

Figure 1. Quantitative analysis of *ADAM 9* mRNA expression in bulk tissues of pancreatic cancer and non-neoplastic pancreas. Expression of *ADAM 9* mRNA was measured using one-step quantitative real-time RT-PCR with gene-specific primers and was normalized to that of 18S rRNA. Expression of *ADAM 9* mRNA did not differ significantly between bulk tissues of pancreatic cancer and non-neoplastic pancreas ($p=0.56$).

neoplastic pancreas (Figure 1, $p=0.56$). Pancreatic cancer tissues typically contain a few carcinoma cells amid a large amount of other structures such as stromal components composed of fibroblasts, endothelial cells and inflammatory cells. Non-neoplastic pancreata included in the present study were typically taken from the distal normal portion of resected pancreata with pancreatic tumors. The histological appearance of these non-neoplastic pancreata showed chronic pancreatitis, possibly secondary pancreatitis due to the occlusion of pancreatic ducts by tumor. These pancreata were also typically composed of various components including inflammatory lesions, stromal lesions and remnant normal structures. These findings suggest that analysis of bulk tissues does not accurately reflect expression levels of *ADAM 9* mRNA in pancreatic cancer cells and normal pancreatic epithelial cells.

Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in human pancreatic cancer cell lines and primary cultures of pancreatic fibroblasts. The present bulk tissue analysis showed no significant difference in the level of *ADAM 9* mRNA between bulk tissues of pancreatic cancer and non-neoplastic pancreas. We performed histological examination of these tissues and found that most of the pancreatic cancer samples and a subset of the non-neoplastic pancreas samples consisted predominantly of stromal components. In order to evaluate the effect of stromal cells in these tissues on *ADAM 9* and *ADAM 15* mRNA

expression, we measured *ADAM 9* and *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts and compared these levels with those in cultured pancreatic cancer cell lines.

Primary cultures of pancreatic fibroblasts showed levels of *ADAM 9* mRNA similar to those of pancreatic cancer cell lines (Figure 2A). These results suggest that the presence of fibroblasts in bulk tissues may affect the expression profiles of *ADAM 9* mRNA in cancer. Pancreatic cancer cell lines also showed significant levels of *ADAM 9* mRNA, but we found no correlation between the histological grade of differentiation and the level of *ADAM 9* mRNA.

The levels of *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts were much lower than those in pancreatic cancer cell lines (Figure 2B). All of the pancreatic cancer cell lines examined in the present study expressed significant levels of *ADAM 15* mRNA, but we found no correlation between the histological grade of differentiation and the level of *ADAM 15* mRNA.

Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in microdissected pancreatic cancer cells and normal pancreatic epithelial cells. On the basis of the results obtained with bulk tissues and cultured cells, we performed LMPC to isolate pancreatic cancer cells and normal pancreatic epithelial cells so as to obtain accurate *ADAM 9* and *ADAM 15* mRNA expression profiles. We then measured *ADAM 9* and *ADAM 15* mRNA expression in these cells with one-step quantitative real-time RT-PCR with gene-specific primers. We typically isolated pancreatic cancer cells from invasive components of pancreatic cancer tissues and normal epithelial cells from normal pancreata or chronic pancreatitis-affected pancreata. Microdissected pancreatic cancer cells showed significantly higher levels of *ADAM 9* mRNA (median, 1.79) than did normal pancreatic epithelial cells (median, 0.85; $p=0.016$) (Figure 3A). Similarly, microdissected pancreatic cancer cells showed significantly higher levels of *ADAM 15* mRNA (median, 0.12) than did normal pancreatic epithelial cells (median, 0.02; $p=0.004$) (Figure 3B). We found no correlation between the histological grade of differentiation and the level of *ADAM 9* or *ADAM 15* mRNA in microdissected pancreatic cancer cells, similar to the results obtained with pancreatic cancer cell lines (Figure 2A).

We measured both *ADAM 9* and *ADAM 15* mRNA in seven microdissected samples of pancreatic cancer cells. We found no significant correlation between *ADAM 9* and *ADAM 15* mRNA levels. None of the samples showed high levels of both *ADAM 9* and *ADAM 15* mRNA. Two of the seven samples of pancreatic cancer cells (28.5%) showed low levels of both *ADAM 9* and *ADAM 15* mRNA. Five of the seven samples (71.5%) showed significant levels of *ADAM 9* or *ADAM 15* mRNA. These results suggest that *ADAM 9* and *ADAM 15* may play independent roles in pancreatic cancer.

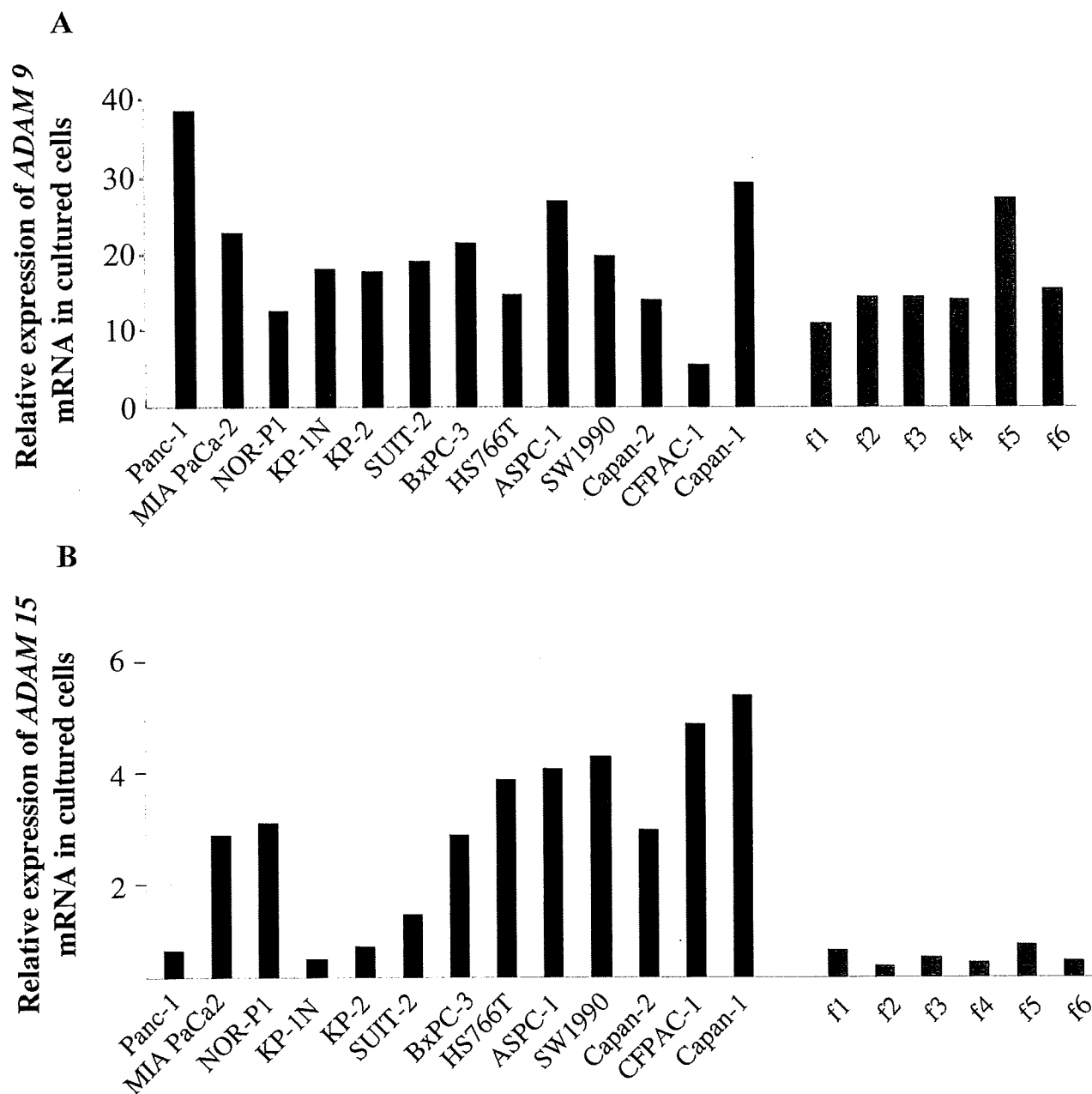


Figure 2. Quantitative analysis of ADAM 9 and ADAM 15 mRNA expression in human pancreatic cancer cell lines (Panc-1 to Capan-1) and primary cultured pancreatic fibroblast (f1-f6). We measured ADAM 9 and ADAM15 mRNA expression in 13 pancreatic cancer cell lines and six cultures of primary cultured pancreatic fibroblast. All of the pancreatic cancer cell lines and the primary cultured pancreatic fibroblasts expressed ADAM 9 and ADAM 15 mRNA. We found no correlation between the histological grade of differentiation and levels of ADAM 9 and ADAM 15 mRNA in cultured pancreatic cancer cells. Primary cultured pancreatic fibroblasts showed levels of ADAM 9 mRNA similar to those of the pancreatic cancer cell lines, but the levels of ADAM 15 mRNA were much lower in primary cultured pancreatic fibroblasts than in pancreatic cancer cell lines.

Five out of nine samples of normal pancreatic epithelial cells (55.6%) showed ADAM 15 mRNA levels below the level corresponding to 0.01 ng of total RNA derived from Capan-1 cells, although these cells showed higher levels of 18S rRNA

than the levels corresponding to 0.08 ng of total RNA derived from Capan-1 cells, suggesting that the relative levels of ADAM 15 mRNA normalized to the levels of 18S rRNA in these samples were lower than 0.125, which was used as the

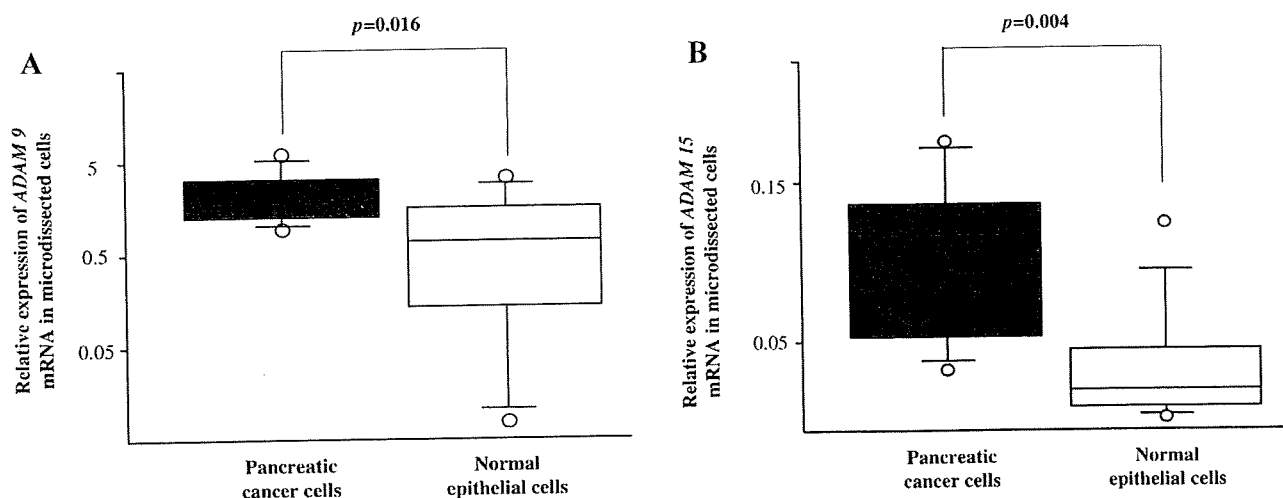


Figure 3. Quantitative analysis of *ADAM 9* and *ADAM 15* mRNA in pancreatic cancer cells and normal pancreatic epithelial cells. We isolated pancreatic cancer cells and normal pancreatic epithelial cells from frozen sections by microdissection and performed one-step quantitative real-time RT-PCR with gene-specific primers to analyze the levels of *ADAM 9* and *ADAM 15* mRNA in these cells. Expression of *ADAM 9* and *ADAM 15* mRNA was normalized to that of 18S rRNA. We found that pancreatic cancer cells showed significantly higher levels of *ADAM 9* and *ADAM 15* mRNA than did normal pancreatic epithelial cells ($p=0.016$ for *ADAM 9*; $p=0.004$ for *ADAM 15*).

value for statistical analysis. In contrast, 9 out of 13 samples of normal pancreatic epithelial cells (79.2%) showed significant levels of *ADAM 9* mRNA. In the present study, cultured pancreatic fibroblasts showed significant levels of *ADAM 9* mRNA but did not show any *ADAM 15* mRNA. These results suggest that *ADAM 15* may not play a major role under normal conditions and may not be involved in tumor-stromal cell interactions; expression of *ADAM 15* may be more specific than that *ADAM 9* to pancreatic cancer cells.

In the present microdissection-based experiments, 8 out of 11 samples (72.7%) were derived from resected tumors with lymph node metastasis. Levels of *ADAM 9* mRNA in these 8 samples (median, 2.17) were higher than those in the samples without lymph node metastasis (median, 1.07), although the difference was not statistically significant ($p=0.07$). Seven out of 11 patients included in the present study (63.6%) survived longer than 15 months, and the levels of *ADAM 9* mRNA in the samples from these patients (median, 1.31) were lower than those in the other samples (median, 2.38), although the difference was not statistically significant ($p=0.06$). These results are consistent with previous reports suggesting that *ADAM 9* may play roles in tumor aggressiveness, progression and prognosis (8, 16).

Discussion

In this study, we performed quantitative analysis of *ADAM 9* and *ADAM 15* mRNA levels in human pancreatic cancer cell lines, primary cultured pancreatic fibroblasts, microdissected

pancreatic cancer cells and normal pancreatic epithelial cells with quantitative real-time RT-PCR. Analysis of bulk tissues showed no significant difference in the levels of *ADAM 9* mRNA between pancreatic cancer and non-neoplastic pancreas. However, using microdissection we found that pancreatic cancer cells showed significantly higher levels of *ADAM 9* mRNA than did normal epithelial cells. Pancreatic cancer tissues typically contain a few ductal carcinoma cells amid a large amount of stromal components. We also confirmed that primary cultured pancreatic fibroblasts express significant levels of *ADAM 9* mRNA. Grutzmann *et al.* (14) performed microdissection and microarray based gene expression analysis and found differential expression of *ADAM 9* between pancreatic cancer cells and normal epithelial cells. However, Tan *et al.* (29) reported no overexpression of *ADAM 9* using microarray analysis of bulk pancreatic cancer tissues. Thus, microdissection is necessary for the accurate analysis of *ADAM 9* in pancreatic cancer.

All of the primary cultured pancreatic fibroblasts analyzed expressed *ADAM 9* and *ADAM 15* mRNA. Levels of *ADAM 9* mRNA were similar to those of cultured pancreatic cancer cell lines. Mazzocca *et al.* (30) reported that *ADAM 9* protein was expressed by stromal liver myofibroblasts in metastatic liver tumors and suggested that *ADAM 9* secreted by stromal liver myofibroblasts may promote carcinoma invasion. Primary cultured pancreatic fibroblasts examined in the present study were obtained from tumors with the histological appearance of invasive ductal carcinoma, indicating that tumor-associated

pancreatic fibroblasts may play an important role in the progression of pancreatic cancer. However, *ADAM 15* may not be involved in tumor-stromal cell interactions because the expression levels of *ADAM 15* mRNA in primary cultures of pancreatic fibroblasts were much lower than those in cultured pancreatic cancer cell lines.

In the present study, we isolated pancreatic cancer cells from tumors for microdissection-based quantitative analysis. Results showed that the levels of *ADAM 9* mRNA were significantly higher in pancreatic cancer cells than in normal epithelial cells. In addition, the levels of *ADAM 9* mRNA appeared to be related to lymph node metastasis and prognosis. *ADAM 9* sheds and activates several growth factors, such as heparin-binding EGF and transforming growth factor- α (10), and also binds several integrins (11-13) and degrades ECM components such as fibronectin and gelatin (12). Mazzocca *et al.* (30) showed that *ADAM 9* promotes carcinoma invasion, suggesting that *ADAM 9* plays a role in pancreatic cancer, contributing to its aggressiveness and poor prognosis.

Microdissection-based quantitative analysis showed that the levels of *ADAM 15* mRNA were significantly higher in pancreatic cancer cells than in normal epithelial cells. The targets of *ADAM 15* ectodomain shedding are EGF family ligands (20). The disintegrin domain of *ADAM 15* binds several integrins (22-24) and degrades ECM components such as Type IV collagen and gelatin (12). *ADAM 15* has been reported to be up-regulated in malignant tumors and related to tumor aggressiveness (17-19). However, Herren *et al.* (31) reported that *ADAM 15* overexpression enhanced cell-cell interactions and reduced cell migration. The role of *ADAM 15* in cancer progression remains controversial. Our results showed that *ADAM 15* mRNA was up-regulated in pancreatic cancer, suggesting that *ADAM 15* may play a role in the progression of pancreatic cancer. However, we found no relation between the level of *ADAM 15* mRNA and the status of lymph node metastasis, prognosis or histological differentiation, possibly due to the small number of samples analyzed. In the present study, levels of *ADAM 15* mRNA were much lower in primary cultured pancreatic fibroblasts than in pancreatic cancer cell lines. Microdissection-based analysis showed that normal pancreatic epithelial cells expressed extremely low levels of *ADAM 15* mRNA but significant levels of *ADAM 9* mRNA, suggesting that the expression of *ADAM 15* may be more specific than the expression of *ADAM 9* to pancreatic cancer cells.

Conclusion

We confirmed upregulation of *ADAM 9* and *ADAM 15* mRNA expression in microdissected pancreatic cancer cells and found that LMPC is indispensable for the accurate analysis of these genes in pancreatic cancer. *ADAM 9* and

ADAM 15 may play roles in the progression of pancreatic cancer and may present promising target genes for the diagnosis and treatment of pancreatic cancer.

References

- 1 Yeo CJ, Abrams RA, Grochow LB *et al*: Pancreaticoduodenectomy for pancreatic adenocarcinoma: postoperative adjuvant chemoradiation improves survival. A prospective, single-institution experience. *Ann Surg* 225: 621-633; discussion 633-636, 1997.
- 2 Seals DF and Courtneidge SA: The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev* 17: 7-30, 2003.
- 3 Blobel CP *et al*: ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 6: 32-43, 2005.
- 4 Lendeckel U, Kohl J, Arndt M *et al*: Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. *J Cancer Res Clin Oncol* 131: 41-48, 2005.
- 5 O'Shea C, McKie N, Buggy Y *et al*: Expression of ADAM-9 mRNA and protein in human breast cancer. *Int J Cancer* 105: 754-761, 2003.
- 6 Karan D, Lin FC, Bryan M *et al*: Expression of ADAMs (a disintegrin and metalloproteases) and TIMP-3 (tissue inhibitor of metalloproteinase-3) in human prostatic adenocarcinomas. *Int J Oncol* 23: 1365-1371, 2003.
- 7 Tannapfel A, Anhalt K, Hausermann P *et al*: Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. *J Pathol* 201: 238-249, 2003.
- 8 Le Pabic H, Bonnier D, Wewer UM *et al*: ADAM12 in human liver cancers: TGF- β -regulated expression in stellate cells is associated with matrix remodeling. *Hepatology* 37: 1056-1066, 2003.
- 9 Shintani Y, Higashiyama S, Ohta M *et al*: Overexpression of ADAM9 in non-small cell lung cancer correlates with brain metastasis. *Cancer Res* 64: 4190-4196, 2004.
- 10 Peduto L, Reuter VE, Shaffer DR *et al*: Critical function for ADAM9 in mouse prostate cancer. *Cancer Res* 65: 9312-9319, 2005.
- 11 Zhou M, Graham R, Russell G *et al*: MDC-9 (ADAM-9/Meltrin gamma) functions as an adhesion molecule by binding the $\alpha(v)\beta(5)$ integrin. *Biochem Biophys Res Commun* 280: 574-580, 2001.
- 12 White JM: ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr Opin Cell Biol* 15: 598-606, 2003.
- 13 Nath D, Slocombe PM, Webster A *et al*: Meltrin gamma (ADAM-9) mediates cellular adhesion through $\alpha(6)\beta(1)$ integrin, leading to a marked induction of fibroblast cell motility. *J Cell Sci* 113(Pt 12): 2319-2328, 2000.
- 14 Grutzmann R, Foerder M, Alldinger I *et al*: Gene expression profiles of microdissected pancreatic ductal adenocarcinoma. *Virchows Arch* 443: 508-517, 2003.
- 15 Alldinger I, Dittert D, Peiper M *et al*: Gene expression analysis of pancreatic cell lines reveals genes overexpressed in pancreatic cancer. *Pancreatol* 5: 370-379, 2005.
- 16 Grutzmann R, Luttges J, Sipos B *et al*: ADAM9 expression in pancreatic cancer is associated with tumour type and is a prognostic factor in ductal adenocarcinoma. *Br J Cancer* 90: 1053-1058, 2004.

- 17 Carl-McGrath S, Lendeckel U, Ebert M *et al*: The disintegrin-metalloproteinases ADAM9, ADAM12, and ADAM15 are upregulated in gastric cancer. *Int J Oncol* 26: 17-24, 2005.
- 18 Kuefer R, Day KC, Kleer CG *et al*: ADAM15 disintegrin is associated with aggressive prostate and breast cancer disease. *Neoplasia* 8: 319-329, 2006.
- 19 Schutz A, Hartig W, Wobus M *et al*: Expression of ADAM15 in lung carcinomas. *Virchows Arch* 446: 421-429, 2005.
- 20 Sahin U, Weskamp G, Kelly K *et al*: Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* 164: 769-779, 2004.
- 21 Fourie AM, Coles F, Moreno V *et al*: Catalytic activity of ADAM8, ADAM15, and MDC-L (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. *J Biol Chem* 278: 30469-30477, 2003.
- 22 Nath D, Slocombe PM, Stephens PE *et al*: Interaction of metargidin (ADAM-15) with alphavbeta3 and alpha5beta1 integrins on different haemopoietic cells. *J Cell Sci* 112(Pt 4): 579-587, 1999.
- 23 Zhang XP, Kamata T, Yokoyama K *et al*: Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin alphavbeta3. *J Biol Chem* 273: 7345-7350, 1998.
- 24 Eto K, Puzon-McLaughlin W, Sheppard D *et al*: RGD-independent binding of integrin alpha9beta1 to the ADAM-12 and -15 disintegrin domains mediates cell-cell interaction. *J Biol Chem* 275: 34922-34930, 2000.
- 25 Sato N, Mizumoto K, Beppu K *et al*: Establishment of a new human pancreatic cancer cell line, NOR-P1, with high angiogenic activity and metastatic potential. *Cancer Lett* 155: 153-161, 2000.
- 26 Ohuchida K, Mizumoto K, Murakami M *et al*: Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. *Cancer Res* 64: 3215-3222, 2004.
- 27 Ohuchida K, Mizumoto K, Yamada D *et al*: Quantitative analysis of MUC1 and MUC5AC mRNA in pancreatic juice for preoperative diagnosis of pancreatic cancer. *Int J Cancer* 118: 405-411, 2006.
- 28 Tachikawa T and Irie T: A new molecular biology approach in morphology: basic method and application of laser microdissection. *Med Electron Microsc* 37: 82-88, 2004.
- 29 Tan ZJ, Hu XG, Cao GS *et al*: Analysis of gene expression profile of pancreatic carcinoma using cDNA microarray. *World J Gastroenterol* 9: 818-823, 2003.
- 30 Mazzocca A, Coppari R, De Franco R *et al*: A secreted form of ADAM9 promotes carcinoma invasion through tumor-stromal interactions. *Cancer Res* 65: 4728-4738, 2005.
- 31 Herren B, Garton KJ, Coats S *et al*: ADAM15 overexpression in NIH3T3 cells enhances cell-cell interactions. *Exp Cell Res* 271: 152-160, 2001.

Received October 30, 2006
Accepted December 29, 2006

The Role of the DNA Damage Checkpoint Pathway in Intraductal Papillary Mucinous Neoplasms of the Pancreas

Yoshihiro Miyasaka,¹ Eishi Nagai,² Hiroshi Yamaguchi,¹ Kei Fujii,¹ Takahiro Inoue,¹ Kenoki Ohuchida,² Tomomi Yamada,³ Kazuhiro Mizumoto,² Masao Tanaka,² and Masazumi Tsuneyoshi¹

Abstract Purpose: Intraductal papillary mucinous neoplasms (IPMN) are known to show a transition from adenoma to carcinoma accompanied by several molecular abnormalities. ATM-Chk2-p53 DNA damage checkpoint activation, which is involved in prevention of the progression of several tumors, was analyzed to evaluate the role of the DNA damage checkpoint in the progression of IPMNs.

Experimental Design: One hundred and twenty-eight IPMNs were classified into four groups (intraductal papillary mucinous adenoma, borderline IPMN, noninvasive intraductal papillary mucinous carcinoma, and invasive intraductal papillary mucinous carcinoma) and stained immunohistochemically using antibody for Thr⁶⁸-phosphorylated Chk2. Expression of ATM, Chk2, and p21^{WAF1} and accumulation of p53 were also analyzed.

Results: Chk2 phosphorylation was shown in all adenomas and showed a significant decreasing trend with the progression of atypia ($P < 0.0001$ by the Cochran-Armitage test for trend). Expression of p21^{WAF1} also exhibited a decreasing tendency ($P < 0.0001$), reflecting DNA damage checkpoint inactivation. p53 accumulation was mostly detected in malignant IPMNs. It was suggested that the DNA damage checkpoint provides a selective pressure for p53 mutation.

Conclusion: Our findings indicate that DNA damage checkpoint activation occurs in the early stage of IPMNs and prevents their progression. It is suggested that disturbance of the DNA damage checkpoint pathway due to Chk2 inactivation or p53 mutation contributes to the carcinogenesis of IPMNs.

Intraductal papillary mucinous neoplasms (IPMN) are pancreatic exocrine tumors composed of dilated main or branch ducts lined by mucin-producing atypical epithelium, which usually proliferates in a papillary fashion (Fig. 1; refs. 1, 2). Based on their architectural and nuclear atypia, the WHO has divided IPMNs into three groups: intraductal papillary mucinous adenoma (IPMA), borderline IPMN (IPMB), and intraductal papillary mucinous carcinoma (IPMC; ref. 1). Borderline lesions and carcinoma are accompanied by less atypical lesions in the vicinity, and transition from adenoma to adenocarcinoma is recognized within lesions. They are considered to exhibit a pattern of progression similar to that seen in colorectal adenocarcinoma [i.e., the adenoma-carcinoma sequence (3, 4)]. Some IPMCs are restricted to epithelium, whereas others are associated with invasive carcinoma. Sohn et al. (5)

investigated 136 patients with IPMNs, including 52 with invasive carcinoma, and concluded that associated invasive carcinoma is the strongest prognostic factor.

Various biological changes associated with the progression of IPMNs have been reported. Biankin et al. (6) reported that the frequency of loss of p16^{INK4A} and Smad4, cyclin D1 overexpression, and p53 accumulation are greater in IPMC and in IPMC with invasive carcinoma. House et al. (7) indicated that IPMNs with invasion tend to have multiple methylated genes, which are related to cell cycle control (*p16*, *p73*, and *APC*), DNA repair (*MGMT* and *hMLH1*), and cell adhesion (*E-cadherin*).

Recently, the DNA damage checkpoint pathway, including the ATM-Chk2-p53 pathway, was reported to be involved in tumorigenesis. The ATM-Chk2 pathway was originally shown to be activated in response to double-strand DNA breaks caused by ionizing radiation (8, 9). Responding to DNA damage, ATM phosphorylates Chk2 at Thr⁶⁸, and activated Chk2 phosphorylates downstream proteins, such as p53, activation of which leads to cell cycle arrest and DNA repair or apoptosis (Fig. 2). Bartkova et al. (10) showed that Chk2 phosphorylation occurred in colon adenoma and the early stage of urinary bladder cancer and decreased in advanced carcinoma. They thus suggested that the ATM-Chk2 DNA damage signaling pathway was activated and delayed or prevented tumor progression in the early stage of tumorigenesis. Gorgoulis et al. (11) reported the activation of the DNA damage checkpoint in precancerous lesions of lung and skin as

Authors' Affiliations: Departments of ¹Anatomic Pathology and ²Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University and ³Department of Medical Information Science, Kyushu University Hospital, Fukuoka, Japan
Received 1/5/07; revised 4/9/07; accepted 5/11/07.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Masazumi Tsuneyoshi, Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-6061; Fax: 81-92-642-5968; E-mail: masazumi@surgpath.med.kyushu-u.ac.jp.

© 2007 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-07-0032

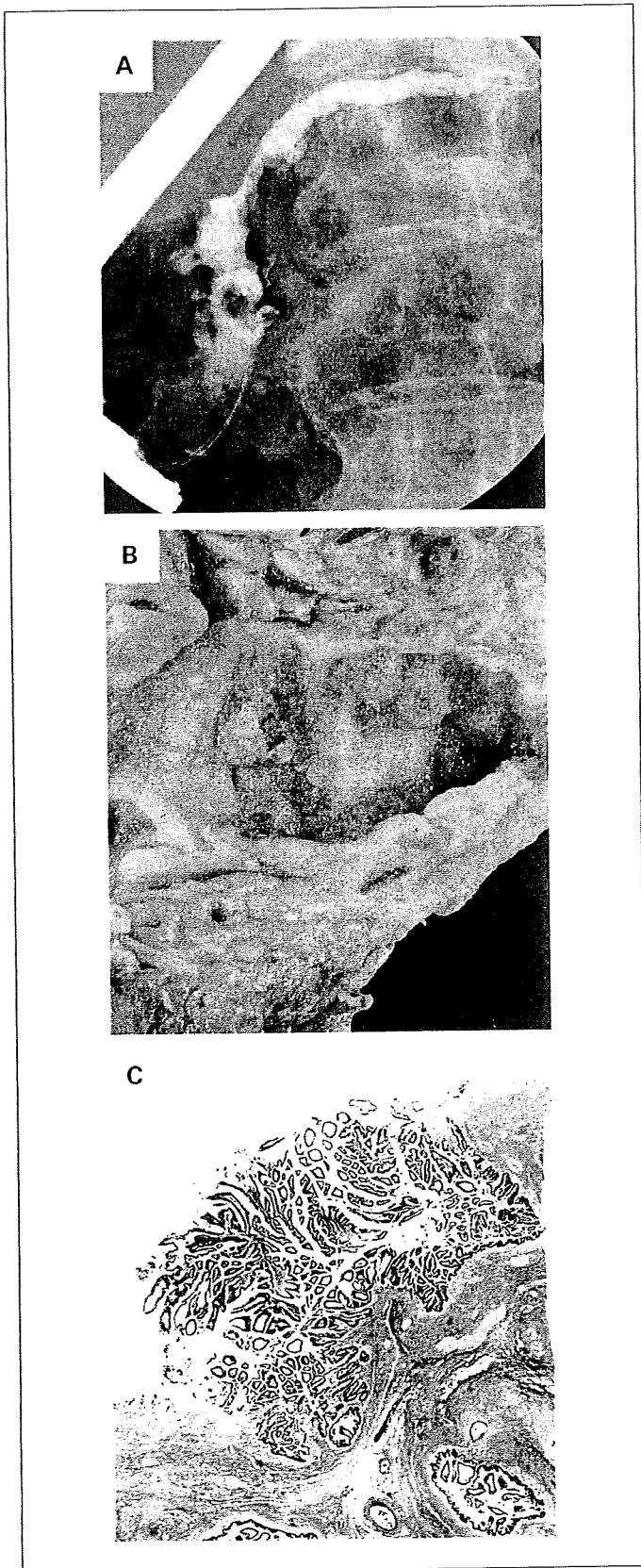


Fig. 1. A to C, a case of main duct type IPMN. A, endoscopic retrograde pancreatography visualizing dilated main pancreatic duct and nodules. B, pancreatoduodenectomy specimen with dilated main pancreatic duct containing multiple nodules. C, photomicrograph showing epithelial papillary projection on the main pancreatic duct wall. The histopathologic diagnosis was noninvasive IPMC.

indicated by phosphorylation of Chk2 and H2AX. It is proposed that frequent inactivation of p53 in carcinomas supports DNA damage checkpoint activation in precursor lesions because activated checkpoint might confer a selective pressure for p53 dysfunction (11, 12). Indeed, aberrations of checkpoint-related genes, such as *ATM*, *Chk2*, and *p53*, have been reported in the literature and their role as tumor suppressors has been widely accepted (13, 14). However, the role of the DNA damage checkpoint pathway in pancreatic neoplasms has not yet been investigated.

The existence of IPMNs with the adenoma-carcinoma sequence prompted us to investigate the status of the DNA damage checkpoint pathway in a large series of IPMNs. Activation of the pathway was evaluated by immunohistochemistry using antibody for Thr⁶⁸-phosphorylated Chk2. We also investigated expression of *ATM*, *Chk2*, and p21^{WAF1}, which is known as a downstream effector of the *ATM*-*Chk2*-p53 pathway, and accumulation of p53. The purpose of this study was to clarify the involvement of the DNA damage checkpoint pathway in the tumorigenesis and progression of IPMNs.

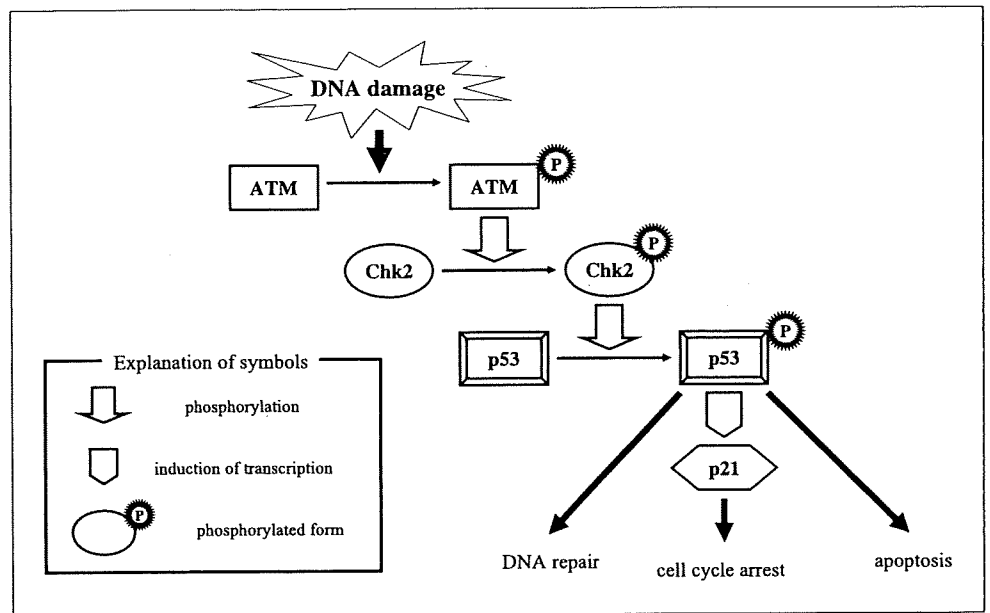
Materials and Methods

Tissue samples. One hundred and twenty-eight cases of IPMN were collected from the files of the Department of Anatomic Pathology of Kyushu University. All samples were obtained by surgery between July 1986 and January 2006. The original H&E slides for each case were reviewed by three pathologists (Y.M., T.I., and M.T.) independently, and the IPMNs were classified into four groups (IPMA, IPMB, noninvasive IPMC, or invasive IPMC) according to the WHO criteria (1). Because of the heterogeneity of the atypia in IPMN, we determined which lesion had the highest degree of architectural and cellular atypia in each case and selected it as a representative section. Normal ductal epithelia in these pancreata, which were available in 119 cases, were also evaluated.

Immunohistochemistry. Serial 3- μ m sections were prepared from the selected paraffin blocks, deparaffinized in xylene, and rehydrated in ethanol. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by microwave in citrate buffer at pH 6.0. A Histofine SAB-PO kit (Nichirei) was used for immunohistochemical labeling. Each section was exposed to 10% nonimmunized rabbit serum (Chk2, *ATM*, p53, and p21^{WAF1}) or goat serum (phospho-Chk2) for 10 min to block nonspecific binding of the antibodies and then incubated with primary antibodies at 4°C overnight. We used the following antibodies obtained from commercial suppliers: rabbit monoclonal anti-phospho-Chk2 (Thr⁶⁸) antibody (80F5, 1:200; Cell Signaling), mouse monoclonal anti-Chk2 antibody (DCS-270, 1:200; MBL), mouse monoclonal anti-*ATM* antibody (ATX08, 1:50; Novus Biologicals), mouse monoclonal anti-p53 (PAb1801, 1:100; Oncogene), and mouse monoclonal anti-p21^{WAF1} (EA10, 1:200; Calbiochem). The sections were conjugated with biotinylated anti-rabbit (phospho-Chk2) or anti-mouse (Chk2, *ATM*, p53, and p21^{WAF1}) immunoglobulin solution for 20 min followed by 20-min incubation with peroxidase-labeled streptavidin. For visualization of the reaction products, 3,3'-diaminobenzidine was used as a chromogen, and then nuclear counterstaining with hematoxylin was done.

Evaluation. Immunohistochemical staining was assessed by the three pathologists independently. To assess the expression of Chk2 and phospho-Chk2, we used the scoring system described by Eymin et al. (15). Scores were calculated by multiplying the percentage of positive cells (0-100) by the intensity score (0-3); when the score was ≥ 10 , the tumor was considered positive for the antibody. Based on published reports, the criteria for staining with other antibodies were as follows: $\geq 5\%$ nuclear and cytoplasmic staining was considered positive for *ATM*

Fig. 2. Schema of the ATM-Chk2-p53 DNA damage checkpoint pathway. In response to DNA damage, activated ATM phosphorylates Chk2 at Thr⁶⁸. Activated Chk2, in turn, phosphorylates p53. Activated p53 induces transcription of downstream proteins, such as p21, which results in cell cycle arrest, DNA repair, and apoptosis.



(16) and $\geq 10\%$ nuclear staining was considered positive for p21^{WAF1} (17) or p53 accumulation (6).

Statistical analysis. Clinicopathologic characteristics were compared among the four groups using the χ^2 test, Fisher's exact test, or ANOVA with Bonferroni correction. The Cochran-Armitage test was used to determine trends of immunohistochemical staining. All analyses were done using Statistical Analysis System for Windows, release 8.2 (SAS Institute, Inc.).

Results

Clinicopathologic data. The 128 IPMNs analyzed in this study comprised 46 IPMAs, 30 IPMBs, 25 noninvasive IPMCs, and 27 invasive IPMCs. The clinicopathologic features of the

patients are summarized in Table 1. The neoplasms showed a tendency to arise in the pancreas head in elderly men. Larger cysts, mural nodules, and main duct type were significantly correlated with malignancy. These findings are consistent with previous reports (5, 18). This group can therefore be considered as an average population of IPMNs.

Chk2 phosphorylation in IPMNs. All of the IPMAs showed nuclear staining for phospho-Chk2, whereas normal ductal epithelia in 31 of 119 (26%) cases were positive for phospho-Chk2, indicating that the DNA damage checkpoint was fully activated in adenoma. With progression of atypia, the rate of phospho-Chk2 staining decreased: 46 of 46 (100%) of IPMAs, 29 of 30 (96.7%) of IPMBs, 21 of 25 (84.0%) of noninvasive

Table 1. Clinicopathologic features

	IPMA (n = 46)	IPMB (n = 30)	IPMC		Total	P
			Noninvasive (n = 25)	Invasive (n = 27)		
Gender						
Male	28	19	14	18	79	
Female	18	11	11	9	49	0.8795
Age (y)						
Mean \pm SD	64.3 \pm 8.5	67.3 \pm 7.4	68.3 \pm 6.4	65.5 \pm 9.1	66.0 \pm 8.1	0.1689
Location						
Head	26	21	20	18	85	
Body or tail	20	9	4	8	41	
Whole pancreas	0	0	1	1	2	0.6780
Distribution						
Main duct type	8	7	9	10	34	
Branch type	36	21	16	11	84	
Combined type	2	2	0	6	10	0.0119*
Size (mm)						
Median (25%, 75%)	25.5 (15.0, 35.0)	26.5 (20.0, 40.0)	36.0 (30.0, 50.0)	33.5 (28.0, 47.0)	30.0 (20.0, 40.0)	0.0003*
Mural nodule						
Present	7	13	15	19	54	
Absent	39	17	10	8	74	<0.0001*

*Statistically significant.

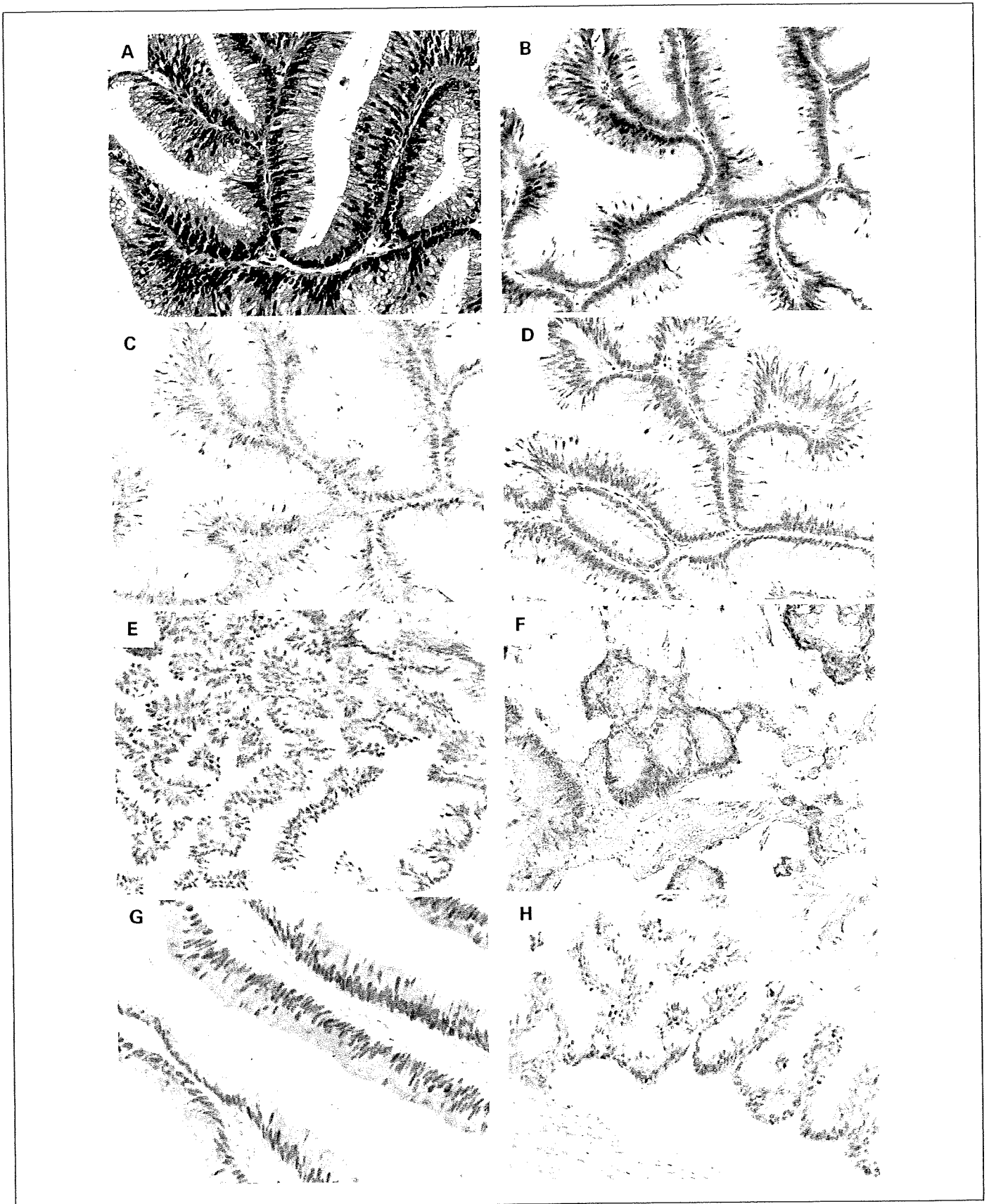


Fig. 3. *A to D*, IPMB. *A*, H&E stain. *B*, positive nuclear stain for phospho-Chk2. *C*, positive nuclear stain for Chk2. *D*, positive nuclear stain for ATM. *E*, noninvasive IPMC showing positive stain for phospho-Chk2. *F*, invasive IPMC showing negative stain for phospho-Chk2. *G*, IPMB showing positive nuclear stain for p21^{WAF1}. *H*, noninvasive IPMC showing positive nuclear stain for p53.

IPMCs, and 16 of 27 (59.9%) of invasive IPMCs (Figs. 3A, B, E, and F and 4A). The Cochran-Armitage test showed a significant decreasing trend between adenoma and invasive carcinoma ($P < 0.0001$). Less atypical lesions around phospho-Chk2-negative areas showed phospho-Chk2 staining. This suggests that activation of Chk2 is gradually disrupted in the transition to malignancy.

ATM and Chk2 protein expression. To investigate the cause of the loss of phospho-Chk2 staining, immunohistochemistry for Chk2 and ATM expression was done. Chk2 showed nuclear staining, whereas ATM showed nuclear and/or cytoplasmic staining. Chk2 expression was preserved in all tumors except one noninvasive IPMC (Figs. 3A and C and 4B), and ATM expression was preserved in all except one case of invasive IPMC (Figs. 3A and D and 4C). Both cases were negative for phospho-Chk2 staining and exhibited positive staining in surrounding less atypical lesions. Normal ductal epithelia in all cases were positive for both antibodies (Fig. 4B and C).

p21^{WAF1} expression and p53 accumulation. To investigate downstream of Chk2, we studied p21^{WAF1} expression and p53 accumulation. Normal epithelia in 7 of 119 (6%) cases were positive for p21^{WAF1}. Forty-one IPMAs (89%), 21 IPMBs (70%), 16 noninvasive IPMCs (64%), and 11 invasive IPMCs (41%) exhibited p21^{WAF1} expression (Figs. 3G and 4D). p21^{WAF1} expression showed a significant decreasing trend between adenoma and invasive carcinoma ($P < 0.0001$). Accumulation of p53 was seen in no normal epithelia (0%), one IPMA (2%), no IPMB (0%), five noninvasive IPMCs (20%), and eight invasive IPMCs (30%) and was shown to have an increasing tendency (Figs. 3H and 4E; $P < 0.0001$).

Discussion

In this study, we showed that Chk2 was phosphorylated in most of the IPMNs, including all cases of adenoma. Although Chk2 was originally reported to be phosphorylated in response to DNA double-strand breaks, recent studies have shown Chk2 phosphorylation in tumors of the lung, urinary bladder, colon, breast, and skin (10, 11, 15, 19). Chk2 phosphorylation has even been detected in preneoplastic lesions of the lung and skin (11). These findings suggest that the DNA damage checkpoint pathway is activated in the early stage of neoplasia, and our present data were consistent with this. However, the mechanism of Chk2 phosphorylation in neoplasms remains to be elucidated. Bartkova et al. (10) proposed that oncogene-induced DNA damage and telomere dysfunction activate the DNA damage pathway, and Gorgoulis et al. (11) mentioned that DNA replication stress due to aberrant stimulation of cell proliferation induces DNA damage. It has been reported that activation of oncogenes, such as *KRAS* mutation or c-erbB-2 overexpression, occurs in the early stage of IPMN (20–22). Recently, mutated *HRAS* was reported to activate the ATM-Chk2 pathway in both human fibroblast cultures and mouse keratinocyte tumor models (23, 24). Mutated *KRAS* might have similar effects on the DNA damage checkpoint activation in IPMNs because *KRAS* has a high degree of sequential analogy with *HRAS* and mutated forms of *HRAS* and *KRAS* have similar abilities to cause morphologic and growth transformation in cultured cells (25, 26). Unexpectedly, even normal ductal epithelia showed positive staining for phospho-Chk2 in some cases, but the proportion of these cases was much lower. It is

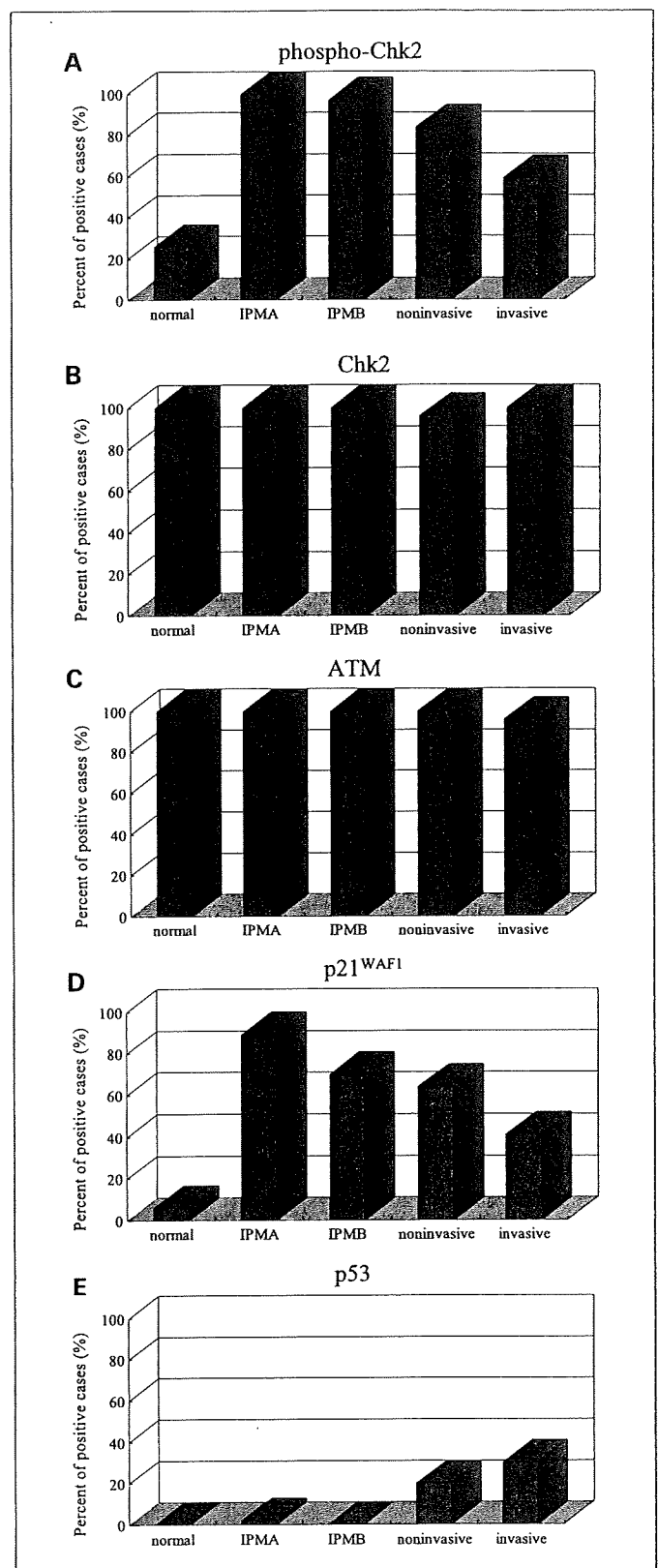


Fig. 4. Results of immunohistochemical analysis. A, phospho-Chk2 staining showed a significant decreasing trend ($P < 0.0001$, Cochran-Armitage test). B, one phospho-Chk2-negative noninvasive IPMC showed Chk2-negative staining. C, one phospho-Chk2-negative IPMC with invasion showed ATM-negative staining. D, p21 expression showed a significant decreasing trend ($P < 0.0001$, Cochran-Armitage test). E, p53 accumulation showed a significant increasing trend ($P < 0.0001$, Cochran-Armitage test).

therefore reasonable to conclude that the DNA damage checkpoint is activated in neoplasms. Although some reports have documented that Chk2 was not phosphorylated in normal tissue (e.g., lung, urinary bladder, and breast), others have documented that Chk2 was phosphorylated in normal bone marrow, testicular, and esophageal tissue (10, 11, 19, 27). Unlike lymphocytes in bone marrow and stem cells in testes, which undergo physiologic DNA double-strand breaks, the process of Chk2 activation in the normal esophageal epithelium remains unclear. There is also no evidence supporting physiologic DNA damage in pancreatic ductal epithelium. Considering the multifocal lesions and the frequent recurrence after margin negative resection of IPMNs, some of the morphologically normal ductal epithelium in the vicinity of the IPMN may already have activated oncogenes and hence show activation of the DNA damage response (5, 28).

In our study, Chk2 showed a decreasing trend of phosphorylation with the progression of IPMNs. In addition, p21^{WAF1} expression decreased and p53 accumulation increased in the transition of IPMN from benign tumor to malignancy. Aberrations of the DNA damage checkpoint pathway are frequently recognized in malignant IPMNs, suggesting that checkpoint pathway disruption contributes to the IPMN progression. Bartkova et al. (10) reported a decrease of Chk2 phosphorylation in advanced urinary bladder carcinoma. Gorgoulis et al. (11) found that some lung cancers and melanomas showed no phospho-Chk2 staining, whereas their precursor lesions did. Our data indicate that, in some cases, loss of phospho-Chk2 staining could be attributed to ATM or Chk2 protein depletion. However, the cause of Chk2 inactivation in the other cases is uncertain. Loss of p53-binding protein 1 expression might prevent Chk2 phosphorylation, as concomitant defects of p53-binding protein 1 expression and Chk2 phosphorylation have been reported in lung cancer and melanoma (11, 19).

Alternatively, it is possible that phosphatases, such as Wip1, are involved in Chk2 dephosphorylation (29).

In the present study, expression of the cyclin-dependent kinase inhibitor p21^{WAF1} exhibited a gradual decline parallel to phospho-Chk2 staining. Down-regulation of p21^{WAF1} according to tumor progression has been reported in gastric and colonic neoplasms, and expression of p21^{WAF1} is regulated by Chk2 via both p53-dependent and p53-independent pathways (30–33). The reduction of p21^{WAF1} expression observed in this study is thought to reflect inactivation of the DNA damage checkpoint pathway in IPMNs. Biankin et al. (34) reported an increasing trend of p21^{WAF1} in intraepithelial neoplasia associated with invasive ductal carcinoma of the pancreas, but the genetic differences between invasive ductal carcinoma and IPMNs might account for this discordance.

p53 abnormalities in IPMNs are documented in the literature (6, 20, 35). Although p53 accumulation is less common in IPMNs than in pancreatic invasive ductal carcinoma, some IPMNs show it (36). Similar to our data, the frequency of p53 accumulation increased in malignant IPMNs. Halazonetis (12) proposed that frequent p53 inactivation in human cancer cells is due to DNA damage checkpoint activation. Without DNA damage checkpoint pathway activation, there would be no selective pressure for p53 dysfunction. In other words, p53 mutation in malignant IPMNs supports activation of the DNA damage checkpoint in IPMNs.

It has been shown that Chk2 activation leads to cellular senescence (32, 33, 37). Senescence is a state of permanent cell cycle arrest accompanied by specific morphologic alterations and has been reported to be induced by certain oncogenes, such as *Ras* (38, 39). Recently, several authors have shown that senescence occurs in premalignant tumor cells and plays a role in limiting carcinogenesis, and they have suggested that attenuation of senescence due to deficiency of its effectors

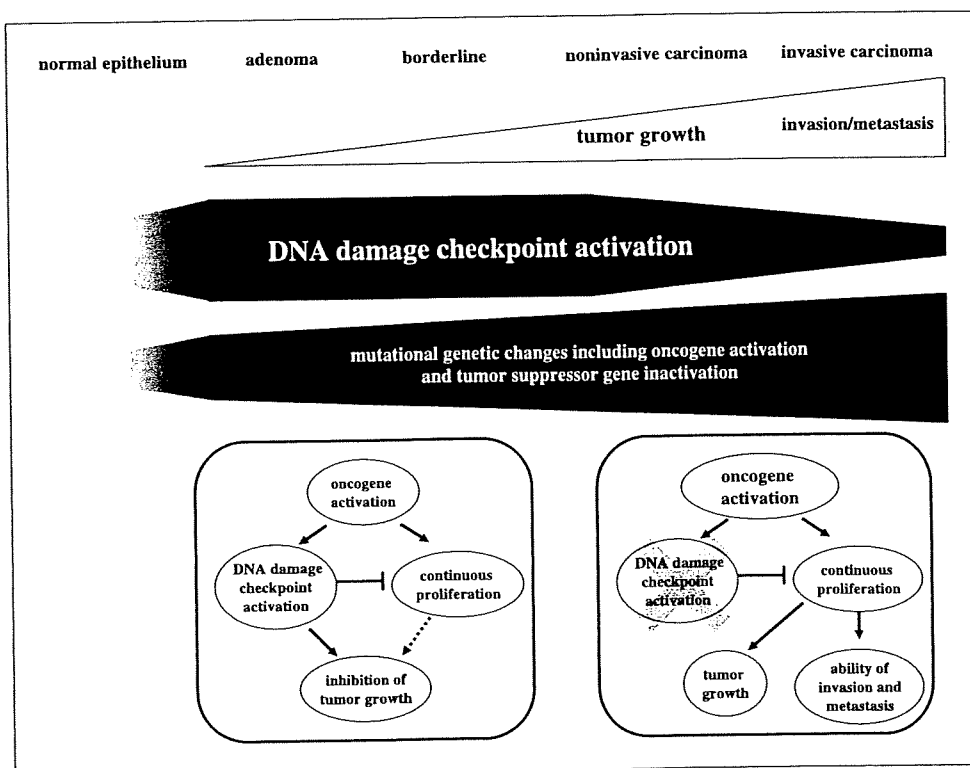


Fig. 5. Schema of hypothesis for the progression of IPMNs. In premalignant IPMNs, DNA damage checkpoint activation inhibits oncogene-induced cell proliferation and tumor growth. Activated oncogenes and disruption of the checkpoint pathway due to dysfunction of checkpoint-related molecules or phosphatase overexpression result in continuous cell proliferation, which leads to explosive tumor growth and acquisition of invasiveness and metastatic abilities.

results in progression to malignancy (40–42). It has been reported that the DNA damage checkpoint pathway is involved in the induction of oncogene-induced senescence and that inactivation of the DNA damage checkpoint due to suppression of mediator proteins, such as ATM, terminates senescence (23, 24). Our data showed that both Chk2 activation and expression of p21^{WAF1}, which is reported to be a senescence effector protein, reached a peak in adenoma lesions and they decreased gradually in the progression to invasive malignancy (33). It is therefore suggested that premalignant IPMNs may undergo senescence and that disruption of senescence might lead to malignancy.

We hypothesize a model for the progression of IPMNs as shown in Fig. 5. In premalignant IPMN, the activated DNA damage checkpoint inhibits oncogene-induced cell prolifera-

tion. Inactivation of the DNA damage checkpoint leads to continuous proliferation of tumor cells. Sustained replication increases the occurrence of molecular abnormalities, and shortage of nutrients due to the increased cell population might provide selective pressure for molecular abnormalities that enable cells to invade or metastasize (43). In other words, disruption of the DNA damage checkpoint results in acquisition of the characteristics of malignancy. Considering the good prognosis of benign and borderline lesions, development of methods to maintain the DNA damage checkpoint would enable us to avoid the use of invasive treatment. Detailed exploration of the molecular abnormalities leading to DNA damage checkpoint inactivation, such as dysfunction of p53-binding protein 1 and overexpression of Wip1, will contribute to progress in the treatment of IPMNs.

References

- Longnecker DS, Adler G, Hruban RH, Kloepfel G. Intraductal papillary-mucinous neoplasms of the pancreas. In: Hamilton SR, Aaltonen LA, editors. Pathology and genetics of tumors of the digestive system. Lyon: IARC Press; 2000. p. 237–40.
- Hruban RH, Takaori K, Klimstra DS, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am J Surg Pathol* 2004;28:977–87.
- Nagai E, Ueki T, Chijiwa K, Tanaka M, Tsuneyoshi M. Intraductal papillary mucinous neoplasms of the pancreas associated with so-called "mucinous ductal ectasia." Histochemical and immunohistochemical analysis of 29 cases. *Am J Surg Pathol* 1995;19:576–89.
- Nijima M, Yamaguchi T, Ishihara T, et al. Immunohistochemical analysis and *in situ* hybridization of cyclooxygenase-2 expression in intraductal papillary-mucinous tumors of the pancreas. *Cancer* 2002;94:1565–73.
- Sohn TA, Yeo CJ, Cameron JL, et al. Intraductal papillary mucinous neoplasms of the pancreas: an updated experience. *Ann Surg* 2004;239:788–97; discussion 97–9.
- Biankin AV, Biankin SA, Kench JG, et al. Aberrant p16(INK4A) and DPC4/Smad4 expression in intraductal papillary mucinous tumours of the pancreas is associated with invasive ductal adenocarcinoma. *Gut* 2002;50:861–8.
- House MG, Guo M, Iacobuzio-Donahue C, Herman JG. Molecular progression of promoter methylation in intraductal papillary mucinous neoplasms (IPMN) of the pancreas. *Carcinogenesis* 2003;24:193–8.
- Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* 1998;282:1893–7.
- Ahn JY, Schwarz JK, Piwnicka-Worms H, Canman CE. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res* 2000;60:5934–6.
- Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005;434:864–70.
- Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005;434:907–13.
- Halazonetis TD. Constitutively active DNA damage checkpoint pathways as the driving force for the high frequency of p53 mutations in human cancer. *DNA Repair (Amst)* 2004;3:1057–62.
- Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004;432:316–23.
- Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000;408:307–10.
- Eymin B, Claverie P, Salon C, et al. p14ARF activates a Tip60-dependent and p53-independent ATM/ATR/CHK pathway in response to genotoxic stress. *Mol Cell Biol* 2006;26:4339–50.
- Arlehang L, Adell G, Knutsen A, Thorstenson S, Sun XF. ATM expression in rectal cancers with or without preoperative radiotherapy. *Oncol Rep* 2005;14:313–7.
- Biankin AV, Kench JG, Biankin SA, et al. Pancreatic intraepithelial neoplasia in association with intraductal papillary mucinous neoplasms of the pancreas: implications for disease progression and recurrence. *Am J Surg Pathol* 2004;28:1184–92.
- Tanaka M, Kobayashi K, Mizumoto K, Yamaguchi K. Clinical aspects of intraductal papillary mucinous neoplasm of the pancreas. *J Gastroenterol* 2005;40:669–75.
- DiTullio RA, Jr., Mochan TA, Venere M, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol* 2002;4:998–1002.
- Sessa F, Solcia E, Capella C, et al. Intraductal papillary-mucinous tumours represent a distinct group of pancreatic neoplasms: an investigation of tumour cell differentiation and K-ras, p53 and c-erbB-2 abnormalities in 26 patients. *Virchows Arch* 1994;425:357–67.
- Hoshi T, Imai M, Ogawa K. Frequent K-ras mutations and absence of p53 mutations in mucin-producing tumors of the pancreas. *J Surg Oncol* 1994;55:84–91.
- Sakai Y, Yanagisawa A, Shimada M, et al. K-ras gene mutations and loss of heterozygosity at the p53 gene locus relative to histological characteristics of mucin-producing tumors of the pancreas. *Hum Pathol* 2000;31:795–803.
- Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 2006;444:633–7.
- Di Micco R, Fumagalli M, Cicalese A, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 2006;444:638–42.
- Kaplan JB. Biological activity of human N-ras and K-ras genes containing the Asn¹⁷ dominant negative mutation. *Oncol Res* 1994;6:611–5.
- Oldham SM, Clark GJ, Gangarosa LM, Coffey RJ, Jr., Der CJ. Activation of the Raf-1/MAP kinase cascade is not sufficient for Ras transformation of RIE-1 epithelial cells. *Proc Natl Acad Sci U S A* 1996;93:6924–8.
- Bartkova J, Bakkenist CJ, Rajpert-De Meyts E, et al. ATM activation in normal human tissues and testicular cancer. *Cell Cycle* 2005;4:838–45.
- Salvia R, Crippa S, Falconi M, et al. Branch-duct intraductal papillary mucinous neoplasms of the pancreas: to operate or not to operate? Results of a prospective protocol on the management of 109 consecutive patients. *Gut*. In press 2006.
- Oliva-Trastoy M, Berthonaud V, Chevalier A, et al. The Wip1 phosphatase (PPM1D) antagonizes activation of the Chk2 tumour suppressor kinase. *Oncogene* 2007;26:1449–58.
- Oya M, Yao T, Tsuneyoshi M. Expressions of cell-cycle regulatory gene products in conventional gastric adenomas: possible immunohistochemical markers of malignant transformation. *Hum Pathol* 2000;31:279–87.
- Dogliani C, Pelosio P, Laurino L, et al. p21/WAF1/CIP1 expression in normal mucosa and in adenomas and adenocarcinomas of the colon: its relationship with differentiation. *J Pathol* 1996;179:248–53.
- Gire V, Roux P, Wynford-Thomas D, Brondello JM, Dulic V. DNA damage checkpoint kinase Chk2 triggers replicative senescence. *EMBO J* 2004;23:2554–63.
- Aliouat-Denis CM, Dendouga N, Van den Wyngaert I, et al. p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res* 2005;3:627–34.
- Biankin AV, Kench JG, Morey AL, et al. Overexpression of p21 (WAF1/CIP1) is an early event in the development of pancreatic intraepithelial neoplasia. *Cancer Res* 2001;61:8830–7.
- Fujii H, Inagaki M, Kasai S, et al. Genetic progression and heterogeneity in intraductal papillary-mucinous neoplasms of the pancreas. *Am J Pathol* 1997;151:1447–54.
- Fukushima N, Mukai K, Sakamoto M, et al. Invasive carcinoma derived from intraductal papillary-mucinous carcinoma of the pancreas: clinicopathologic and immunohistochemical study of eight cases. *Virchows Arch* 2001;439:6–13.
- d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003;426:194–8.
- Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature* 2004;432:307–15.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997;88:593–602.
- Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. *Nature* 2005;436:642.
- Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAF600-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005;436:720–4.
- Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 2005;436:725–30.
- Karpinetz TV, Foy BD. Tumorigenesis: the adaptation of mammalian cells to sustained stress environment by epigenetic alterations and succeeding matched mutations. *Carcinogenesis* 2005;26:1323–34.

Anti-patched-1 Antibodies Suppress Hedgehog Signaling Pathway and Pancreatic Cancer Proliferation

MASAFUMI NAKAMURA¹, MAKOTO KUBO¹, KOSUKE YANAI¹, YOSHIKO MIKAMI¹, MIO IKEBE¹, SHUNTARO NAGAI¹, KOJI YAMAGUCHI², MASAO TANAKA² and MITSUO KATANO¹

Departments of ¹Cancer Therapy and Research, and ²Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract. *Background: The hedgehog (Hh) signaling pathway is aberrantly activated in many human carcinomas including pancreatic cancer and regulates tumor cell growth. Over-production of sonic hedgehog (Shh), a ligand of the Hh signaling pathway, increases the Hh signaling activity through transmitting the signal to patched-1 (Ptch1), the receptor of the Hh signaling pathway. Materials and Methods: α -Ptch1 antibodies were raised against an oligo-peptide, designed according to the Ptch1 amino-acid sequence. The specificity of α -Ptch1 was examined by immunoblotting and immuno-fluorescence, and biological effects were detected by RT-PCR and cell proliferation assay using two pancreatic cancer cell lines, Panc1 and SUI-2. Results: α -Ptch1 recognized a 160 kDa protein as shown by immunoblotting and cell surface staining of pancreatic cancer cells. Incubation with α -Ptch1 suppressed Hh signaling activity and proliferation of pancreatic cancer cells. Conclusion: These results provide a new strategy for controlling Hh dependent development of pancreatic cancer and other Hh related carcinomas.*

Pancreatic cancer is one of the most lethal human carcinomas with overall 5-year survival rate of <5%, partially because of the difficulty of diagnosis at an early stage (1). Despite the complete surgical removal of the tumor, most patients develop the disease again as metastases or local recurrence (2). Thus, it is a task of great urgency to establish a new medical strategy for the treatment of pancreatic cancer.

Abbreviations: Hh, hedgehog; Shh, sonic hedgehog; Ptch1, patched-1; Smo, smoothened; α -Ptch1, antibodies against patched1.

Correspondence to: Masafumi Nakamura, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan. Tel: +81 92 642 6941, Fax: +81 92 642 6221, e-mail: mnaka@surg1.med.kyushu-u.ac.jp

Key Words: Pancreatic cancer, hedgehog signaling pathway, patched-1, sonic hedgehog, antibodies.

Recently, the hedgehog (Hh) signaling pathway has been reported to be aberrantly activated in pancreatic cancer in a ligand dependent manner (3-9). Without ligand stimuli, transmembrane protein patched-1 (Ptch1) suppresses another transmembrane protein smoothened (Smo), resulting in the silencing of the signal. In pancreatic cancer cells, aberrantly over produced sonic hedgehog (Shh), one of three Hh ligands, binds Ptch1 and inhibits the suppressive effect of Ptch1 on Smo, which activates Gli (glioma-associated oncogene) to transcribe Hh target genes (6). The activated Hh signaling pathway promotes tumor cell growth, metastases and invasion. The Smo antagonist cyclopamine has been shown to inhibit the Hh signaling pathway, and suppress tumor cell growth *in vitro* (6) and in xenografts (4). Cyclopamine also increased the E-cadherin level and reduced the invasive ability of pancreatic cancer cells *in vitro*, and inhibited the metastatic spread of immortal pancreatic cells stably over-expressing Gli1 in a xenograft model (2). These findings indicate that the Hh signaling pathway is a useful therapeutic target in human pancreatic cancer. However cyclopamine is an alkaloid and not precisely targeting the hedgehog pathway.

Antibodies which recognize an oligopeptide of Ptch1 on the cancer cell surface and have the ability to silence Shh stimuli to Ptch1 were raised in the present study. The aim was to find a new strategy for controlling pancreatic cancer and other Hh dependent carcinomas by precisely controlling the Hh activity to avoid the side effects. The antibodies might be also useful as a tool of marking and targeting cancer-cell surface *in vivo* for the diagnosis and the drug-delivery system of anti-cancer drugs.

Materials and Methods

Cell culture, reagents and antibodies. Human pancreatic cancer cell lines (Panc1 and SUI-2), and human breast carcinoma cell lines (SK-BR-3, BT-474, MCF-7 and MDA-MB-231) were maintained at 37°C under a humidified atmosphere of 5% CO₂ and 95% air in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies)

and antibiotics (100 units/mL penicillin, and 100 µg/mL streptomycin Meijiseika, Tokyo, Japan). Anti-Ptch1 anti-serum (α -Ptch1) was prepared by Sigma (Deisenhofen, Germany) by the KLH (keyhole limpet hemacyamin)-MBS method (10), briefly, an oligo-peptide customized according to the amino-acid sequence of Ptch1 (KADYPNIQH) as an antigen was conjugated to the KLH by cystein and injected to rabbits. Rabbit serum was collected before (pre-serum) and after (α -Ptch1) immunization. Another anti-Ptch1 serum (G-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoblotting. Whole-cell extraction was performed with M-PER[®] Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and the whole-cell extract (100 µg) was separated by electrophoresis on SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schnell BioScience, Dassel, Germany). Blots were then incubated with pre-serum (1:500), α -Ptch1 (1:500) or G-19 (1:500) as primary antibodies overnight at 4°C. Then the blots were incubated with horseradish peroxidase-linked secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h. Immunocomplexes were detected with an ECL[®] plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad).

Immunostaining of cell line. Panc1 cells (2×10^4 /well) were seeded onto pre-underlaid Poly-L-lysine coated cover glass (Asahi Techno Glass Corporation, Chiba, Japan) in 24-well plates and were incubated overnight in 10% FBS-RPMI. Immunostaining was performed as previously described with some modification (11). In brief, cells were fixed in 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100 and incubated with primary antibodies (α -Ptch1 or pre-serum) followed by secondary antibodies. After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), samples were examined by Laser confocal microscopy (Nikon ECLIPS E600, Tokyo, Japan). Exposure time for recording was manually fixed to be the same for pre-serum and α -Ptch1. The antibodies and dilutions used were as follows: pre-serum (1/200) or α -Ptch1 (1/200) and AlexaFluor 488 goat anti-rabbit IgG (1:400; Molecular Probes, Eugene, OR, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed according to a method described previously (9). Briefly, total RNA was extracted from each cell line with the guanidinium thiocyanate-phenol-chloroform single-step method. For the reverse transcription reaction, pd(N)6 Random Hexamer (GE Healthcare UK Ltd, Buckinghamshire, England) were used for priming. *Gli1* sense (5'-TCT GCC CCC ATT GCC CAC TTG -3') and antisense (5'-TAC ATA GCC CCC AGC CCA CTT G-3') primers yielded a 480-bp product. *Ptch1* sense (5'-CGG CGT TCT CAA TGG GCT GGT TTT-3') and antisense (5'-GTG GGG CTG CTG TCT CGG GTT CG-3') primers yielded a 376-bp product. β -Actin sense (5'-CCA GGC ACC AGG GCG TGA TG-3') and antisense (5'-CGG CCA GCC AGG TCC AGA CG-3') primers gave rise to a 436-bp product. Amplification conditions comprised an initial denaturation for 2 min at 94°C followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min. Amplification of each

gene was in the linear range. The RT-PCR products were separated on ethidium bromide-stained 2% agarose gels. Semi-quantitative analysis was done with Molecular Imager FX Pro (Bio-Rad).

Proliferation assay. Cells (2×10^4 /well) seeded in 48-well plates in complete culture medium were incubated overnight. After a few brief washes with RPMI, the medium was changed to 2% FBS-RPMI containing α -Ptch1 or pre-serum. After incubation for 4 days, cells were harvested by trypsinization and viable cells were counted by flow cytometry [Coulter Counter (Beckman Coulter, Fullerton, CA, USA)].

Results

Immunoblotting analysis of α -Ptch1. An extract of Panc1, a pancreatic cancer cell line, was prepared as a source of Ptch1 protein, and subjected to immunoblotting. α -Ptch1 recognized a 160 kDa band of the Panc1 extract (Figure 1A) in contrast to the pre-serum control. Extracts of 4 breast cancer cell lines, SK-BR-3, BT-474, MCF-7 and MDA-MB-231, and Panc1, were subjected to immunoblotting with α -Ptch1 or G-19. α -Ptch1 visualized the same 160 kDa bands in all 5 extracts of the cancer cell lines, consistent with the band recognized by G-19 (Figure 1B). These results indicated that α -Ptch1 antibodies have affinity with Ptch1.

Cell surface protein recognition. The localization of the target protein recognized by α -Ptch1 was checked by immunofluorescence. α -Ptch1 exclusively stained the cell surfaces (Figure 2 middle) in the same pattern as phase contrast images (Figure 2 right). When the α -Ptch1 and phase contrast images were changed to green and red, respectively, overlying of both images showed a yellow color because of the colocalization of the two images (data not shown). In contrast pre-serum stained almost nothing (Figure 2 left). Since Ptch1 is located on the cell membrane, these images confirmed that α -Ptch1 specifically recognized Ptch1.

α -Ptch1 and activity of the Hh signal pathway in pancreatic cancer cells. The effect of α -Ptch1 on Hh signaling activity in pancreatic cancer cells was analyzed by RT-PCR. Because Gli1 and Ptch1 are not only the components of Hh signaling but also target genes of Gli1 trans-activation, the mRNA levels of Gli1 and Ptch1 as markers of the activity of Hh signaling pathway were examined. Panc1 cells were incubated with α -Ptch1, and subjected to RT-PCR. α -Ptch1 suppressed the expression levels of both Gli1 and Ptch1 in contrast to pre-serum, but did not suppress β -actin, an internal control (Figure 3).

Pancreatic cancer cell proliferation. Panc1 proliferation was suppressed by α -Ptch1 in comparison to pre-serum (Figure 4a). The suppressive effect of α -Ptch1 was further examined using a different pancreatic cancer cell line, SUIT-2. α -Ptch1 significantly suppressed proliferation of SUIT-2 in

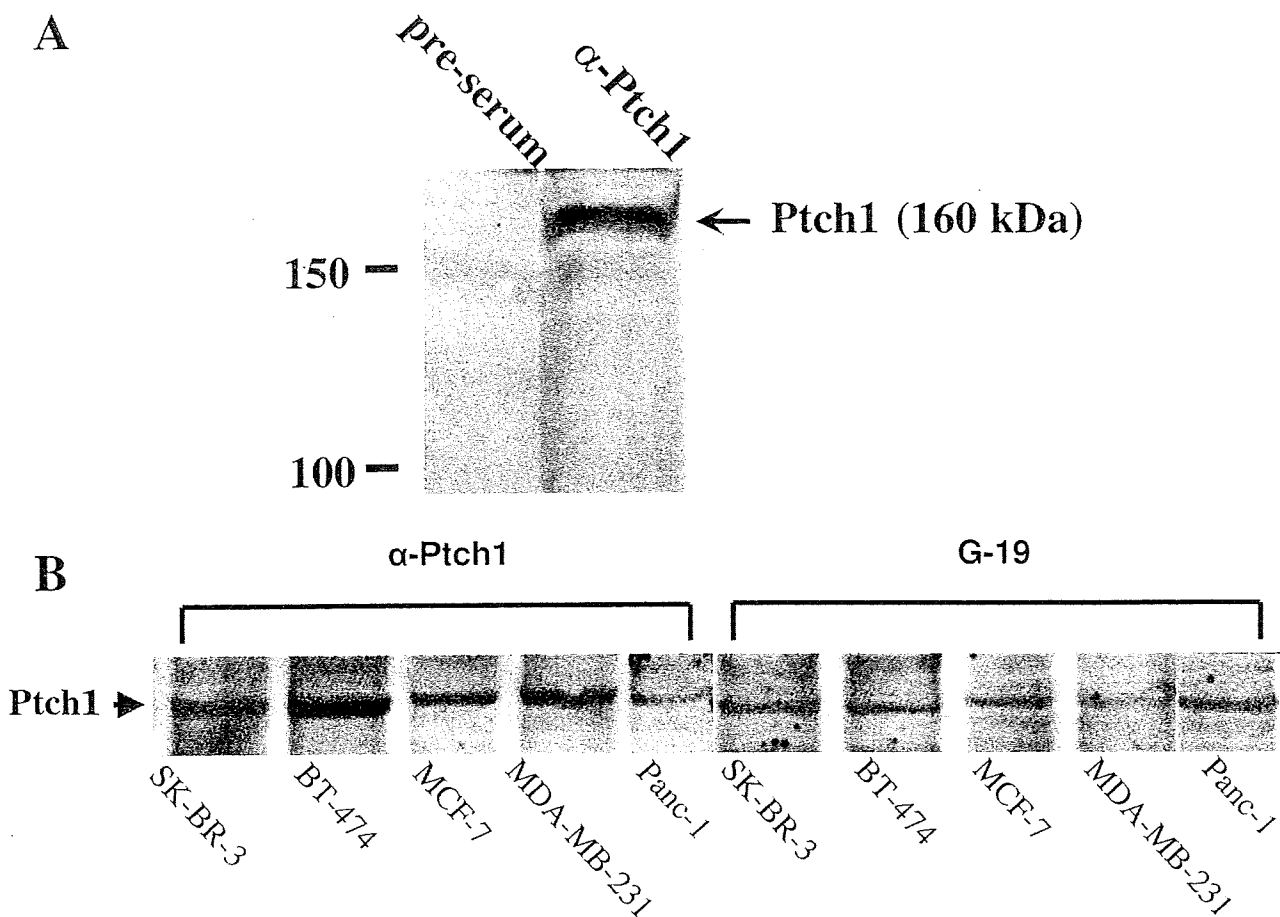


Figure 1. α -Ptch1 recognized 160 kDa protein in immunoblotting analysis. (A) The extract of Panc1 was analyzed by immunoblotting with control pre-serum or α -Ptch1 as first antibodies. α -Ptch1 recognized a 160 kDa band in the Panc1 extract, but pre-serum did not. (B) Multiple extracts of cancer cell lines, as indicated, were analyzed by immunoblotting with α -Ptch1 or G-19. Both α -Ptch1 and G-19 recognized the same 160 kDa band in all extracts.

comparison to pre-serum in a dose dependent manner (Figure 4B). Proliferation of a colon cancer cell line DLD1 was not attenuated by α -Ptch1 (data not shown), consistent with the fact that the Hh signaling pathway is not activated in most colon cancers (7, 12).

Discussion

Ectopic activation of the Hh signaling pathway has been demonstrated in pancreatic cancer development (4) and Shh over-production is the major course of this activation (3). We have previously reported that inflammatory stimuli induced over-expression of Shh through NF- κ B activation in pancreatic cancer (6). Suppression of the aberrantly activated Hh signaling pathway attenuated proliferation, invasion and metastases of pancreatic cancer (2). In the present study, antibodies raised against Ptch1 had a suppressive effect on Hh signaling activity

and pancreatic cancer cell proliferation. Ptch1 is transmembrane protein, located on the plasma membrane (13, 14). Shh binds Ptch1 and transmits signals to the sterol sensing domain (SSD) (15), which suppresses Ptch1 and inhibits Smo (16, 17). Because we aimed to suppress the activity of the Hh signaling pathway and proliferation of cancer cells, the target sequence of α -Ptch1 was located in one of the two extra-cellular arms, the putative docking site of the Hh ligand and Ptch1 to avoid stimuli from Shh to the SSD (13, 14, 18-20). As shown by RT-PCR, α -Ptch1 had the ability to suppress Hh signaling activity in pancreatic cancer cells.

We examined two pancreatic cancer cell lines for confirmation of α -Ptch1 effect on Hh activity and cancer proliferation. α -Ptch1 suppressed the proliferation of Panc1 less than that of SUIT-2, consistent with the fact that Panc1 is less dependent on the Hh signaling pathway than other pancreatic cancer cell lines (2).

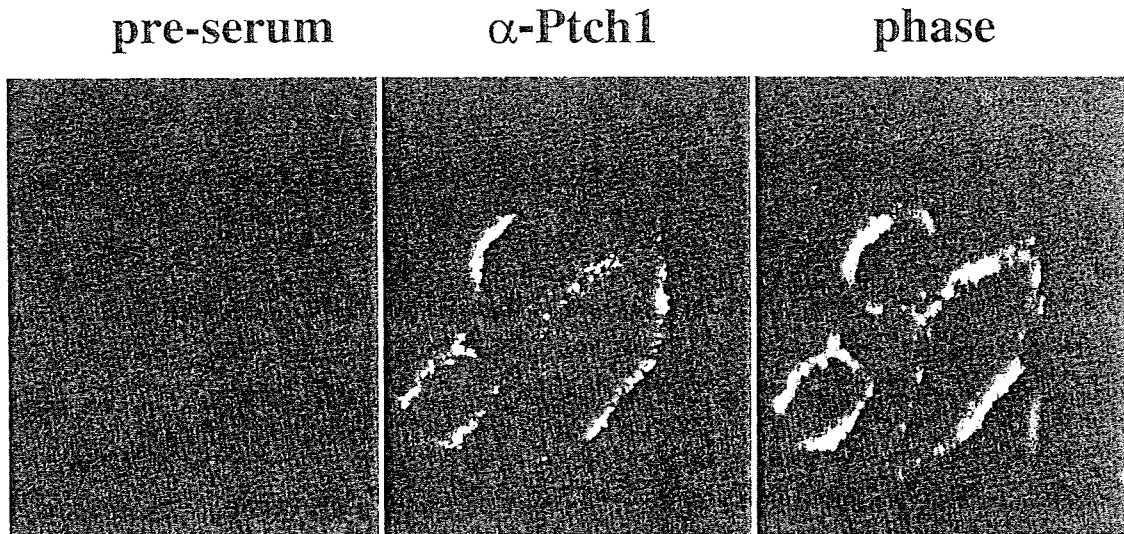


Figure 2. α -Ptch1 recognized a cell surface protein. Panc1 cells were stained with control pre-serum (left) or α -Ptch1 (middle) as first antibodies and subjected to immunofluorescence using laser confocal microscopy. α -Ptch1 exclusively showed cell surface images, in a similar pattern to phase contrast images (right) in contrast to the pre-serum.

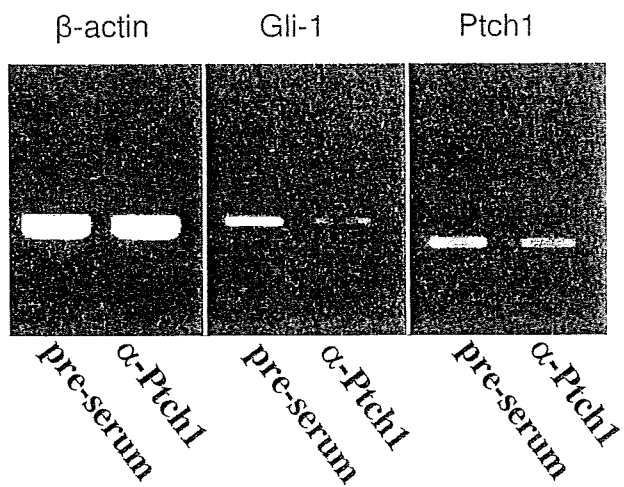


Figure 3. α -Ptch1 antibodies suppressed the Hh signal pathway activity in pancreatic cancer cells. Panc1 cells were incubated with control pre-serum or α -Ptch1, and subjected to RT-PCR with primers for Gli1 (480 bp) and Ptch1 (376 bp) with β -actin (436 bp) as internal control, as indicated. α -Ptch1 suppressed Gli1 and Ptch1 mRNA expression compared with pre-serum, while no significant differences were detected in the levels of β -actin mRNA expressions with both sera.

The effects of α -Ptch1 on Hh signaling activity and cancer cell growth highlight the significance of the Hh pathway and Ptch1 as targets of pancreatic cancer treatment. Further research for the identification of small molecules inhibiting the function of target peptides may shed new light on pancreatic cancer treatment and other Hh related carcinomas.

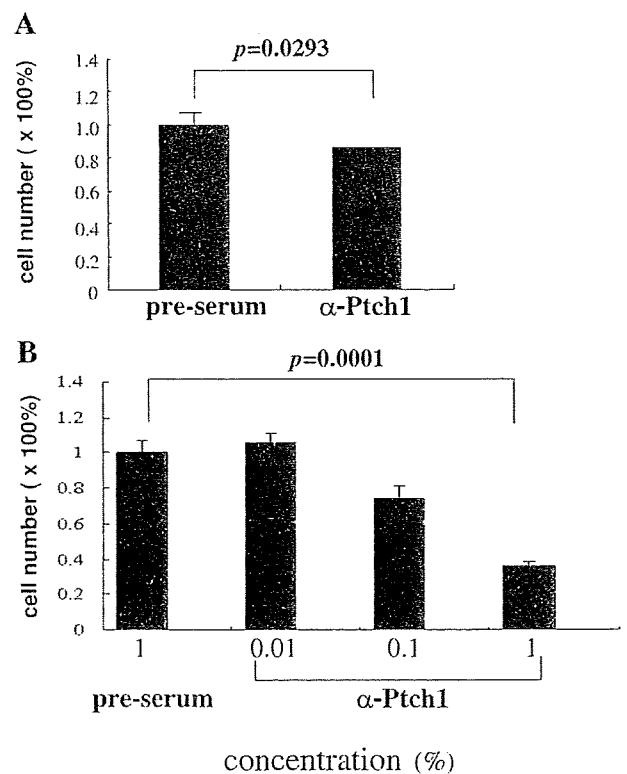


Figure 4. α -Ptch1 suppressed pancreatic cancer cell proliferation. Panc1 (A) and SUIT-2 (B) were incubated with 1% (or the indicated concentrations) of α -Ptch1 or control pre-serum for 4 days and cell numbers were counted by flow-cytometry. Cell numbers relative to those of pre-serum are indicated. Proliferation of Panc1 was suppressed by α -Ptch1 in contrast to pre-serum (A). Proliferation of SUIT-2 was significantly suppressed by α -Ptch1 in contrast to pre-serum in a dose dependent manner (B).

Acknowledgements

This study was supported by General Scientific Research Grants 18390350, 18659372 and 17591414 from the Ministry of Education, Culture, Sports, and Technology of Japan. We thank Kaori Nomiya for skillful technical assistance.

References

- 1 Jemal A, Siegel R, Ward E *et al*: Cancer statistics, 2006. *CA Cancer J Clin* 56: 106-130, 2006.
- 2 Feldmann G, Dhara S, Fendrich V *et al*: Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer Res* 67: 2187-2196, 2007.
- 3 Berman DM, Karhadkar SS, Maitra A *et al*: Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 425: 846-851, 2003.
- 4 Thayer SP, di Magliano MP, Heiser PW *et al*: Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 425: 851-856, 2003.
- 5 Kubo M, Nakamura M, Tasaki A *et al*: Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. *Cancer Res* 64: 6071-6074, 2004.
- 6 Nakashima H, Nakamura M, Yamaguchi H *et al*: Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res* 66: 7041-7049, 2006.
- 7 Akiyoshi T, Nakamura M, Koga K *et al*: Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. *Gut* 55: 991-999, 2006.
- 8 Tasaki A, Akiyoshi T, Koga K *et al*: Immunohistochemical staining of hedgehog pathway-related proteins in human thymomas. *Anticancer Res* 25: 3697-3702, 2005.
- 9 Yanai K, Nagai S, Wada J *et al*: Hedgehog signaling pathway is a possible therapeutic target for gastric cancer. *J Surg Oncol* 95: 55-62, 2007.
- 10 Nakashima T, Sekiguchi T, Kuraoka A *et al*: Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. *Mol Cell Biol* 13: 6367-6374, 1993.
- 11 Nakamura M, Zhou XZ and Lu KP: Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. *Curr Biol* 11: 1062-1067, 2001.
- 12 van den Brink GR, Bleuming SA, Hardwick JC *et al*: Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet* 36: 277-282, 2004.
- 13 Goodrich LV, Johnson RL, Milenkovic L *et al*: Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev* 10: 301-312, 1996.
- 14 Ingham PW, Nystedt S, Nakano Y *et al*: Patched represses the Hedgehog signalling pathway by promoting modification of the Smoothed protein. *Curr Biol* 10: 1315-1318, 2000.
- 15 Pepinsky RB, Rayhorn P, Day ES *et al*: Mapping sonic hedgehog-receptor interactions by steric interference. *J Biol Chem* 275: 10995-11001, 2000.
- 16 Kuwabara PE and Labouesse M: The sterol-sensing domain: multiple families, a unique role? *Trends Genet* 18: 193-201, 2002.
- 17 Martin V, Carrillo G, Torroja C and Guerrero I: The sterol-sensing domain of Patched protein seems to control Smoothed activity through Patched vesicular trafficking. *Curr Biol* 11: 601-607, 2001.
- 18 Stone DM, Hynes M, Armanini M *et al*: The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 384: 129-134, 1996.
- 19 Johnson RL, Rothman AL, Xie J *et al*: Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* 272: 1668-1671, 1996.
- 20 Carpenter D, Stone DM, Brush J *et al*: Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc Natl Acad Sci USA* 95: 13630-13634, 1998.

Received April 23, 2007

Revised August 1, 2007

Accepted August 14, 2007

Local pancreatic resection with preoperative endoscopic transpapillary stenting

Masahiko Hirota, M.D., Keiichiro Kanemitsu, M.D., Hiroshi Takamori, M.D., Akira Chikamoto, M.D., Toshiyuki Ohkuma, M.D., Hiroyuki Komori, M.D., Nobutomo Miyanari, M.D., Takatoshi Ishiko, M.D., Hideo Baba, M.D.*

Department of Gastroenterological Surgery, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto-city, 860-0811 Japan

Manuscript received October 24, 2006; revised manuscript January 20, 2007

Abstract

Background: Pancreatic fistula, although not common, can cause serious complications after pancreatectomy. During local pancreatectomy, injury to the main pancreatic duct (in addition to the accessory and side branch ducts) increases the risk of pancreatic fistula formation. Nonetheless, local pancreatic resection maintains the advantage of preserving pancreatic parenchyma.

Methods: In this study, we reviewed the cases of 5 patients who underwent preoperative endoscopic transpapillary pancreatic stenting to help prevent refractory fistula development after local pancreatic resection.

Results: Stenting was successful in all 5 patients, and none developed a refractory grade C postoperative pancreatic fistula.

Conclusions: These results suggest that in selected patients, preoperative endoscopic pancreatic stenting may be an effective prophylactic measure to lower the risk of refractory grade C fistula formation after local pancreatic resection. © 2007 Excerpta Medica Inc. All rights reserved.

Keywords: Pancreatic resection; Postoperative pancreatic fistula; Pancreatic stenting

Postoperative pancreatic fistula (POPF), although not common, can cause serious complications after pancreatectomy. The incidence of POPF ranges from 0% to 24% after pancreatoduodenectomy, to 10% to 38% after distal pancreatectomy [1–9]. During local pancreatectomy, injury to the main pancreatic duct (in addition to the accessory and side branch ducts) increases the risk of POPF formation. In response to conservative treatment, including no oral intake (pancreatic rest) and adequate drainage, most POPFs resolve transiently and spontaneously in conjunction with a decrease in the output of pancreatic leakage. However, patients with refractory POPF can develop further complications such as intraperitoneal abscess, sepsis, and lethal hemorrhage. For this reason, prophylactic strategies such as pancreatic transaction with modern equipment, fibrin glue sealing of the pancreatic stump, prolamine occlusion of main pancreatic duct, octreotide administration, and Roux-en-Y pancreatojejunostomy have been employed to decrease the inci-

dence of POPF [10–13]. However, none of these procedures eliminates the risk of POPF development.

Local pancreatic resection is indicated for benign or low-grade malignant neoplasms, including intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, serous cystadenoma, and islet cell tumor. It also can be applied to duodenal neoplasms that invade into the pancreas. Although local pancreatic resection maintains the advantage of preserving pancreatic parenchyma, POPF can develop. Sugiyama et al reported the case of a patient in whom preoperative endoscopic pancreatic stenting prevented the development of POPF following local resection of the pancreatic body tumor [14]. Also, Abe et al reported the efficacy of preoperative endoscopic pancreatic stenting for preventing POPF formation after distal pancreatectomy [15]. Together, these reports suggest that preoperative endoscopic pancreatic stenting may be an effective prophylactic against POPF development, even after local resection of lesions in the pancreatic head. POPF is defined by any measurable volume of fluid put out from a drain on or after postoperative day 3 with amylase activity at least 3 times higher than serum amylase [16]. The International Study

* Corresponding author. Tel.: +81-96-373-5213; fax: +81-96-371-4378

E-mail address: hdebaba@kaiju.medic.kumamoto-u.ac.jp

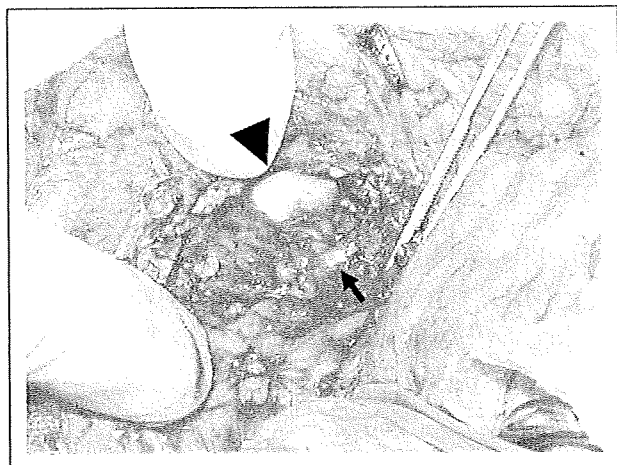


Fig. 1. Photo after local pancreatic resection in case no. 1. The common bile duct (arrow head) and main pancreatic duct (arrow) are exposed on the resection plane. The main pancreatic duct can be detected by the blue color of the indwelled pancreatic stent visible through the wall.

Group on Pancreatic Fistula definition further classified POPFs into 3 grades. Grade A is common transient fistula. Grade B requires a change in management or adjustment in the clinical pathway. If the drains are not functioning to fully drain the fistula, repositioning of the drains is required. Grade C is a refractory POPF that requires a major change in clinical management and aggressive clinical intervention. In this study, we reviewed the cases of 5 patients who underwent prophylactic preoperative endoscopic transpapillary pancreatic stenting to prevent refractory POPF formation after local resection of lesions in the pancreatic head region.

Patients and Methods

Between the 2000 and 2006, 5 patients underwent local resection in the pancreatic head region. All 5 patients underwent prophylactic preoperative endoscopic transpapillary pancreatic stenting to prevent the formation of POPF. There is no control patient who underwent local pancreatic resection without stenting. The group included 4 men and 1 woman, and the mean age was 41 years (range 21 to 69 years). Patients' lesions included 2 cases of insulinoma (one

with noninvasive cancer), 2 cases of IPMN, and 1 case of a duodenal gastrointestinal stromal tumor. Five to 7 days prior to surgery, after informed consents were obtained, each patient underwent placement of an endoscopic transpapillary pancreatic stent (5 F, 5 cm).

During the pancreatic transection, the pancreatic parenchyma was ligated with a nonabsorbable suture. Although fibrin glue was not applied to the pancreatic stump, 1 or 2 closed drains were placed around it. Octreotide was not administered postoperatively.

Results

Preoperative endoscopic transpapillary pancreatic stenting was performed successfully in all 5 patients. No endoscopic sphincterotomy or balloon dilatation was required by the patients, and none developed acute pancreatitis after stent placement.

During local pancreatectomy (Fig. 1), injury to the main pancreatic duct (in addition to the accessory and side branch ducts) increases the risk of pancreatic fistula formation. Postoperatively, none of the 5 patients developed refractory grade C POPF or any other major complications, including intra-abdominal abscess or hemorrhage, pseudocyst, or sepsis (Table 1). Two developed minor and transient biochemical POPF (grades A and B). One of the above 2 patients with transient POPF underwent replacement of closed drains around the pancreatic stump because they dislodged postoperatively. Another patient showed transient bile leakage from the cystic duct stump, which was confirmed by cholangiography via a nasobiliary drainage tube. Although patients had a 60% morbidity rate, no incidence of grade C POPF seems to be worthwhile when taking the possible risk of pancreatic duct injury in local pancreatectomy into consideration. The pancreatic stents were removed postoperatively between 2 and 8 weeks. For the first 2 patients, the stents were removed during the same hospital stay as the operation. For the other 3 patients, the stents were removed after readmission or in an outpatient clinic. No pancreatic stent occlusions were found by macroscopic observation of stent lumens.

Comments

Branch-type IPMN is characterized by low potential for malignancy. Insulinoma is essentially a benign neoplasm,

Table 1
Characteristics of patients

Diagnosis	Age/sex	Duration of stent-indwelling	Duration of hospital stay*	Amylase concentration in drain fluid (U/L)		Complications
				~2nd POD	3rd POD~	
Case no. 1: intraductal papillary mucinous adenoma	69/M	6 wk	13 wk	ND	478	POPF grade A
Case no. 2: insulinoma	21/F	6 wk	7 wk	1,322	74	None
Case no. 3: duodenal gastrointestinal stromal tumor	23/M	3 wk	4 wk	133	ND	None
Case no. 4: insulinoma	36/M	9 wk	5 wk	705	199	Minor bile leakage
Case no. 5: intraductal papillary mucinous noninvasive carcinoma	57/M	13 wk	3 wk	4200	1,702	POPF grade B Dislodgement of drain

POD = postoperative day; ND = not determined; POPF = postoperative pancreatic fistula.

* Duration of hospital stay in Japan, especially in our institute, is generally longer compared with other countries.

and although gastrointestinal stromal tumor is a malignant neoplasm, its growth is not massively invasive nor does it disseminate to lymph nodes. Many minimally invasive surgical procedures, including duodenum-preserving pancreatic head resection [17,18], pancreatic head resection with segmental duodenectomy [19], dorsal/ventral pancreatectomy [20], resection of the lower portion of the head of the pancreas [21], and single-branch resection [22], have been devised for the clinical treatment of such benign or low-grade malignant lesions of the pancreas. POPF continues to be the most troublesome and sometimes lethal complication following partial resection of the pancreas.

In patients with local pancreatic neoplasms, the pancreas is usually soft and maintains normal exocrine function. The standard surgical technique for preventing pancreatic juice leakage from the resection plane of the remnant pancreas is closure by suturing the resection plane [23]. However, the branch pancreatic ducts that communicate with the main duct cross the resection plane, and because of their size, the stumps of these small ducts cannot all be identified and ligated during transection. Thus, closure of the resection plane by suturing may not occlude small pancreatic ducts completely, and leakage from these ducts can lead to POPF formation.

Endoscopic pancreatic stenting has been used successfully to treat pancreatic ductal stricture, pancreatic stones, pancreatic divisum, and pancreatic duct disruption secondary to acute pancreatitis or pancreatic trauma [24,25]. Only a few reports have described the use of endoscopic pancreatic stenting for the treatment or prophylaxis of POPF development after pancreatic surgery [14,15,25,26]. Observations during endoscopic treatment for pancreatic fistula [24–27] demonstrate that a pancreatic stent allows the resection plane to seal by decompression of the pancreatic duct [24,27].

In addition to reducing pancreatic juice leakage from the resection plane, preoperative endoscopic transpapillary pancreatic stenting also prevents injury to the main pancreatic duct in 2 ways. First, the anatomy of the main pancreatic duct is clarified by palpating the stent, and/or by viewing its color. Second, the installed stent can prevent dislocation of the main pancreatic duct during tumor retraction. Although early complications associated with the stent placement are noteworthy, none of the 5 patients described here developed acute pancreatitis after stent placement. Furthermore, no pancreatic stent occlusion was found in our series, although stent occlusions can induce the development of POPF.

In conclusion, preoperative endoscopic pancreatic stenting in selected patients may be an effective prophylactic to prevent refractory grade C POPF formation following local pancreatic resection. Further investigation of additional cases will be required to evaluate the benefit of this approach with greater precision.

References

- [1] Shrikhande SV, Qureshi SS, Rajneesh N, et al. Pancreatic anastomoses after pancreaticoduodenectomy: do we need further studies? *World J Surg* 2005;29:1642–9.
- [2] Lillemore KD, Cameron JL, Kim MP, et al. Does fibrin glue sealant decrease the rate of pancreatic fistula after pancreaticoduodenectomy? Results of a prospective randomized trial. *J Gastrointest Surg* 2004;8:766–74.
- [3] Kazanjian KK, Hines OJ, Eibl G, et al. Management of pancreatic fistulas after pancreaticoduodenectomy: results in 437 consecutive patients. *Arch Surg* 2005;140:849–54.
- [4] Schlitt HJ, Schmidt U, Simunek D, et al. Morbidity and mortality associated with pancreatogastrostomy and pancreatojejunostomy following partial pancreatoduodenectomy. *Br J Surg* 2002;89:1245–51.
- [5] Nakao A, Fujii T, Sugimoto H, et al. Is pancreatogastrostomy safer than pancreatojejunostomy? *J Hepatobiliary Pancreat Surg* 2006;13:202–6.
- [6] Muscari F, Suc B, Kirzin S, et al. French Association for Surgical Research. Risk factors for mortality and intraabdominal complications after pancreatoduodenectomy: multivariate analysis in 300 patients. *Surgery* 2006;139:591–8.
- [7] Balzano G, Zerbi A, Cristallo M, et al. The unsolved problem of fistula after left pancreatectomy: the benefit of cautious drain management. *J Gastrointest Surg* 2005;9:837–42.
- [8] Okabayashi T, Kobayashi M, Sugimoto T, et al. Postoperative pancreatic fistula following surgery for gastric and pancreatic neoplasm; is distal pancreaticosplenectomy truly safe? *Hepatogastroenterology* 2005;52:233–6.
- [9] Sledzianowski JF, Duffas JP, Muscari F, et al. Risk factors for mortality and intraabdominal morbidity after distal pancreatectomy. *Surgery* 2005;137:180–5.
- [10] Suzuki Y, Fujino Y, Tanioka Y, et al. Randomized clinical trial of ultrasonic dissector or conventional division in distal pancreatectomy for non-fibrotic pancreas. *Br J Surg* 1999;86:608–11.
- [11] Takeuchi K, Tsuzuki Y, Ando T, et al. Distal pancreatectomy: is staple closure beneficial? *Aust N Z J Surg* 2003;73:922–5.
- [12] Suc B, Msika S, Fingerhut A, et al. Temporary fibrin glue occlusion of the main pancreatic duct in the prevention of intraabdominal complications after pancreatic resection. *Ann Surg* 2003;237:57–65.
- [13] Li-Ling J, Irving M. Somatostatin and octreotide in the prevention of postoperative pancreatic complications and the treatment of enterocutaneous pancreatic fistulas: a systemic review of randomized controlled trials. *Br J Surg* 2001;88:190–9.
- [14] Sugiyama M, Abe N, Yamaguchi Y, et al. Preoperative endoscopic pancreatic stenting for safe local pancreatic resection. *Hepatogastroenterology* 2001;48:1625–7.
- [15] Abe N, Sugiyama M, Suzuki Y, et al. Preoperative endoscopic pancreatic stenting for prophylaxis of pancreatic fistula development after distal pancreatectomy. *Am J Surg* 2000;191:198–200.
- [16] Bassi C, Dervenis C, Butturini G, et al. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005;138:8–13.
- [17] Beger H, Krautzbecker W, Bittner R, et al. Duodenal-preserving resection of the head of the pancreas in patients with severe chronic pancreatitis. *Surgery* 1985;97:467–73.
- [18] Kimura W, Nagai H. Study of surgical anatomy for duodenal-preserving resection of the pancreas. *Ann Surg* 1995;221:359–63.
- [19] Nakao A. Pancreatic head resection with segmental duodenectomy and preservation of the gastroduodenal artery. *Hepatogastroenterology* 1998;45:533–5.
- [20] Takada T. Ventral pancreatectomy: resection of the ventral segment of the pancreas. *J Hepatobiliary Pancreat Surg* 1993;1:36–40.
- [21] Nakagohri T, Asano T, Takayama W, et al. Resection of the inferior head of the pancreas: report of a case. *Surg Today* 1996;26:640–4.
- [22] Sata N, Koizumi M, Tsukahara M, et al. Single-branch resection of the pancreas. *J Hepatobiliary Pancreat Surg* 2005;12:71–5.
- [23] Fernandez-del Castillo C, Rattner DW, Warshaw AL. Standards for pancreatic resection in the 1990s. *Arch Surg* 1995;130:295–300.
- [24] Haltunen J, Weckman L, Kempainen E, et al. The endoscopic management of pancreatic fistulas. *Surg Endosc* 2005;19:559–62.
- [25] Saeed ZA, Ramirez FC, Hepps KS. Endoscopic stent placement for internal and external pancreatic fistulas. *Gastroenterology* 1993;105:1213–7.
- [26] Sarzen CD. Endoscopic management of pancreatic duct leak complicated by retrograde abscess. *Am J Gastroenterol* 1995;90:2039–41.
- [27] Kazarek RA, Ball TJ, Patterson DJ, et al. Transpapillary stenting for pancreaticocutaneous fistulas. *J Gastrointest Surg* 1997;1:357–61.