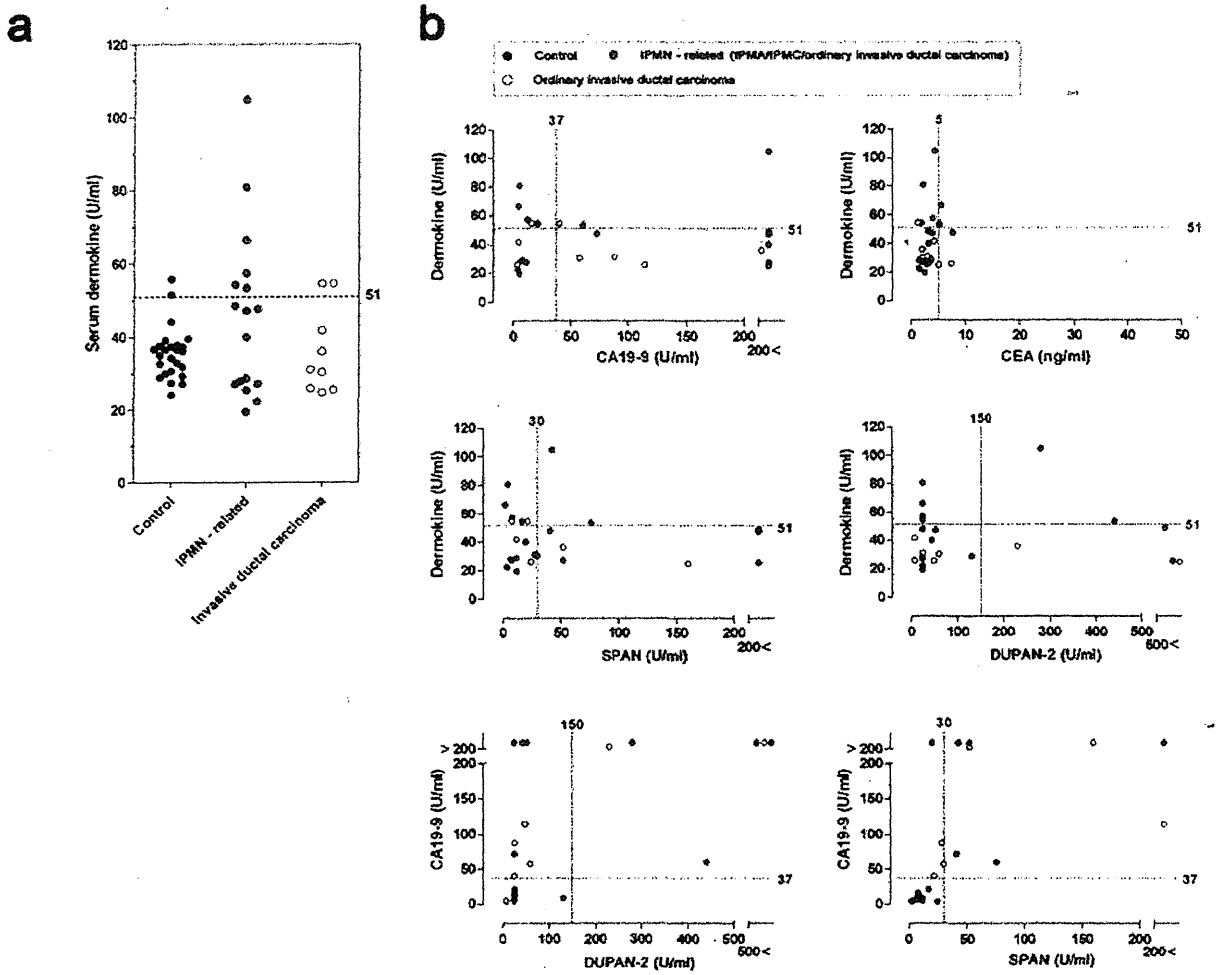

Figure 4. *Dermokine (DK) Enzyme-Linked Immunosorbent Assay (ELISA). a; Serum from 26 patients with pancreatic neoplasms (Intraductal papillary mucin-producing adenoma (IPMA)/ Intraductal papillary mucin-producing carcinoma (IPMC)/invasive carcinoma derived from IPMN, n=17; ordinary invasive ductal carcinoma, 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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(BMI), and postoperative complications.[9–11] Limitations in these studies, such as unspecified preoperative diabetic status of the patients and inconsistent standards used for the diagnosis of postoperative diabetes, make it difficult to reliably identify risk factors for postoperative diabetes.

Although the mass of pancreatic beta cells has been identified as an important determinant of plasma glucose levels in rodents, dogs, monkeys, and humans,[12–15] to our knowledge, very few studies have directly investigated the volume reduction of human pancreatic parenchyma as a risk factor for diabetes, and no previous study systematically quantified resection volumes in a population of patients. To study potential risk factors for new-onset diabetes in preoperatively non-diabetic patients, we sought to reliably quantify the volume reduction of human pancreatic parenchyma and to determine its longitudinal metabolic consequences following DP using multi-detector row computed tomography (MDCT) imaging volumetry.

Methods

Patients

A series of 98 consecutive patients who underwent DP at our institution between January 2005 and December 2011 was originally chosen from our prospectively maintained clinical database for this retrospective study. Data from 37 (38 %) of these candidates were excluded due to preoperative diabetes, as defined either by the WHO criteria of FPG≥126 mg/dl detected on two or more separate days, or this abnormal FPG level detected once and plasma glucose≥ 200 mg/dl measured 2 h after a 75-g glucose drink, or based on their treatment with oral anti-diabetic agents or insulin. The final study population consisted of 61 non-diabetic patients who had undergone DP.

Clinical data on pre- and postoperative patient status were obtained from existing medical records. Family histories of type 2 diabetes in first-degree relatives were also obtained. The preoperative data used for this study had been recorded within 14 days prior to surgery. Nutritional status and pancreatic endocrine functions were assessed based on measurements of body weight, serum albumin, FPG, and serum hemoglobin A1c (HbA1c). HbA1c values represent the National Glycohemoglobin Standardization Program (NGSP) equivalent values (in percent) and in all cases were converted from previous Japan Diabetes Society standard substance and measurement methods (JDS HbA1c, in percent) using the following formula: NGSP HbA1c (%)=JDS HbA1c (%)+0.4 %. The percent resected volume (PRV) of pancreatic parenchyma, excluding tumor volume, was determined from abdominal MDCT measurements. Patient data were collected until the time of diagnosis of new-onset diabetes or tumor recurrence. All 61 patients were followed up for at least 3 months.

For evaluating postoperative course, we defined and graded postoperative pancreatic fistula (POPF) using the classification methods of the International Study Group of Pancreatic Fistula,[16] with POPF grade B or C defined as clinically important pancreatic fistula. Postoperative complications were designated as level I to V based on the Clavien classification.[17]

Determination of PRV of the Pancreatic Parenchyma

PRVs were determined retrospectively, using preoperative MDCT images in all patients. Continuous 0.8-mm 64-row MDCT images were acquired following administration of intravenous contrast material prior to surgery. MDCT data were transferred to a computer workstation (Aquarius; Elk, Osaka, Japan) for measurement of pancreas volume. To delineate the actual pancreatic resection lines, we compared preoperative CT with postoperative CT.

Excluding tumors, cystic lesions, any dilation in the pancreatic duct and bile duct, and vessels, we outlined the borders of the pancreatic parenchyma and the resection lines on every CT slice, and we then computed the resected and remnant areas of pancreatic parenchyma for each slice (Fig. 1). The volume (in milliliters) of the pancreatic parenchyma per slice was calculated as the product of the pancreas area (in square millimeters) times the slice thickness (in millimeters). Resected and remnant volumes of the pancreatic parenchyma were computed as the sum of the slice volumes. PRV was determined using the following formula: $PRV(\%) = [resected\ volume\ of\ normal\ pancreas \div total\ volume\ of\ normal\ pancreas] \times 100$. PRV was calculated in 52 cases.

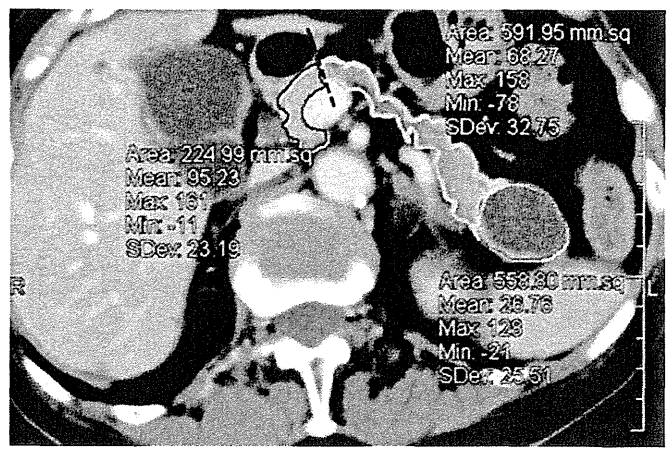

Fig. 1 MDCT pancreas volumetry. Outlined areas are the remnant parenchyma (*black outline*), resected parenchyma (*light gray outline*), and tumor (*dark grey outline*), excluding vessels. The *dashed line* is the pancreatic resection line. To determine percent resected volume, the volume (in milliliters) of the pancreatic parenchyma per slice was calculated as the product of the pancreas area (in square millimeters) times the slice thickness (in millimeters)

In the remaining nine cases, this value could not be measured accurately because of pancreatic edema or tumors with unclear borders that had invaded peri-pancreatic organs.

Definition of Postoperative New-Onset Diabetes

Postoperative new-onset diabetes was diagnosed retrospectively based on the WHO criteria of FPG≥126 mg/dl detected on two or more separate days, or this abnormal FPG level detected once and plasma glucose≥200 mg/dl measured in 2 h after a 75-g glucose drink. The onset day of diabetes was defined as the latter day on which abnormal blood test results were detected. In this study population, no patient was administered anti-diabetic therapy with oral agents or insulin before the development of diabetes, as defined by the criteria of this study.

Statistical Analysis

Patient characteristics are reported as means±standard deviation (SD), and results are presented as means±standard error (SE) or, where indicated, medians (range). Categorical variables are expressed numerically as percentages. For analyses of repeated measurements of body weight, serum albumin, FPG, and serum HbA1c prior to and 3, 6, and 12 months after surgery, we used an analysis of variance (ANOVA) and the Mauchly test, which evaluates the sphericity assumption. We used the Student's *t* test or Mann–Whitney test for continuous variables and Fisher's exact test for categorical variables. A multiple logistic regression analysis yielding odds ratios and 95 % confidence intervals (CIs) was used to identify risk factors for postoperative new-onset diabetes (with $p<0.05$). The optimal HbA1c and PRV cutoffs for predicting the occurrence of postoperative new-onset diabetes were estimated using receiver operating characteristic (ROC) curves. All analyses were performed using JMP 9.0 for Macintosh (SAS Institute Inc, Cary, NC, USA).

Results

Patients' Characteristics

Physiological characteristics of the study patients are outlined in Table 1. While no patient met the WHO criteria for diabetes preoperatively, nine had impaired fasting glucose (IFG), defined as FPG of 110–125 mg/dl. The indications for DP included pancreatic tumors in 55 of the 61 patients (90 %, 25 malignant and 30 benign tumors), alcohol-induced chronic pancreatitis in three patients, autoimmune pancreatitis mimicking pancreatic cancer in two patients, and a pseudocyst following acute pancreatitis in one patient.

Table 1 Clinical characteristics of 61 non-diabetic patients who underwent distal pancreatectomy

Male patients	24 (39)
Age (years)	62±14
BMI (kg/m^2)	21.2±3.8
Preoperative HbA1c (%)	5.8±0.41
Preoperative IFG	9 (16)
Preoperative albumin (mg/dl)	4.0±0.64
Preoperative total cholesterol (mg/dl)	182±45
Preoperative pancreatic alpha-amylase (IU/l)	59 (4–264)
Operative time (min)	333±88
Intraoperative blood loss (ml)	427 (5–3524)
Malignancy	25 (41)
Percent resected volume (%)	38±17
POPF ≥ grade B	16 (26)
Postoperative complication ≥ Clavien's grade II	22 (36)
Postoperative hospital stay (days)	18 (7–58)
Mortality (%)	0

Values are means±SD, medians (range), or *n* (%)

HbA1c hemoglobin A1c, *IFG* impaired fasting glucose, *FPG* fasting plasma glucose, *POPF* postoperative pancreatic fistula

Three patients had a first-degree family history of type 2 diabetes.

Pancreas Volumetry

MDCT imaging volumetric data showed a wide range of volumes of whole, remnant, and resected pancreatic parenchyma and of tumors in patients with or without new-onset diabetes (Table 2). While the mean PRV for all 61 cases was 38 % (range 9–85 %), the mean PRV for the new-onset diabetes group was 49 %, which was significantly higher than the PRV of 32 % for the non-diabetic group.

Sequential Changes in Diabetic and Nutritional Status After Surgery

We compared four physiological parameters in new-onset diabetic versus non-diabetic patients at four time points: before and 3, 6, and 12 months after surgery. Three months after surgery, there were significant increases in FPG and HbA1c in both groups (Table 3). Disturbances in glucose control occurred within the first 3 months after surgery, and did not significantly progress after that time in either group.

During the post-DP follow-up period (median 26 months, range 3–88 months), 22 patients (36 %) developed new-onset diabetes (median onset time 8 months, range 0.5–42 months). In most of the 39 patients without new-onset diabetes, FPG and HbA1c increased significantly during the follow-up period; however, values remained stable in eight

Table 2 CT volumetry in DP patients

	All patients	New-onset diabetes group	No new-onset diabetes group	*p* value
Number of patients with PRV data	52	20	32	
Whole normal parenchyma (ml)	56.6 (16.0–128.2)	54.6 (27.0–89.4)	56.6 (16.0–128.2)	0.58
Remnant normal parenchyma (ml)	36.5 (4.4–116.4)	25.1 (4.4–65.8)	38.6 (6.6–116.4)	0.047
Resected normal parenchyma (ml)	18.7 (3.5–57.7)	25.8 (9.9–57.7)	16.5 (3.5–55.3)	0.004
Tumor or cystic lesion (ml)	5.4 (0–543.7)	4.1 (0–38.7)	7.4 (0.2–543.7)	0.11
PRV (%)	38±17 (9–85)	49±15 (20–85)	32±15 (9–59)	< 0.001

Values are medians (range) or means±SD (range). *p* values were obtained using Mann–Whitney *U* test, except for use of Student's *t* test for PRV

CT computed tomography, *DP* distal pancreatectomy

of these 39 patients (change in HbA1c≤0.1 %), and one patient displayed improvement in glycemic control, as exhibited by a 0.4 % decrease in HbA1c.

While we observed significant between-group differences in the changes in FPG (Fig. 2a) (p=0.003) and HbA1c (Fig. 2b) (p<0.001) over time, there were no significant differences in changes in body weight (Fig. 2c) (p=0.36) or serum albumin (Fig. 2d) (p=0.58). Because Mauchly tests for the sphericity assumption were not significant for these factors (P=0.21, 0.82, 0.28, and 0.30, respectively), the reported p values are for univariate ANOVA.

Risk Factors for Postoperative New-Onset Diabetes

Univariate analyses identified three statistically significant risk factors for postoperative new-onset diabetes: preoperative HbA1c≥5.7 %, PRV>44 %, and age (Table 4). Multivariate logistic regression analysis also identified HbA1c≥ 5.7 % [odds ratio 15.6 (95 % CI 2.80–147), p=0.001] and PRV>44 % [odds ratio 11.3 (95% CI 2.12–92.1), p=0.004] as independent risk factors for postoperative new-onset diabetes (Table 5). Regarding family history, one of three patients with first-degree family history of type 2 diabetes, of whom PRV was 85 %, developed new-onset diabetes at 2 months after surgery. We assessed the sensitivity and specificity of the HbA1c and PRV parameters using the ROC curves. The areas under the ROC curves were 0.831 for HbA1c and 0.793 for PRV. Using these curves, HbA1c of 5.7 % and PRV of 44 % were determined to be the cutoffs for predicting the occurrence of postoperative diabetes. The sensitivity, specificity, and positive and negative predictive values derived from these curves were 0.82, 0.64, 0.56, and 0.86 for HbA1c and 0.75, 0.81, 0.71, and 0.84 for PRV, respectively.

Discussion

We report here two major findings from this study of patients who underwent DP. First, in the majority of preoperatively non-diabetic patients, DP led to disturbances in glucose metabolism, and there was a 36 % incidence of

Table 3 FPG and serum HbA1c before and 3, 6, and 12 months after surgery

	All patients (n=61)	*p* value	New-onset diabetes group (n=22)	*p* value	No new-onset diabetes group (n=39)	*p* value
FPG (mg/dl)						
Before surgery	96±1.9		101±3.8		93±1.9	
3 months after surgery	109±2.8	<0.001[a]	121±5.3	0.008[a]	102±2.6	0.006[a]
6 months after surgery	111±4.9	0.29[b]	133±11.6	0.17[b]	99±2.4	0.75[b]
12 months after surgery	114±5.8	0.19[b]	137±12.2	0.15[b]	100±3.0	0.55[b]
HbA1c (%)						
Before surgery	5.8±0.05		6.1±0.06		5.6±0.06	
3 months after surgery	6.2±0.10	<0.001[a]	6.7±0.19	0.003[a]	5.9±0.07	0.002[a]
6 months after surgery	6.3±0.24	0.73[b]	7.0±0.51	0.81[b]	5.9±0.07	1.00[b]
12 months after surgery	6.4±0.18	0.13[b]	7.1±0.36	0.49[b]	5.9±0.06	0.059[b]

Values are means±SE. Data were analyzed using Student's paired *t* test for each group

[a] Differences compared to values before surgery

[b] Differences compared to values at 3 months after surgery

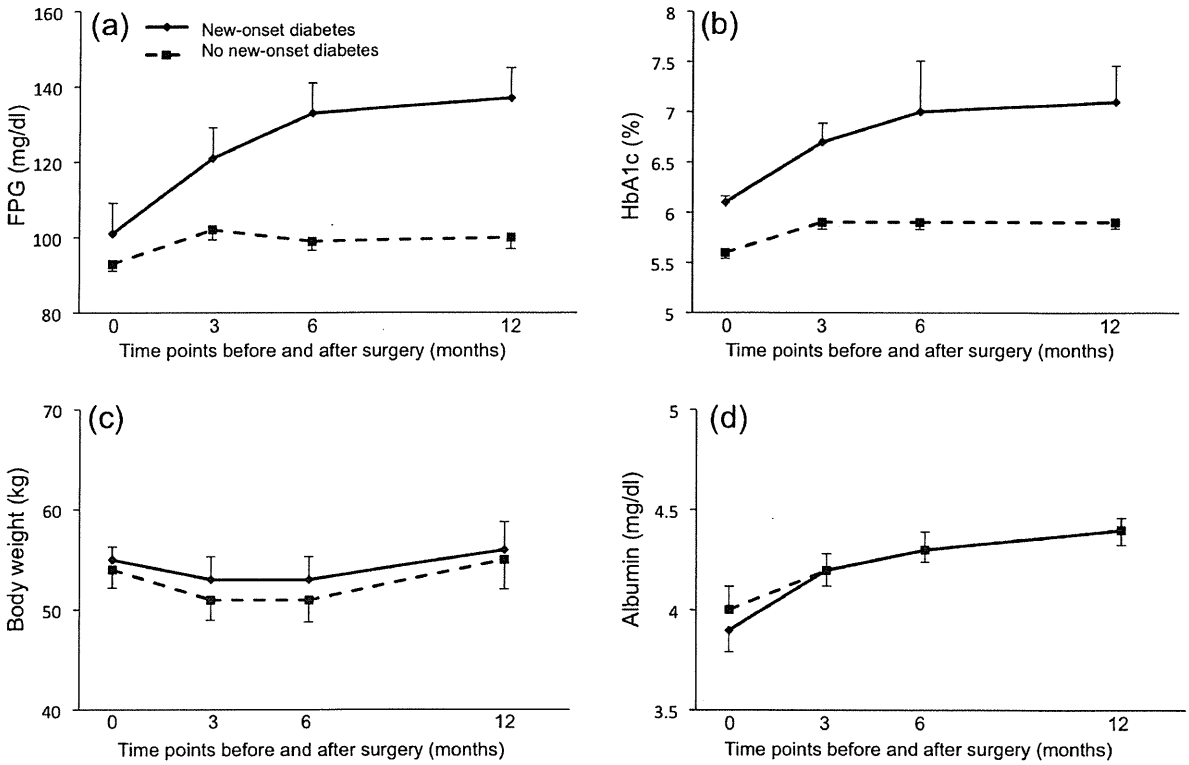

Fig. 2 Changes in parameters before and 3, 6, and 12 months after surgery: **a** FPG, **b** HbA1c, **c** body weight, and **d** serum albumin. Values from patients who developed new-onset diabetes (*solid lines*) were compared with non-diabetic patients (*dashed lines*) using ANOVA, with evaluation of the sphericity assumption by the Mauchly test. Postoperatively, there were significant between-group differences in changes in FPG ($p=0.003$) and HbA1c levels ($p<0.001$). No significant between-group difference in body weight ($p=0.36$) or albumin ($p=0.58$) was observed

new-onset diabetes postoperatively. Second, in DP patients, PRV and preoperative HbA1c were independent risk factors for new-onset diabetes.

Our results enable us to provide evidence-based preoperative counseling and individualized postoperative surveillance. Prior to surgery, we can now offer patients specific information about their individual risk of postoperative diabetes. Postoperatively, appropriate surveillance may detect the development of impaired glucose metabolism [i.e., impaired glucose tolerance (IGT), IFG, and diabetes] at an early stage and enable early intervention. Intensive glucose control has been reported to decrease the risks of major cardiovascular events and death in patients with newly diagnosed type 2 diabetes.[18] Also, in patients with IGT who are pre-diabetic, the early introduction of anti-diabetic agents has been reported to diminish the development of type 2 diabetes.[19] The American Diabetes Association recommends intensive annual monitoring, lifestyle modification, and sometimes use of anti-diabetic agents in patients with IGT, IFG, or HbA1c of 5.7–6.4 % for the prevention and delay of developing type 2 diabetes.[20] Therefore, early detection and intervention for endocrine insufficiency are essential for DP patients.

Prior reports have estimated the incidence of new-onset diabetes after DP at between 9 and 38 % of preoperatively non-diabetic patients.[4,6,8,21] The numerous limitations of these studies (such as unspecified preoperative diabetic status of study patients, inconsistent standards for diagnosis of postoperative diabetes, and selection of cohorts of patients with chronic pancreatitis) make it difficult to evaluate the basis for this wide range of diabetes incidence. In the current study, the incidence of postoperative new-onset diabetes in preoperatively non-diabetic patients was 36 %. We attribute this relatively high measure of incidence to our application of a definitive classification system and close follow-up.

The results of this study identify PRV>44 % as an independent risk factor for postoperative new-onset diabetes in preoperatively non-diabetic DP patients. Although beta cell mass has previously been reported to be significantly related to plasma glucose control,[14,22,23] volumetric assessments in relation to postoperative endocrine function of the pancreas remain scarce. Previous studies in large animals[13,15] and humans[22] have demonstrated that a 50 % loss in beta cells elevates plasma glucose. The DP procedure is often referred to as a "hemi-pancreatectomy," with an estimated 50 % reduction in pancreatic volume after transection on the superior mesenteric vein (SMV).[9,11] In our study, the median PRV in 29 patients with transection on the SMV was 46 %, but we observed a wide range of values in these cases (PRV from 18 to 67 %), as well as among all

Table 4 Univariate analysis of risk factors for postoperative new-onset diabetes

	New-onset diabetes group (n=22)	No new-onset diabetes group (n=39)	p value
Male patients	9 (41)	15 (38)	1.00[a]
Age (years)	67±3.0	59±2.2	0.025[b]
BMI (kg/m^2)	22±0.78	20±0.59	0.075[b]
Preoperative HbA1c≥5.7 %	18 (82)	14 (36)	0.001[a]
Preoperative IFG	6 (27)	3 (8)	0.059[a]
Preoperative albumin (mg/dl)	3.9±0.13	4.0±0.10	0.59[b]
Preoperative total cholesterol (mg/dl)	176±9.6	186±7.3	0.42[b]
Preoperative pancreatic alpha-amylase (IU/l)	58 (6–243)	59 (4–264)	0.98[c]
Operative time (min)	312±19	347±15	0.14[b]
Intraoperative blood loss (ml)	372 (5–1125)	527 (5–3524)	0.17[c]
Malignancy	8 (36)	17 (44)	0.79[a]
PRV>44 %	15 (75)	6 (19)	<0.001[a]
POPF ≥ grade B	6 (27)	10 (26)	1.00[a]
Postoperative complication ≥ Clavien's grade II	7 (32)	15 (38)	0.78[a]
Postoperative hospital stay (days)	16 (7–54)	18 (7–58)	0.60[c]
Adjuvant chemotherapy	7 (32)	15 (38)	0.78[a]

Values are means±SE, medians (range) or n (%)

HbA1c hemoglobin A1c, *FPG* fasting plasma glucose, *POPF* postoperative pancreatic fistula

[a] p values were obtained using Fisher's exact test

[b] p values were obtained using Student's t test

[c] p values were obtained using Mann–Whitney U test

cases of DP (PRV from 9 to 85 %). Variations in PRV can also be attributed to differences in the patients' pancreatic sizes and shapes, as well as differences in pancreatic tumor characteristics (i.e., location and the involvement of the main pancreatic duct that causes atrophy of the distal pancreas). Our use of MDCT-based measurements of pancreas volume resulted in more precise PRV values and thereby provides evidence that greater resection of pancreatic tissue increases the incidence of new-onset diabetes in preoperatively non-diabetic DP patients. Thus, although pancreatic resection must be tailored to suit the tumor character (benign or malignant), location, and extent of tumor invasion, our data suggest that parenchyma-sparing pancreatectomies (such as middle pancreatectomy or tumor enucleation) are more likely to maintain postoperative pancreatic endocrine function and reduce the risk of diabetes.

In this study, we frequently observed a delay in diabetes onset in the 22 new-onset diabetics (median time 8 months, range 0.5–42 months), with only five showing signs of diabetes within 3 months. However, both groups of DP patients displayed significant increases in FPG and HbA1c levels within 3 months following surgery, but without further increases thereafter. These data lead us to hypothesize that, while surgical reduction of pancreatic parenchyma volume quickly impairs glucose metabolism, the observed lag in diabetes onset depends on other factors, such as the amount and overall health of the remaining endocrine pancreas that control plasma glucose.

In patients with insufficient functional reserve of the remnant pancreas to compensate for beta cell deficit (with severity depending on the volume of the pancreas removed), overt diabetes would develop in the early postoperative period (within 3 months postoperatively). The functional reserve of the endocrine pancreas could be estimated based on the preoperative HbA1c value, which was identified as a risk factor predictive of postoperative diabetes in this study. In patients with late-onset diabetes (later than 3 months after surgery), beta cell compensation would considerably influence the diabetes onset time. In the field of islet cell transplants, although obese individuals are generally at high risk of diabetes, it has been reported that the high demand for insulin in obese donors without diabetes promotes the necessary increase in islet cell hypertrophy and proliferation.[24] Islet cells that remain after pancreatectomy are likely in a similar situation that stimulates islet hypertrophy and thus a compensatory increase in insulin secretion in the endocrine pancreas. Eventually, however, this pre-diabetic state may progress to overt diabetes once the endocrine pancreas is exhausted and fails to control glucose homeostasis. Also,

Table 5 Multivariate logistic regression analysis of risk factors for postoperative new-onset diabetes

	Odds ratio	95 % CI	p value
Age	1.03[a]	0.96–1.11	0.42
Preoperative IFG	1.52	0.18–14.7	0.69
Preoperative HbA1c≥5.7 %	15.6	2.80–147	0.001
PRV>44 %	11.3	2.12–92.1	0.004

[a] Odds ratio by 1 year post-DP

additional factors that vary the timing of late-onset postoperative diabetes include normal progression of underlying diseases, such as pancreatitis, as well as acquired risk factors for type 2 diabetes (e.g., weight gain or aging).

Our analyses identified preoperative HbA1c as a second risk factor for postoperative new-onset diabetes. HbA1c is increasingly viewed as a superior index of chronic hyperglycemia relative to plasma glucose (which varies during the day), and the American Diabetes Association recently added HbA1c≥6.5 % to its diagnostic criteria for the detection of early diabetes, with slightly lower HbA1c values (from 5.7 to 6.4 %) categorized as signaling an increased risk of diabetes.[1] In our study, the cutoff value at which HbA1c became a risk factor was 5.7 %. Among the study's 61 patients (none of whom met the WHO criteria for diabetes, FPG≥126 mg/dl), preoperative HbA1c was ≥6.5 % in two patients and between 5.7 and 6.4 % in another 30 patients. Of these 32 individuals, 18 patients (56 %) developed postoperative diabetes. However, it is noteworthy that four additional patients with HbA1c<5.7 %, but relatively high PRV (from 47 to 61 %), also developed diabetes.

The metabolic consequences of pancreatic resection are multifaceted and can be affected by glucoregulatory hormone concentrations, the balance between production and utilization of glucose, changes in insulin sensitivity and nutritional status, surgical complications,[25,26] and tumor character (malignant or benign). Despite decreases in insulin secretion, some studies have reported post-pancreatectomy improvements in glucose control in malignant cases.[27,28] Because malignant pancreatic tumors are known to produce substances that impair the action of insulin and decrease insulin sensitivity, a patient's diabetic status can sometimes improve after tumor removal.[29,30] Of the nine patients in our study who showed stable glycemic control (eight with HbA1c≤0.1 %) or improved control (one with HbA1c reduced by 0.4 %) during the post-DP follow-up period, five had malignant tumors. The one patient with improved glycemic control had a highly advanced cancer, as well as preoperative HbA1c and BMI levels of 6.2 % and 28.7 kg/mm^2, respectively, indicating pre-diabetes. This patient lost 13 % of his body weight within 3 months after surgery, and it is likely that this loss helped suppress the progression of type 2 diabetes. Our results did not demonstrate any influence of malignant tumors on postoperative endocrine function.

Conclusion

Adequate preoperative functional reserve of the endocrine pancreas (HbA1c<5.7 %) and maximizing the volume of the pancreatic parenchyma preserved are two key determinants of successful postoperative glycemic control. Our findings enable reliable preoperative evaluation of the risk of developing diabetes and to perform postoperative surveillance appropriately. Late-onset diabetes needs to be recognized as a common sequela of DP, and longitudinal follow-up and preventive intervention (weight control and anti-diabetic agents for pre-diabetic patients) should be introduced in high-risk patients.

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