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ORIGINAL ARTICLE – PANCREATIC TUMORS

Perioperative Intra-Arterial and Systemic Chemotherapy for Pancreatic Cancer

Hiroshi Takamori, MD[1], Keiichiro Kanemitsu, MD[1], Masahiko Hirota, MD[1], Osamu Ikeda, MD[2], Hiroshi Tanaka, MD[1], Toru Beppu, MD[1], Yasuyuki Yamashita, MD[2], Natsuo Oya, MD[3], and Hideo Baba, MD[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; [2]Department of Diagnostic Radiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; [3]Department of Radiation Oncology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

ABSTRACT

Background. Even after curative resection of pancreatic cancer, there is a high probability of systemic recurrence. This indicates that subclinical metastases are already present at the time of operation. The purpose of this study was to assess the feasibility and outcomes of patients who received a novel multimodality therapy combining pancreatic resection and intraoperative radiation therapy (IORT) with pre- and postoperative chemotherapy for pancreatic cancer.

Methods. For eligible patients with pancreatic cancer, 5-FU was administered at a dose of 125 mg/m^2/day on days 1–5 every week as a continuous pancreatic and hepatic arterial infusion, and gemcitabine was infused intravenously at a dose of 800 mg/m^2 per day once per week for 2 weeks for preoperative chemotherapy. Pancreatic resection combined with IORT was performed 1 week after preoperative chemotherapy. Postoperative chemotherapy was performed in the same way as preoperative chemotherapy. We performed an intention-to-treat analysis for all enrolled patients.

Results. This study enrolled 44 patients. The most common toxicities were hematological and gastrointestinal events. Grade 3/4 hematological toxicities were observed during preoperative chemotherapy, although there were no grade 3/4 nonhematological events. Postoperative chemotherapy-related toxicities were more critical and frequent than preoperative ones. There were no pre- or postoperative chemotherapy-associated deaths. Median overall survival was 36.5 months with 30.5% overall 5-year survival.

Conclusions. This multimodality therapy is feasible and promises to contribute to survival. It should be evaluated in a phase III setting.

Pancreatic adenocarcinoma remains a lethal disease, with an overall 5-year survival rate ranging from 0.4 to 5%.[1,2] Even after curative resection of pancreatic cancer, there is a high probability of systemic and/or local recurrence.[3–5] This indicates that subclinical metastases are already present in most patients at the time of operation, even if preoperative radiological imaging or intraoperative examination revealed no metastatic lesions. Therefore, a multimodality strategy, including not only local control but also treatment of micrometastases, is required for patients with pancreatic cancer. For local control, beginning in 1984 we introduced extended radical pancreatectomy combined with intraoperative radiation therapy (IORT).[6] This approach provided the best control of local recurrence, but there was no survival benefit because of blood-borne metastases.[5] To treat unresectable pancreatic cancer, we introduced a combination of chemotherapy using 5-fluorouracil (5-FU) pancreatic and hepatic arterial continuous infusion and systemic gemcitabine administration; this combined therapy was well tolerated, with a 1-year survival rate of 50.9%.[7]

We studied a novel multimodality therapy combining pancreatic resection and IORT with pre- and postoperative chemotherapy using 5-FU intra-arterial continuous infusion and systemic gemcitabine administration in patients with potentially resectable pancreatic cancer. The purpose of this study was to evaluate the feasibility and outcomes of this multimodality therapy.

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H. Takamori, MD
e-mail: h-takamori@fc.kuh.kumamoto-u.ac.jp

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PATIENTS AND METHODS

Patients

All patients were advised of the investigational nature of the study and gave their written, informed consent to participate before the beginning of the study. All patients underwent a standard pretreatment evaluation that included a physical examination, a thin-section, contrast-enhanced, multiphase spiral computed tomography (CT) of the abdomen, and ultrasonography. The absence of liver metastasis was confirmed by CT during arterial portography combined with CT-assisted hepatic arteriography (CTAP + CTHA), as described previously.[8] The absence of lung metastasis was confirmed by chest CT. The protocol required patients with potentially resectable disease as assessed by a physical examination and the following objective radiographic criteria: (1) no evidence of remote metastases; (2) no evidence of tumor extension to the celiac axis or the superior mesenteric artery. We included only patients in whom it was technically possible to resect and reconstruct the superior mesenteric vein (SMV) or the portal vein (PV), if the tumor involved SMV or PV. We excluded cases in which the tumor was 1 cm or smaller in diameter, because of the very low possibility of systemic spreading of the disease. Patients were required to have an Eastern Cooperative Oncology Group performance status of ≤ 2.

Perioperative Chemotherapy

The treatment schema is shown in Fig. 1. The pre- and postoperative chemotherapy consisted of the combination of 5-FU arterial continuous infusion and systemic gemcitabine administration. In all cases, the catheter for arterial infusion was introduced from the femoral artery under local anesthesia. After the closure of the distal tip of the catheter, a side hole was made at an appropriate site in the celiac axis to allow the distribution of 5-FU to both the pancreatic tumor and the liver preoperatively, and in the hepatic artery to distribute the drug to the whole liver postoperatively. An arterial port was implanted in the subcutaneous tissue. 5-FU was administered at a dose of 125 mg/m^2 per day on days 1–5 each week as continuous infusion through the arterial port for 2 weeks during preoperative chemotherapy and for 8 weeks during postoperative chemotherapy. Gemcitabine was infused intravenously for 30 min at a dose of 800 mg/m^2 once weekly for a total of 2 doses preoperatively and for a total of 18 doses postoperatively. The doses of these drugs were based on our preliminary results for the combination chemotherapy using 5-FU intra-arterial infusion and systemic gemcitabine for unresectable pancreatic cancer.[7]

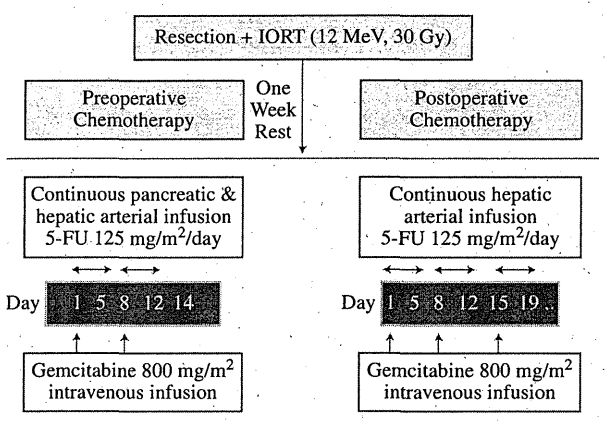

FIG. 1 Treatment schema. 5-FU was administered on days 1–5 every week as a continuous arterial infusion combined with gemcitabine infused once weekly for 2 weeks followed by pancreatic resection combined with IORT. Postoperative chemotherapy was performed in the same way as preoperative chemotherapy

In cases of grade 3 or higher toxicity according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0, drug infusion was interrupted until recovery. History, physical examination, and complete blood counts (CBCs) were repeated weekly before infusion of the drugs. Chemistry profiles were performed every 2 weeks. The catheter and port for arterial infusion were removed after the completion of intra-arterial infusion of 5-FU.

Surgery

Patients with cancer of the head of the pancreas underwent a substomach-preserving pancreaticoduodenectomy (SSPPD), a pylorus-preserving pancreaticoduodenectomy (PPPD), or a Kausch-Whipple resection: the last of these was performed if a tumor directly invaded the duodenum or antrum of the stomach, or if a distal gastrectomy had been performed before. Patients with cancer of the body or tail of the pancreas underwent a distal pancreatectomy. Patients underwent resection with reconstruction of SMV or PV if a tumor was thought during surgery to involve these vessels. For IORT, a dose of 30 Gy with a 12 MeV of electron beam was delivered to the operative field using a special pentagon applicator following dissection, as described previously.[6]

Hospital death was defined as death during hospitalization. Major surgical complications included any occurrence of anastomotic leak, postoperative intra-abdominal or gastrointestinal hemorrhage or fistula, intra-abdominal abscess, pneumonia, catheter-related sepsis, thromboembolic events, and reoperation. Pancreatic fistula was assessed according to an international study group (ISGPF) definition.[9]

Toxicity and Outcome Evaluation

Toxicities were graded according to NCI-CTC version 3.0. Survival was calculated from the day of surgery and estimated by the Kaplan–Meier method. The first site of disease recurrence was documented for outcome analysis.

All patients were evaluated every 3–4 months by physical examination as well as by chest and abdominal CT after surgery. For those without any recurrence after 2 years, follow-up was at 6-month intervals. Cytologic or histologic confirmation of disease recurrence was not required.

RESULTS

Patient Characteristics

From May 2001 through September 2008, 44 patients were enrolled in this study. The patients' characteristics are outlined in Table 1. The primary pancreatic lesion was located in the head in 33 patients, in the body in 9, and in the tail in 2. All patients underwent pancreatic resection. Pancreatic ductal adenocarcinoma was confirmed in all patients histologically. R0 resection was performed in 37 patients, R1 resection in 5 (11.4%), and R2 resection in 2 (4.5%). The median tumor size was 3 (range, 1.3–8.7) cm. Lymph node metastases were identified in 30 patients (68.2%), including para-aortic lymph node metastases in 3 patients. Resection and reconstruction of SMV or PV were necessary in 22 patients (50%), although 13 (29.5%) were proven to have histological portal invasion. Thirty-four patients received IORT after resection. All of the patients began postoperative chemotherapy after recovery from surgery, although 20 patients (45.5%) were completely treated according to the postoperative schedule. The mean pre- and postoperative doses of total 5-FU administered per patient were 2.8 and 5.2 g. The mean pre- and postoperative doses of total gemcitabine were 5.2 and 14.3 g.

Toxicities of Pre- and Postchemotherapy and Surgery

All 44 patients were included in the toxicity analysis. The overall toxicity profiles related to pre- and postoperative chemotherapy are outlined in Table 2. The most common toxicities were hematological and gastrointestinal events.

Nineteen patients (43.2%) experienced grade 3/4 neutropenia during preoperative chemotherapy. All preoperative chemotherapy-related toxicities abated after discontinuation of drug infusion. Forty-three patients underwent surgery 1 week after the completion of preoperative chemotherapy. Only one patient experienced a delay in surgery because of grade 4 neutropenia. Five major complications occurred in five patients after surgery, including grade C pancreatic fistula in two patients, intra-abdominal abscess in one, and cerebral infarction in one. Three patients recovered from complications by means of conservative therapies. One patient underwent reoperation for grade C pancreatic fistula. Hospital death was observed in one patient because of liver failure after intra-abdominal bleeding caused by pancreatic fistula.

TABLE 1 Patient characteristics

Characteristics	No. of patients	%
Total no. of patients	44	
Median age (yr)	65 (37–79)	
Male/female	26/18	
Site of primary lesion		
Head	33	75
Body	9	20.5
Tail	2	4.5
Pancreatectomy		
PPPD	16	36.4
SSPPD	13	29.5
PD	4	9.1
DP	11	25
Stage		
Ia	3	6.8
Ib	1	2.3
11a	10	22.7
11b	26	59.1
III	1	2.3
IV	3	6.8
Histologic differentiation		
Well	16	36.4
Moderately	22	50
Poorly	5	11.4
Adenosquamous	1	2.3
Tumor size (cm)		
1.0–2.0	6	13.6
2.1–4.0	33	75
>4.1	5	11.4
Nodal involvement		
Present	30	68.2
Absent	14	31.8
Portal vein invasion		
Present	13	29.5
Absent	31	70.5
Residual tumor		
R0	37	84.1
R1	5	11.4
R2	2	4.5

PPPD pylorus-preserving pancreaticoduodenectomy; *SSPPD* substomach-preserving pancreaticoduodenectomy; *PD* pancreaticoduodenectomy; *DP* distal pancreatectomy

TABLE 2 Pre- and postoperative chemotherapy-related grade 3/4 toxicities

	Preoperative	Postoperative
Hematological		
Anemia	0	4 (9.1)
Leukopenia	8 (18.2)	14 (31.8)
Neutropenia	19 (43.2)	24 (54.5)
Thrombocytopenia	2 (4.5)	3 (6.8)
Others		
Perforation of small intestine	0	1 (2.3)
Liver abscess	0	3 (6.8)
Cardiac ischemia/infarction	0	2 (4.5)
Renal dysfunction	0	1 (2.3)
Cholangitis	0	3 (6.8)
Appetite loss	0	1 (2.3)

Percentages are shown in parentheses

Toxicities are defined by NCI-CTC for Adverse Events v3.0

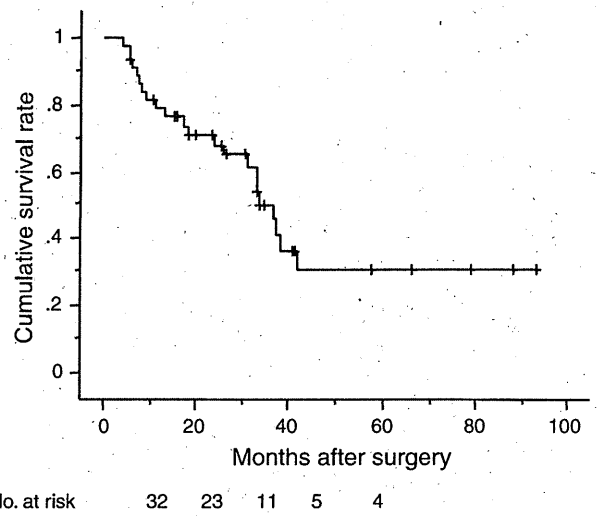

FIG. 2 Overall survival curve for all patients

TABLE 3 Outcomes after this multimodality therapy for patients with pancreatic cancer

	No.
Cancer deaths	17
Liver metastases	4
Lung metastases	1
Pleural dissemination	1
Peritoneal dissemination	6
Local recurrence	4
Bone metastases	1
Non-cancer-related deaths	5
Alive	22
Total	44

Postoperative chemotherapy was initiated between 3 and 12 weeks after surgery. Twenty-four patients (54.5%) experienced grade 3/4 neutropenia during postoperative chemotherapy, although these toxicities abated after drug infusion was interrupted. Perforation of the small intestine in one patient occurred 1 year after pancreatic resection. This patient underwent emergency surgery and recovered. Grade 3/4 cardiac ischemia occurred in two patients and liver abscess in three patients (6.8%) during postoperative chemotherapy. No intra-arterial catheter-related toxicity occurred in any of the patients. Neither pre- nor postoperative chemotherapy-associated death was observed.

Survival and Outcome

The median follow-up period was 28.2 (range, 5.5–93.3) months. The 1, 3, and 5-year actuarial overall survival rates in all the patients were 78.8, 50.3, and 30.5%, respectively (Fig. 2). The median survival time was 36.5 months.

At last follow-up, 22 of the 44 patients (50%) had died. Seventeen (38.6%) had died as a result of recurrence. There were five (11.4%) non-cancer-related deaths, including one hospital death. Twenty-two patients (50%) remained alive. The median time of tumor recurrence was 24.0 months from the day of surgery. Liver metastases were observed in four patients (9.1%), peritoneal dissemination in six (13.6%), lung metastases in one (2.3%), pleural dissemination in one, bone metastases in one, and local recurrence in four (Table 3). Eight patients survived more than 32.3 months. The two patients with R2 resection died of peritoneal dissemination within 12 months.

DISCUSSION

To our knowledge, this is the first report of perioperative intra-arterial and systemic chemotherapy for pancreatic cancer. This treatment was clearly operator-dependent. Grade 3/4 neutropenia was relatively frequent during perioperative chemotherapy, although the toxicities abated after interruption of drug infusion. Grade 3/4 nonhematological toxicities were observed during postoperative chemotherapy. Liver abscess occurred in three patients. This was thought to be influenced by regurgitated cholangitis, because all of the patients underwent hepaticojejunostomy after PD. Perforation of the small intestine occurred in one patient 3 months after completion of postoperative chemotherapy. Cardiac ischemia required hospitalization for two patients. However, the relationship between these events and chemotherapy was unclear. Toxicities were more critical and frequent during postoperative chemotherapy than during preoperative. Intra-arterial infusion was acceptable

for perioperative chemotherapy, because no catheter-related toxicity was observed.

Practical and theoretical advantages of preoperative treatment of pancreatic cancer were proposed as an early treatment for micrometastases and optimized patient selection for surgery.[10–12] Circulating tumor cells in the blood proved to be present in 28% of patients with pancreatic cancer, and the prevalence increased with tumor stages.[13] Moreover, complications, which occurred after 30–45% of major pancreatic resections, delayed the initiation of postoperative chemotherapy.[14,15] These are supported to introduce preoperative chemotherapy for pancreatic cancer.

The rationale for intra-arterial infusion of chemotherapeutic agents appears to be promising from the point of view of the drug-concentration response, because most liver metastases (>3 mm) have an arterial blood supply.[16,17] Locoregional adjuvant chemotherapy has been reported to have 3-year survival rates ranging from 48 to 54%, and lower recurrence rates of liver metastases ranging from 8 to 17% for pancreatic cancer compared with no-adjuvant studies.[3,4,18,19] This study also showed that liver metastases diminished to 9.1%, indicating that intra-arterial chemotherapy might be effective to prevent liver metastases.

We adopted pancreatic resection combined with IORT for local control in this series. Local recurrence was observed in only four patients (9.1%). Single-institution experiences suggest that local failure rates were lower in radiation groups (10–26%) than in no-radiation groups (50–80%).[20–24] This indicated that resection combined with IORT could provide good control of local recurrence.

Recently, a phase III randomized trial (CONCO 001 study) demonstrated that adjuvant gemcitabine significantly delayed the development of recurrence after resection of pancreatic cancer, with a median survival time of 22.1 months.[25] Evans et al. reported on a phase II trial of neoadjuvant gemcitabine-based chemoradiation for stage I/II pancreatic cancer.[26] The median survival time of 36.5 months in our study is similar to the 34 months in the Evans group trial despite a greater proportion of patients with node-positive (68.2%) and R2 resection (4.5%) in our study than in the Evans group trial. Because our perioperative chemotherapy is complicated, it will be necessary to clarify which adjuvant treatment is most effective for pancreatic cancer to simplify treatment.

In conclusion, this perioperative chemotherapy for pancreatic cancer is feasible and promises to contribute to survival. It should be evaluated in a phase III setting.

REFERENCES

1. Bramhall SR, Allum WH, Jones AG, Allwood A, Cummins C, Neoptolemos JP. Treatment and survival in 13560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. *Br J Surg.* 1995;82:111–5.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin.* 2008;58:71–96.
3. Griffin JF, Smalley SR, Jewell W, Paradelo JC, Reymond RD, Hassanein RE, Evans RG. Patterns of failure after curative resection of pancreatic carcinoma. *Cancer.* 1990;66:56–61.
4. Sperti C, Pasquali C, Piccoli A, Pedrazzoli S. Recurrence after resection for ductal adenocarcinoma of the pancreas. *World J Surg.* 1997;21:195–200.
5. Takamori H, Hiraoka T, Kanemitsu K, Tsuji T. Pancreatic liver metastases after curative resection combined with intraoperative radiation for pancreatic cancer. *Hepato-Gastroenterol.* 2004;51: 1500–3.
6. Hiraoka T, Uchino R, Kanemitsu K, et al. Combination of intraoperative radiation with resection of cancer of the pancreas. *Int J Pancreatol.* 1990;7:201–7.
7. Takamori H, Kanemitsu K, Tsuji T, et al. 5-Fluorouracil intra-arterial infusion combined with systemic gemcitabine for unresectable pancreatic cancer. *Pancreas.* 2005;30:223–6.
8. Takamori H, Ikeda O, Kanemitsu K, et al. Preoperative detection of liver metastases secondary to pancreatic cancer. Utility of combined helical computed tomography during arterial portography with biphasic computed tomography-assisted hepatic arteriography. *Pancreas.* 2004;29:188–92.
9. Bassi C, Dervenis C, Butturini G, et al. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery.* 2005;138:8–13.
10. Evans DB, Rich TA, Byrd DR, et al. Preoperative chemoradiation and pancreaticoduodenectomy for adenocarcinoma of the pancreas. *Arch Surg.* 1992;127:1335–9.
11. Wayne JD, Abdalla EK, Wolff RA, Crane CH, Pisters PW, Evans DB. Localized adenocarcinoma of the pancreas: the rationale for preoperative chemoradiation. *Oncologist.* 2002;7:34–45.
12. Pisters PW, Wolff RA, Janjan NA, et al. Preoperative paclitaxel and concurrent rapid fractionation radiation for resectable pancreatic adenocarcinoma: toxicities, histologic response rates, and event-free outcome. *J Clin Oncol.* 2002;20:2537–44.
13. Z'graggen K, Centeno BA, Fernandez-del Castillo C, Jimenez RE, Werner J, Warshaw AL. Biological implications of tumor cells in blood and bone marrow of pancreatic cancer patients. *Surgery.* 2001;129:537–46.
14. Sohn TA, Yeo CJ, Cameron JT, et al. Resected adenocarcinoma of the pancreas–616 patients: results, outcomes, and prognosis indicators. *J Gastrointest Surg.* 2000;4:567–79.
15. Frederick WAI, Wolff RA, Crane CH. Preoperative (neoadjuvant) therapy for resectable adenocarcinoma of the pancreas. In: Evans DB, Pisters PWT, Abbruzzese JL, Pollock RE, editors. MD Anderson Solid Tumor Oncology Series. New York: Springer; 2001. p. 243–54.
16. Archer SG, Gray BN. Vascularization of small liver metastases. *Br J Surg.* 1989;76:545–8.
17. Ackermann NB. The blood supply of experimental liver metastases. IV. Changes in vascularity with increasing tumor growth. *Surgery.* 1974;75:589–96.
18. Ishikawa O, Ohigashi H, Sasaki Y, et al. Liver perfusion chemotherapy via both the hepatic artery and portal vein to prevent hepatic metastasis after extended pancreatectomy for adenocarcinoma of the pancreas. *Am J Surg.* 1994;168:361–4.
19. Beger HG, Gansauge F, Büchler MW, Link KH. Intraarterial adjuvant chemotherapy after pancreaticoduodenectomy for pancreatic cancer: significant reduction in occurrence of liver metastasis. *World J Surg.* 1999; 23:946–949.
20. Breslin TM, Hess KR, Harbison DB, et al. Neoadjuvant chemoradiotherapy for adenocarcinoma of the pancreas: treatment variables and survival duration. *Ann Surg Oncol.* 2001;8:123–32.
21. Spitz FR, Abbruzzese JL, Lee JE, et al. Preoperative and postoperative chemoradiation strategies in patients treated with

pancreaticoduodenectomy for adenocarcinoma of the pancreas. *J Clin Oncol.* 1997;15:928–37.
22. Tepper JE, Nardi G, Suit H. Carcinoma of the pancreas: review of MGH experience from 1963–1973. Analysis of surgical failure and implications for radiation therapy. *Cancer.* 1976;37:1519–25.
23. Kayahara M, Nagakawa T, Ueno K, Ohta T, Takeda T, Miyazaki I. An evaluation of radical resection for pancreatic cancer based on the mode of recurrence as determined by autopsy and diagnostic imaging. *Cancer.* 1993;72:2118–23.
24. Whittington R, Bryer MP, Haller DG, Solin LJ, Rosato EF. Adjuvant therapy of resected adenocarcinoma of the pancreas. *Int J Radiat Oncol Biol Phys.* 1991;21:1137–43.
25. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer. A randomized controlled trial. *JAMA.* 2007;297:267–77.
26. Evans DB, Varadhachary GR, Crane CH, et al. Preoperative gemcitabine-based chemoradiation for patients with resectable adenocarcinoma of the pancreas. *J Clin Oncol.* 2008;26: 3496–502.

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Dickkopf-1 is overexpressed in human pancreatic ductal adenocarcinoma cells and is involved in invasive growth

Nobuyasu Takahashi[1,2], Tsuyoshi Fukushima[1], Kenji Yorita[1], Hiroyuki Tanaka[1], Kazuo Chijiiwa[2] and Hiroaki Kataoka[1]

[1] Section of Oncopathology and Regenerative Biology, Department of Pathology, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, Japan
[2] Department of Surgical Oncology and Regulation of Organ Function, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, Japan

The protein products of the *Dickkopf* (*DKK*) genes are antagonists of Wnt glycoproteins, which participate in tumor development and progression by binding to frizzled receptors. In this study, the expression of DKK-1 was analyzed in a panel of 43 human cultured carcinoma cell lines. DKK-1 expression was consistently and significantly upregulated in pancreatic carcinoma cell lines. Low level of DKK-3 expression was also seen. In contrast, the expression of DKK-2 and -4 was not detectable in most pancreatic carcinoma cell lines. The overexpression of DKK-1 was confirmed in surgically resected human pancreatic cancer tissues, in which the mRNA level was evaluated in paired samples from cancerous and noncancerous pancreatic tissues. In ductal adenocarcinomas (23 cases), DKK-1 mRNA levels were significantly upregulated compared to corresponding noncancerous tissues in a statistically significant level. To test the biological role of DKK-1 in pancreatic carcinoma cells, we performed a knockdown of DKK-1 in SUIT-2 human pancreatic adenocarcinoma cell line and S2-CP8, its metastatic subline, using a retroviral short hairpin RNA expression vector. DKK-1 knockdown resulted in reduced migratory activity of SUIT-2 *in vitro*. The *in vitro* growth rate and Matrigel invasion were also suppressed by DKK-1 knockdown in S2-CP8 cells. Collectively, the evidence suggests that, despite of its presumed antagonistic role in Wnt signaling, DKK-1 may have a role in the aggressiveness of pancreatic carcinoma cells and could, therefore, serve as a novel biomarker of pancreatic cancer.

The *Dickkopf* (*DKK*) genes are Wnt antagonists that were originally identified as inducers of head formation in Xenopus.[1] Wnt glycoproteins participate in tumor development and progression by binding to frizzled receptors and signaling through the canonical and noncanonical Wnt pathways.[2]

Key words: DKK-1, wnt signal, pancreatic cancer, ductal adenocarcinoma

Abbreviations: DAC: ductal adenocarcinoma; DKK: Dickkopf; DMEM: Dulbecco's modified Eagle's medium; EC: endocrine carcinoma; FBS: fetal bovine serum; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; GSK-3β: glycogen synthase kinase-3β; HSP70: heat shock protein 70; IPMC: intraductal papillary mucinous carcinoma; JNK: c-Jun N-terminal kinase; MCAC: mucinous cystadenocarcinoma; PanIN: pancreatic intraepithelial neoplasia; PBS: phosphate buffered saline; RT-PCR: reverse-transcriptase/polymerase-chain reaction; SCA: serous cystadenoma; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA: short hairpin RNA; SPN: solid pseudopapillary neoplasm; TBS-T: Tris-buffered saline with 0.05% Tween 20.

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Correspondence to: Hiroaki Kataoka, Section of Oncopathology and Regenerative Biology, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan, Fax: +81-985-85-6003, E-mail: mejina@med.miyazaki-u.ac.jp

The *DKK* gene family consists of *DKK-1*, *-2*, *-3*, *-4*, and a unique DKK-3-related gene *soggy*.[2] The expression of these genes is temporally and spatially regulated, and all DKK proteins show distinct and elevated expression patterns in tissues that mediate the epithelial-mesenchymal transformation.[3] This finding suggests that they may participate in the epithelial to mesenchymal transition that is important not only in embryogenesis, but also in cancer progression.[4] DKK-1 is a secreted protein that has been clearly defined as a direct inhibitor of Wnt binding to LRP5/6 coreceptors of frizzled.[2] Evidence for the potential involvement of DKK-1 inactivation in human cancers is accumulating. The *DKK-1* gene is frequently hypermethylated in colorectal cancers, whereas overexpression of DKK-1 reduced the growth of colorectal carcinoma cells.[5,6] The expression of DKK-1 is also reportedly downregulated in melanoma cells,[7] lung cancer cells with neuroendocrine differentiation[8] and in a metastatic subline of hepatocellular carcinoma cell line.[9] In contrast, overexpression of DKK-1 has also been found in some tumors, such as Wilms' tumor, hepatoblastoma, hepatocellular carcinoma and ovarian endometrioid adenocarcinoma.[10–13] Moreover, gene expression profiles have revealed that *DKK-1* is overexpressed in nonsmall cell lung carcinoma and esophageal squamous cell carcinoma, serving as a serologic and prognostic biomarker.[14] DKK-1 may also have roles in bone metastases of breast and prostatic carcinomas.[15,16] Therefore, the function and role of DKK-1 in cancer appears to depend on the histological types of the cancer cells and the tissue microenvironment.

Pancreatic cancer has the worst prognosis of any major malignancy and its incidence is increasing.[17] Delayed diagnosis and an intrinsic biological aggressiveness contribute to the abysmal prognosis of this disease. The identification of sensitive biomarkers and further understanding of the cellular biology of pancreatic ductal adenocarcinoma (DAC) cells are required. In this study, expression analysis of a panel of human carcinoma cell lines showed that DKK-1 was consistently upregulated in pancreatic carcinoma cell lines. Using surgically resected tissues, we confirmed the upregulation of DKK-1 mRNA levels in DAC of the pancreas. We also utilized a short hairpin RNA (shRNA)-based knockdown system to examine the role of DKK-1 in cultured pancreatic carcinoma cells.

Material and Methods

Cell culture and cell growth assay

Human pancreatic adenocarcinoma cell line SUIT-2[18] and its metastatic sublines, S2-VP10 and S2-CP8,[19] were kindly provided by Dr. Takeshi Iwamura (Junwakai Memorial Hospital). S2-CP8 and S2-VP10 were established by Cutis-Pulmonary metastasis-culture (8 times) and Vein-Pulmonary metastasis-culture (10 times), *via* injection of parental SUIT-2 cells subcutaneously (S2-CP8) or intravenously (S2-VP10) into nude mice.[19] Other 6 human pancreas cancer cell lines (SUIT-4, AsPC-1, MIA PaCa-2, PANC-1, HPAF and BxPC-3), 13 colon cancer cell lines (RCM-1, RCM-2, RCM-3, CaCo-2, HCT116, SW837, DLD-1, LoVo, WiDr, Colo205, COCM-1, CaR-1 and Colo320DM), 11 lung cancer cell lines (LC-1/sq, RERF-LC-AI, LC2/ad, RERF-LC-KJ, HLC-1, A549, PC14, Lu135, Lu139, T3M-11 and MS-1), 3 gastric cancer cell lines (MKN28, MKN45 and KATO III), 1 duodenal cancer cell line (HUTU80), 2 hepatocellular carcinoma cell lines (HepG2 and HuH7), 3 breast cancer cell lines (MCF-7, BT-20 and SKBR3), 1 cervical cancer cell line (HeLa), 1 renal cell carcinoma cell line (MRT-1) and 1 prostate cancer cell line (PC-3) were used in this study. RCM-1, RCM-2, RCM-3, COCM-1, LC-1/sq, LC-2/ad and MRT-1 were established in our laboratory. SUIT-4, HPAF, BxPC-3, HUTU 80, MCF-7, BT-20 and SKBR-3 were also provided by Dr. T. Iwamura, Junwakai Memorial Hospital. HLC-1 was kindly provided by the Department of Pathology, Keio University. LoVo, Hela, RERF-LC-AI, RERF-LC-KJ, A549, PC-14, Lu139, MS-1, T3M11 and Lu139 were obtained from Riken Cell Bank (Tsukuba, Japan). WiDr, CaCO-2, SW837, Colo205, KATO III, HepG2, HuH7, AsPC-1, PANC-1, MIA PaCa-2 and PC-3 were obtained from Dainihon Seiyaku (Osaka, Japan). IBL (Fujioka, Japan) provided MKN-28 and -45. DLD-1, CaR-1 and Colo320DM were received from the Health Science Research Resources Bank (Osaka, Japan). HCT116 was purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) or a mixture of RPMI 1640 and Ham's F-12 containing 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), penicillin G (100 U/ml) and 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid, pH 7.25, at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of tissue samples

All fresh tumor tissues were obtained from surgical specimens of patients with pancreatic tumors in the University of Miyazaki Hospital, Miyazaki, Japan. Informed consent was obtained from the patients and the protocol was approved by the ethical board of the Faculty of Medicine, University of Miyazaki. A total of 36 cases were evaluated in this study. Tissue samples were crashed and frozen in liquid nitrogen in the operating room as soon as the tumor was resected, and stored at −80°C freezer until analysis. Total cellular RNA was extracted with Trizol reagent (Gibco BRL, Gaithersburg, MD), followed by DNase I (Roche Applied Science, Indianapolis, IN) treatment and phenol-chloroform-isoamylalcohol extraction.

Immunohistochemistry and *in situ* hybridization

Formalin-fixed and paraffin-embedded sections were subjected to antigen retrieval by autoclaving for 5 min in 10 mM citrate buffer, pH 6.0. After peroxidase blocking, the sections were blocked in 3% bovine serum albumin (BSA) and 10% goat serum in phosphate-buffered saline (PBS) for 1 hr at room temperature. Subsequently, the sections were incubated with anti-DKK-1 rabbit polyclonal antibody (5 μg/ml in 1% BSA/PBS; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Negative controls consisted of an omission of the primary antibodies. The sections were rinsed in PBS and incubated with Envision-labeled polymer (DAKO) for 30 min at 37°C. After washing, the sections were visualized with nickel, cobalt-3,39-diaminobenzidine (Pierce, Rockford, IL) and counter stained with haematoxylin. For *in situ* hybridization (ISH) study, formalin-fixed and paraffin-embedded sections (4-μm thick) were fixed in 4% paraformaldehyde/PBS, dehydrated and used for ISH reaction with a fully automated ISH apparatus (Ventana, Yokohama, Japan) as described previously. A 708-bp cDNA fragment corresponding to bases 182–889 of the human DKK-1 cDNA sequence[20] was used as a template to generate digoxigenin-labeled RNA probes. The same amount of each antisense or sense probe (200 ng/slide) was used. The reaction was visualized with BlueMap Kit (Roche, GmbH, Penzberg, Germany) and counterstained with nuclear fast red.

Knockdown of DKK-1

The knockdown vector was constructed using an shRNA expression retroviral vector pSINsi-hU6 (TAKARA Bio, Shiga, Japan) as described previously.[21] The target sequence of the *DKK-1* gene was 5′-GGAATAAGTACCAGACCATTG-3′ and its scramble control was 5′-GAGGAATCATCAGACGCATTA-3′. For infection of retroviral vectors, Amphopack-293 packaging cells cultured in 6-well plates were incubated with 1 μg of recombinant retroviral vector

and 3 μl of TransFectin (Bio-Rad, Hercules, CA) for 12 hr. After 48 hr of cultivation, the supernatant containing the retroviral particles was collected, filtered through a 0.45-μm filter, and used to infect target cells. Cultured SUIT-2 cells were trypsinized and resuspended in the viral supernatant in the presence of 5 μg/ml of polybrene (Aldrich, Milwaukee, WI) for 12 hr. The cells were then incubated with a 1:1 mixture of fresh medium and viral supernatant with magnetofection reagent (CombiMag™, OZ Biosciences, Marseille, France), and placed on a magnetic plate for an additional 12 hr. This process was repeated 3 times. The transfected cells were subcultured at an appropriate density in fresh DMEM containing 0.5 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). G418-resistant cell pools were readily established within 2 weeks.

Reverse-transcriptase/polymerase-chain reaction (RT-PCR) and quantitative real-time RT-PCR

Three micrograms of total RNA was reverse-transcribed with a mixture of oligo dT and random primer using 200 units of SuperScript™ Reverse Transcriptase (Gibco BRL), and 1/90 of the resultant cDNA was processed for each PCR reaction with 0.1 μM of both forward and reverse primers and 2.5 units of HotStar™ Taq DNA Polymerase (Qiagen, Tokyo, Japan). The PCR products were analyzed by 1.5% agarose gel electrophoresis. The following primers were used for conventional RT-PCR: DKK-1 (247-bp product), 5′-AGGAAG CGCCGAAAACGCTGCATG-3′ (forward) and 5′-AGGCA-CAGTCTGATGACCGGAGAC-3′ (reverse); DKK-2 (334 bp), 5′-GCAGTGATAAGGAGTGTGAAGTT-3′ (forward) and 5′-AATGCAGTCTGATGATCGTAGGC-3′ (reverse); DKK-3 (349 bp), 5′-AGGCAGAAGAA GCTGCTGCTAA-3′ (forward) and 5′-AGCTGGTCTCCACAGCACTCACT-3′ (reverse); DKK-4 (315 bp), 5′-ACGGACT GCAATACCAGA AAGTT-3′ (forward) and 5′-CAAAGTCCAG GGCCA-CAGTCAA-3′ (reverse); c-Myc (338 bp) 5′-TCCAGC TTG TACCTGCAGGATCTGA-3′ (forward) and 5′-CCT CCAG-CAGAAGGTGATCCAGACT-3′ (reverse); cyclin D1 (319 bp) 5′-GGTCCTGCCGTCCATGCGGA-3′ (forward) and 5′-CGGGGT CATTGCGGCCAGGT-3′ (reverse); glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (300 bp), 5′-GTGAAGGTCGGAGTCAACG-3′ (forward) and 5′-GGTGAAGACGCCAGTGGACTC-3′ (reverse).

Quantitative real-time RT-PCR using SYBR green was performed in a LightCycler™ (Roche Applied Science) according to the manufacturer's instructions. Levels of internal control, β-actin mRNA, were measured as described previously.[21]

In vitro motility and invasion assays

A monolayer wounding (scratch) assay was performed to evaluate the *in vitro* motility of cultured cells. Cells were allowed to form a monolayer on a culture dish, and a wound was made by scratching the monolayer with a pipette tip. After the scratched cells were removed, the cells were cultivated for the indicated time periods. *In vitro* invasive capability was evaluated using the Matrigel invasion assay performed with Chemotaxicells (polycarbonate filter, pore size 8 μm) (Kurabo, Osaka, Japan) coated with 25 μg/filter of Matrigel (Gibco BRL). Cells (1×10^5) in 100 μl of DMEM/0.1% BSA were placed in the upper compartment and incubated for 48 hr. As a chemoattractant, 1% FBS was added to the lower compartment. After incubation, the cells on the upper surface of the filter were wiped off with a cotton swab, and the cells on the lower surface were stained with hematoxylin. Migration activity on Type IV collagen was quantified by counting the cells in 10 randomly selected fields (200× original magnification).

Immunoblot analysis

To detect DKK-1 protein, subconfluent cultured cells were washed 3 times with PBS and cultured with serum-free DMEM for 72 hr. The serum-free conditioned media were harvested and dialyzed against 2.5 mM Tris-HCl (pH 7.6). The dialyzed samples were lyophilized and stored at −80°C until analyzed. Equal amounts of proteins were electrophoresed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to Immobilon membrane (Millipore, Bedford, MA), and detected by immunoblotting as described previously.[21] Anti-human DKK-1 rabbit polyclonal antibody (Santa Cruz Biotechnology) was used as the primary antibody. To evaluate β-catenin and glycogen synthase kinase-3β (GSK-3β), cellular proteins were extracted at 80% confluency with cell lysis buffer (CelLytic™-M; Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitor and phosphatase inhibitor mixtures (Sigma-Aldrich) on ice. To examine the cellular localization of β-catenin, cellular proteins were extracted and fractionated in cytosolic and nuclear fractions using CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich). After centrifugation (16,000*g* for 10 min), equal amounts of proteins were separated by SDS-PAGE and subjected to immunoblot analysis as described.[21] After blocking with 5% phosphatase-free BSA in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T), the membrane was incubated with primary antibody diluted in Can Get Signal™ (TOYOBO, Osaka, Japan) solution1 for 2 hr at room temperature. The following primary antibody were used: anti-β-catenin (Santa Cruz Biotechnology) and anti-GSK-3β (BD Transduction Laboratories, San Jose, CA) mouse monoclonal antibodies; anti- phospho-β-Catenin, anti-phospho-GSK-3β, anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-lamin A/C, anti-heat shock protein 70 (HSP70) rabbit polyclonal antibodies (Cell Signaling Technology, Danvers, MA). Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies diluted in Can Get Signal solution for 1 hr at room temperature. The labeled proteins were visualized as described.[21]

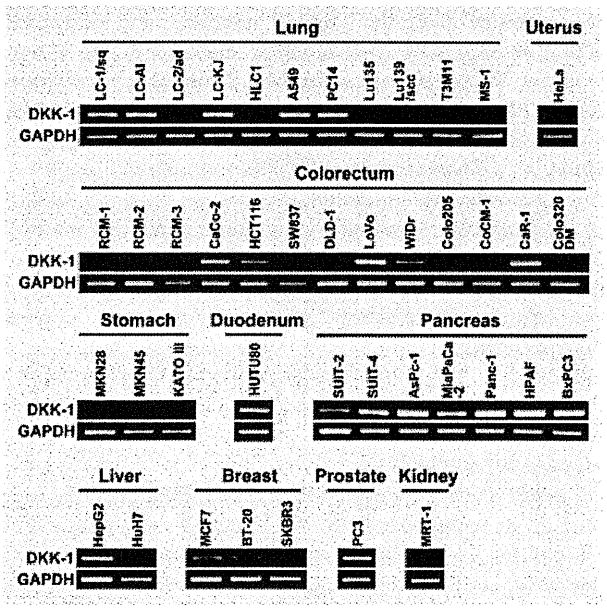

Figure 1. DKK-1 expression in human tumor cell lines (RT-PCR: 30 cycles of amplification). GAPDH mRNA was used as the internal control.

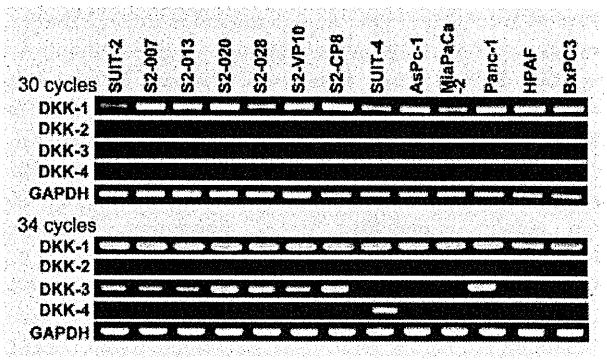

Figure 2. Expression of DKK-1, -2, -3 and -4 in human pancreas carcinoma cell lines. Representative results of 2 RT-PCR experiments (30 cycles and 34 cycles of amplification) are shown.

Subcutaneous injection of tumor cells in nude mice

All animal work was carried out under protocols approved by the University of Miyazaki Animal Research Committee, in accordance with international guiding principles for biomedical research involving animals. Injections of 5×10^6 cells/0.2 ml PBS were subcutaneously administered at the abdominal flank of 6-week-old male nude mice (Balb/cAJc1-nu). Tumor volume was estimated by the formula $V = L \times W \times W/2$ (V: volume [mm^3]; L: length [mm]; W: width [mm]). All mice were observed everyday and sacrificed at 8 weeks postimplantation. Their lungs were excised and fixed in 4% paraformaldehyde for 24 hr. The number of metastatic lesions was counted in the largest cross-sectional specimens from both lungs of each mouse.

Statistics

Comparisons between 2 paired groups or 2 unpaired groups were performed with the Wilcoxon signed rank test or Mann–Whitney *U*-test, respectively, using Statview 5.0 (Brainpower, Calabasas, CA). Significance was set at $p < 0.05$.

Results

Consistent upregulation of DKK-1 expression in cultured human pancreatic carcinoma cell lines

DKK-1 expression was characterized in a panel of cultured human carcinoma cell lines; lung (11 lines), uterine (1), colorectal (13), gastroduodenal (4), pancreatic (7), 2 hepatocellular (2), breast (3), prostate (1) and renal (1) (Fig. 1). Among the 43 cell lines examined, DKK-1 mRNA was detectable in 27, and its expression level was distinct in 20. Remarkably, all pancreatic carcinoma cell lines (7/7) showed distinct levels of DKK-1 mRNA, suggesting that DKK-1 may be consistently upregulated in pancreatic cancer (Fig. 1). Pulmonary nonsmall cell lung carcinoma cell lines also showed relatively consistent DKK-1 expression. In contrast, none of the small cell lung carcinoma or gastric carcinoma cell lines expressed DKK-1 mRNA (0/4 and 0/3, respectively). Detection was also low in colorectal carcinoma cell lines (5/13).

The expression of the other *DKK* family genes such as *DKK-2*, *DKK-3* and *DKK-4* was tested in a panel of human pancreatic carcinoma cell lines (Fig. 2). In this panel, in addition to 7 pancreatic cell lines, 6 subclones of SUIT-2 with different histopathological differentiation and metastatic capabilities were also included.[19,22] Notably, all pancreatic carcinoma cell lines examined abundantly expressed DKK-1 mRNA. In contrast, DKK-2 and DKK-4 expression was hardly detectable in most cell lines. A low level of DKK-3 mRNA was observed in 68% (8/13) of pancreatic cancer cell lines. These data suggested that DKK-1 may have an important role in the biology of pancreatic cancer and may also serve as a novel biomarker of this disease.

Upregulation of DKK-1 in pancreatic cancer tissue *in vivo*

Next, we asked whether DKK-1 is also upregulated in pancreatic cancer tissue *in vivo* by examining DKK-1 mRNA levels in surgically resected pancreatic cancer tissues and corresponding normal tissues. Among 23 cases of invasive DAC (6 cases of well-differentiated tubular adenocarcinoma, 14 cases of moderately differentiated tubular adenocarcinoma, 2 cases of poorly differentiated tubular adenocarcinoma and 1 case of metastatic liver tumor from pancreatic moderately differentiated tubular adenocarcinoma), 17 showed significantly upregulated expression of DKK-1 in cancer tissue compared with corresponding normal tissue (Fig. 3*a*). Quantitative real-time RT-PCR analyses also confirmed this trend, and the difference was statistically significant (Fig. 3*c*). Intraductal papillary mucinous carcinoma (IPMC) (4 cases) also showed increased DKK-1 expression (Figs. 3*b* and 3*c*). We also examined DKK-1 mRNA levels in 2 cases of mucinous

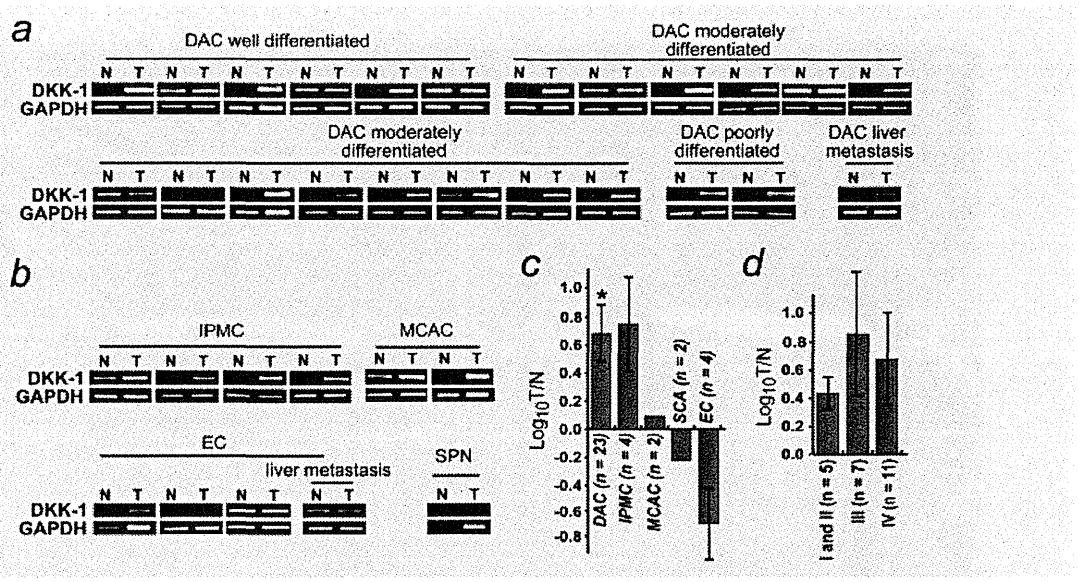

Figure 3. DKK-1 expression in pancreatic tumor tissues and corresponding normal pancreatic tissues. (*a*) RT-PCR (30 cycles) of pancreatic ductal adenocarcinoma (DAC, 23 cases). N, normal tissue; T, tumor tissue. (*b*) RT-PCR (30 cycles) of intraductal papillary mucinous carcinoma (IPMC, 4 cases), mucinous cystadenocarcinoma (MCAC, 2 cases), endocrine carcinomas (EC, 4 cases) and solid and pseudopapillary neoplasm (SPN, 1 case). (*c*) Quantitative real-time RT-PCR analysis of the tumor tissues. The data were normalized by β-actin mRNA level and the ratio of DKK-1 in tumor tissue to that in corresponding normal tissue is indicated as a logarithmic scale. SCA, serous cystadenoma. Values are mean of $\log_{10}$T/N ± standard error. *, DKK-1 level in the tumor tissue is significantly higher than that in the corresponding normal tissue, $p < 0.01$ (Wilcoxon signed rank test). (*d*) Relationship between DKK-1 expression ($\log_{10}$T/N) and disease stage.

cystadenocarcinoma (MCAC) and 1 case showed increased DKK-1 expression (Fig. 3*b*). In contrast, solid pseudopapillary neoplasm (SPN) (1 case), serous cystadenoma (SCA) (2 cases) and endocrine carcinoma (EC) (4 cases) did not show enhanced expression of DKK-1 (Figs. 3*b* and 3*c*). Although there may be a trend that advanced DAC (Stage III/IV) showed higher DKK-1 mRNA levels than Stage I/II, the difference was not statistically significant (Fig. 3*d*).

To verify the overexpression of DKK-1 in pancreatic DAC cells *in vivo*, we performed an immunohistochemical analysis using surgically resected DAC tissues. Immunoreactive DKK-1 proteins were, in fact, overexpressed in DAC cells compared with non-neoplastic ductal epithelium (Figs. 4*a* and 4*b*). This finding was further confirmed by ISH for DKK-1 mRNA in the serial sections (Figs. 4*c* and 4*d*). The specific mRNA signal was observed in DAC cells but not in normal ductal epithelial cells, showing similar staining pattern to that in the immunohistochemistry. The DAC cells at the invasion front tended to show increased levels of DKK-1 mRNA, and no signal could be identified in the endocrine cells of Langerhans islands (Figs. 4*e* and 4*f*).

Reduced cellular invasiveness by knockdown of DKK-1 in human pancreatic carcinoma cell lines

To test the biological role of DKK-1 in pancreatic carcinoma cells, we attempted to knockdown of DKK-1 in the human pancreas carcinoma cell line SUIT-2 using a retroviral vector expressing DKK-1 shRNA (Fig. 5*a*). A significant reduction in the levels of both DKK-1 protein and mRNA was observed in DKK-1 knockdown cell pools (SUIT2-KD) compared with controls treated with scrambled shRNA (SUIT2-scr). Subsequent real-time RT-PCR revealed that the mRNA level of DKK-1 in SUIT2-KD was suppressed by 17% compared with levels in SUIT2-scr (not shown). The knockdown of DKK-1 did not alter cellular proliferation (Fig. 5*b*). However, migratory activity was suppressed, as judged by a monolayer wounding assay (Fig. 5*c*). Matrigel invasion assay was also performed using SUIT-scr and SUIT2-KD, but invaded cells through Matrigel were hardly visible in both cases (data not shown).

To test the effect on cellular invasiveness, *DKK-1* gene silencing was also performed using S2-CP8, a metastatic subline of SUIT-2 established by Cutis-Pulmonary metastasis-culture (8-cycle selection).[19] S2-CP8 cells show loss of E-cadherin expression and are more invasive than SUIT-2.[23] DKK-1 was more highly expressed in S2-CP8 than in SUIT-2 cells (Fig. 2). Both mRNA and protein levels of DKK-1 were significantly downregulated by shRNA (Fig. 6*a*), and real time RT-PCR revealed a 72.3% reduction in mRNA levels (not shown). Interestingly, *in vitro* growth rate in serum (10% FBS)-containing medium was significantly suppressed by *DKK-1* silencing in S2-CP8 (Fig. 6*b*). As S2-CP8 cells easily lost their cohesiveness and were not suitable for the monolayer wounding assay, we used the Matrigel invasion assay to

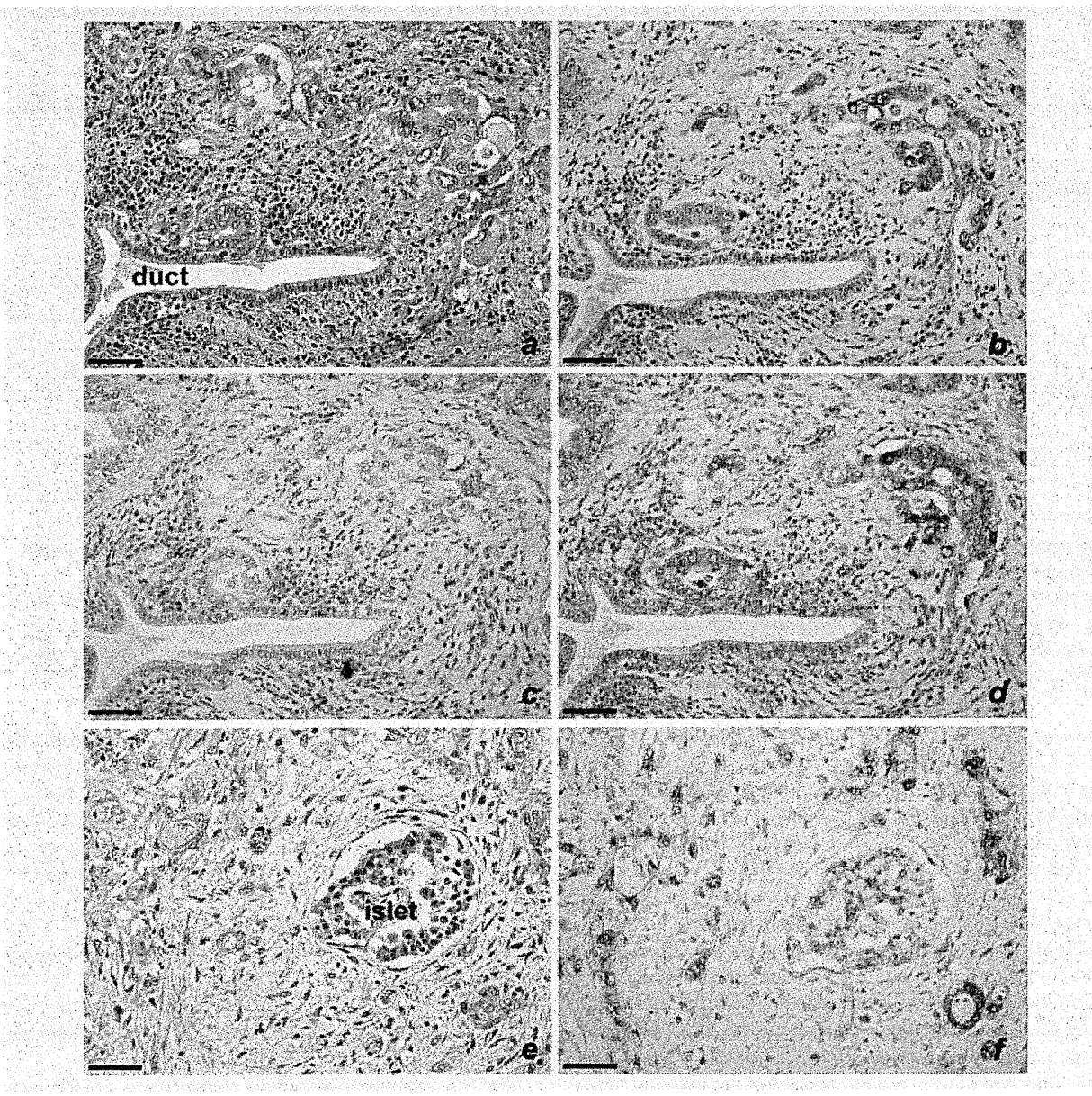

Figure 4. Expression of DKK-1 in pancreatic DAC cells *in vivo*. Serial tissue sections of 2 pancreatic DAC cases (*a–d* and *e–f*) were stained with hematoxylin and eosin (*a*, *e*), immunostained for DKK-1 (*b*), and used for *in situ* hybridization with DKK-1 sense probe (negative control) (*c*) and antisense probe (*d*, *f*). Significantly increased DKK-1 protein and mRNA levels were observed in carcinoma cells compared with non-neoplastic epithelium of the pancreatic duct (duct) (*a–d*). DAC cells at the invasion front also showed enhanced DKK-1 mRNA levels (*e*, *f*). islet, Langerhans islet. Bar, 50 μm.

test the effect of DKK-1 knockdown on cellular invasion. In this assay, the cells were cultured in serum-free medium containing 0.1% BSA in order to minimize the growth advantage of the control (S2CP8-scr) cells and 1% FBS was used as a chemoattractant in the lower well. As shown in Figure 6*c*, knockdown of DKK-1 expression resulted in significantly reduced cellular invasiveness. In an attempt to analyze a possible molecular mechanism underlying DKK-1 konckdown-induced phenotype, we examined the phosphorylation of β-catenin and GSK-3β, both of which are involved in canonical Wnt signaling, and JNK. However, the phosphorylation status of these proteins was not altered by the *DKK-1* silencing (Fig. 6*d*). Moreover, nuclear β-catenin level and mRNA levels of possible transcriptional targets of canonical Wnt/β-catenin

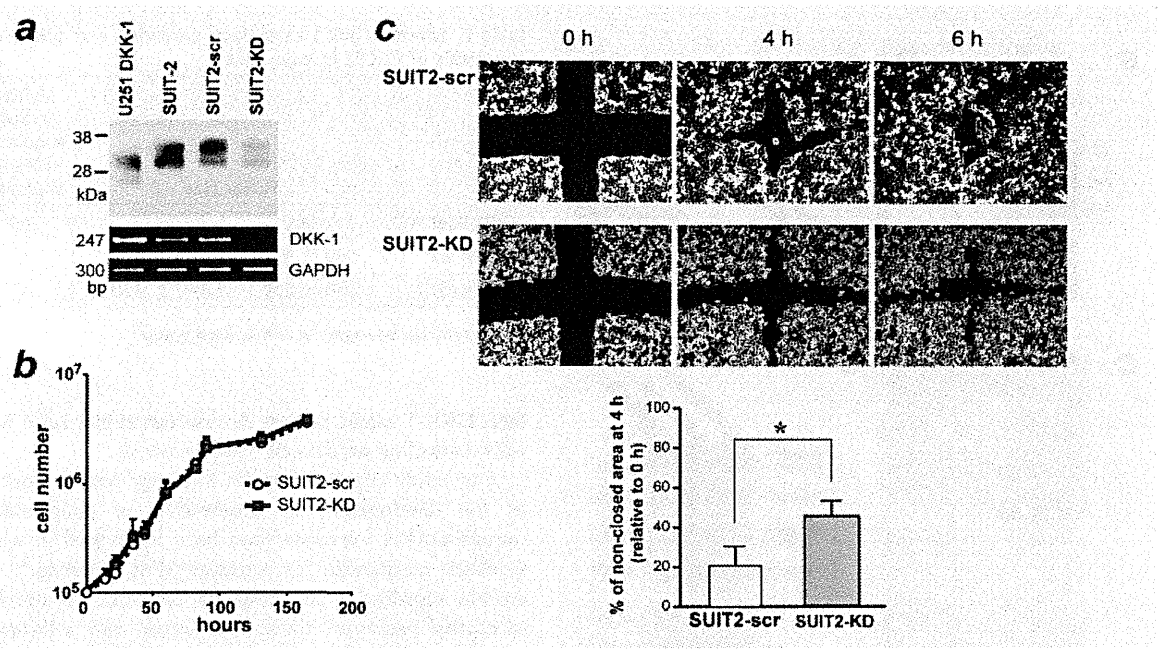

Figure 5. Effect of DKK-1 knockdown on SUIT-2 cells. (*a*) Immunoblot (upper panel) and RT-PCR (lower panel) analyses. Both DKK-1 protein and mRNA levels were significantly decreased in a SUIT-2 cell pool infected with a DKK-1 shRNA retroviral vector (SUIT2-KD) compared with parental (SUIT-2) and scrambled control (SUIT2-scr) cells. As a control, U251 human glioblastoma cells with engineered overexpression of DKK-1 in a plasmid vector (U251 DKK-1) were also used in the analyses. (*b*) Knockdown of DKK-1 did not alter the *in vitro* cellular growth of SUIT-2 cells. (*c*) Cell scratch assay. The confluent monolayer was wounded by scratching with a pipette tip. Photographs were taken of the wound 0, 4 and 6 hr after scratching. Knockdown of DKK-1 suppressed cellular restitution of the wounded area. The ratio (%) of nonclosed area at 4 hr to that at 0 hr was calculated and shown as a bar graph ($n = 4$). Values are mean ± standard deviation. $^*p < 0.05$ (Mann–Whitney *U*-test).

signaling, such as c-Myc and cyclin D1, were not altered either.

Finally, we tested the effect of DKK-1 knockdown on the *in vivo* metastatic capability of S2-CP8 in a nude mouse model. After subcutaneous implantation of S2CP8-KD or S2CP8-scr cells, the cells were examined for pulmonary metastasis. There was no significant difference in either tumor growth rate or incidence of pulmonary metastasis between S2CP8-KD and S2CP8-scr cells, though there may be a tendency toward decreases in tumor size and number of metastases per each lung following DKK-1 knockdown (Table 1).

Discussion

The family of human DKK proteins is composed of DKK-1, DKK-2, DKK-3, DKK-4 and soggy.[2] DKK-1 and DKK-4 suppress the Wnt-induced signaling that is frequently involved in tumor progression.[24] Consequently, reduced expression in tumor cells and tumor-suppressive activity have been reported for DKK-1. For example, DKK-1 expression is significantly reduced in gastrointestinal cancers and malignant melanoma,[5–7,25] and mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1.[26,27] However, DKK-1 is upregulated in some tumor types including esophageal and nonsmall cell lung carcinomas, despite its Wnt inhibitory activity.[10–14] In this study, we show that DKK-1 is also consistently overexpressed in pancreatic carcinomas, particularly DAC, and may promote invasive growth of the cancerous cells.

To date, little is known regarding the expression and function of DKK-1 in the pancreas. Many attempts of expression profiling have been made in order to identify molecules involved in the progression of pancreatic carcinomas.[28–34] Enhanced expression of DKK-1 has not been found in most studies. However, in one report of global gene expression, the *DKK-1* gene is included on a list of 217 known genes that were highly expressed in pancreatic DAC, without further validation of the expression.[30] We confirmed in this study that DKK-1 mRNA is in fact upregulated in cultured human pancreatic carcinoma cell lines *in vitro* and also in pancreatic DAC cells *in vivo*. Furthermore, using retroviral expression of DKK-1 shRNA, we showed that knockdown of DKK-1 suppresses the migration and invasion of human pancreatic DAC cell line SUIT-2 and its metastatic subline S2-CP8 *in vitro*. Notably, *DKK-1* silencing also suppressed the cell growth in S2-CP8. In accordance with our finding, Yamabuki *et al.* showed that overexpression of DKK-1 in NIH3T3 and

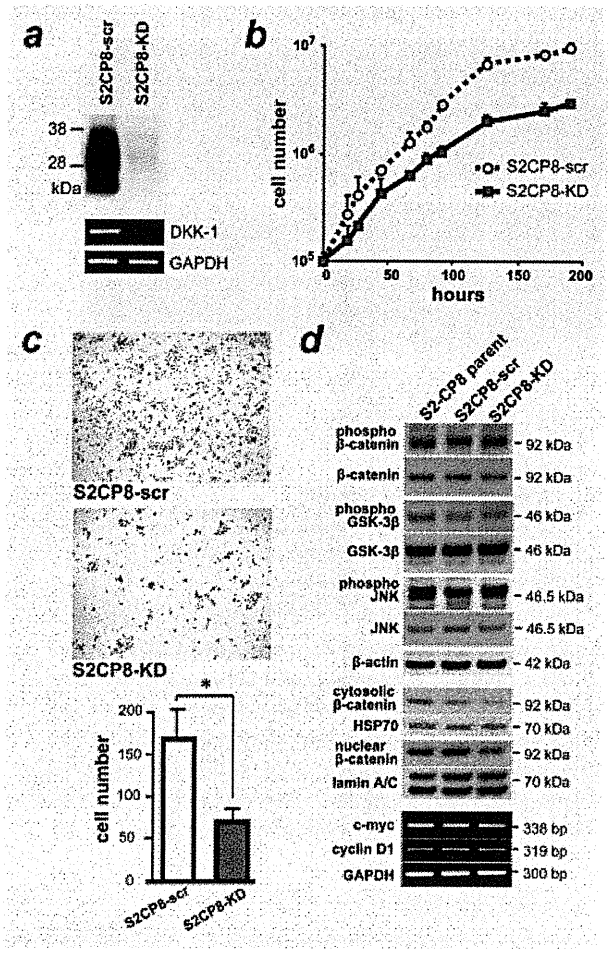

Figure 6. Effect of DKK-1 knockdown on S2-CP8 cells. (*a*) Immunoblot (upper panel) and RT-PCR (lower panel) analyses indicated that both DKK-1 protein and mRNA levels were significantly decreased in S2-CP8 cell pools infected with a DKK-1 shRNA retroviral vector (S2CP8-KD) compared with scrambled control (S2CP8-scr) cells. (*b*) Knockdown of DKK-1 in S2-CP8 cells resulted in reduced growth rate and saturation density. (*c*) Effects of DKK-1 knockdown on invasiveness of S2-CP8 cells through Matrigel. Values are means ± standard deviation of triplicate experiments. $*p < 0.001$, Mann–Whitney *U* test. (*d*) Immunoblot analysis for the effects of DKK-1 knockdown on phosphorylation of β-catenin, GSK-3β and JNK. The β-actin signal is also shown as an internal blot control of each sample. The levels of cytosolic and nuclear β-catenin were also analyzed, and HSP70 and lamin A/C were used as internal blot controls. The effects of DKK-1 knockdown on c-Myc and cyclin D1 mRNA levels were also analyzed by RT-PCR (30 cycles).

COS-7 cells results in significantly enhanced Matrigel invasion *in vitro*.[14] However, it has also been reported that overexpression of DKK-1 in human hepatocellular carcinoma cell line M-H7402 suppresses cell growth and migration.[9] Therefore, DKK-1 might possess diverse functional roles in tumor cells depending on the cell types involved.

Table 1. Effects of DKK-1 knockdown on growth and pulmonary metastasis of S2-CP8 in nude mice

	Tumor size		Number of pulmonary metastasis (8 weeks after implantation)		
Cells injected[1]	2 weeks	4 weeks	0	<9	≥10
S2CP8-scr[2]	590 ± 470	1,710 ± 880	1	1	3
S2CP8-KD[2]	300 ± 230	1,540 ± 920	0	4	1

[1]Subcutaneous injection. [2]$n = 5$ in each group.

Our study suggests that DKK-1 may have a positive role in the development or progression of pancreatic DAC, though DKK-1 expression has been implicated in a negative feedback mechanism for activated Wnt signaling.[2,5] Wnt/β-catenin signaling is an important factor in the development of normal pancreatic tissue.[35] However, only a limited number of reports have been published regarding the role of Wnt signaling in pancreatic cancer,[36–38] and the function of DKK-1 in the pancreatic cancer has not been clarified. Interestingly, a recent study of a mutant mouse model indicates that activation of β-catenin, a critical member of canonical Wnt signaling, results in SPN of the pancreas, while it blocks the formation of pancreatic intraepithelial neoplasia (PanIN) in the presence of an activating mutation in K-ras,[38] suggesting that canonical Wnt signaling may suppress the development of PanIN. As PanIN is a precursor lesion of DAC, the upregulation of DKK-1 may have a role in the development of PanIN and, thereby, DAC of the pancreas *via* its inhibitory effect on the canonical pathway. Alternatively, DKK-1 may have Wnt/β-catenin-independent functions. For example, ectopic expression of DKK-1 in HeLa cells did not alter cellular β-catenin localization or expression of Wnt target genes.[39] In lung and esophageal cancer cells that overexpress the *DKK-1* gene, there was no relationship between the mRNA expression patterns of DKK-1 and LRP5/6, the binding target of DKK-1 in Wnt signaling, suggesting that there may be unknown binding partners and/or receptors of DKK-1.[14] In accordance with this hypothesis, phosphorylation of β-catenin or GSK-3β and expression of Wnt target genes were not altered by DKK-1 knockdown in S2-CP8 in our study. JNK may also be a candidate for the target of DKK-1 activity.[40] However, JNK phosphorylation level was not altered by DKK-1 knockdown in S2-CP8 cells. Another possibility regarding the function of cancer cell-derived DKK-1 is its effect on the stromal cells, as Wnt signaling may also be activated in the stroma of pancreatic cancer.[37] Whatever the underlying biological function may be, further clinicopathological study will be required to test the prognostic impact of DKK-1 expression using a large number of pancreatic DAC cases.

In summary, although the detailed function of DKK-1 in pancreatic carcinogenesis and progression of DAC is unknown, our results suggest a role for DKK-1 in the promotion of invasive growth of pancreatic cancer cells, and that it could serve as a novel tumor marker for pancreatic carcinoma.

References

1. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 1998;391:357–62.
2. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 2006;25:7469–81.
3. Monaghan AP, Kioschis P, Wu W, Zuniga A, Bock D, Poustka A, Delius H, Niehrs C. Dickkopf genes are co-ordinately expressed in mesodermal lineages. *Mech Dev* 1999;87: 45–56.
4. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* 2007;98: 1512–20.
5. Gonzalez-Sancho JM, Aguilera O, Garcia JM, Pendas-Franco N, Pena C, Cal S, Garcia de Herreros A, Bonilla F, Munoz A. The Wnt antagonist DICKKOPF-1 gene is a downstream target of β-catenin/TCF and is downregulated in human colon cancer. *Oncogene* 2005;24:1098–103.
6. Aguilera O, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM. Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene* 2006; 25:4116–21.
7. Kuphal S, Lodermeyer S, Bataille F, Schuierer M, Hoang BH, Bosserhoff AK. Expression of Dickkopf genes is strongly reduced in malignant melanoma. *Oncogene* 2006;25:5027–36.
8. Osada H, Tomida S, Yatabe Y, Tatematsu Y, Takeuchi T, Murakami H, Kondo Y, Sekido Y, Takahashi T. Roles of achaete-scute homologue 1 in DKK1 and E-cadherin repression and neuroendocrine differentiation in lung cancer. *Cancer Res* 2008;68:1647–55.
9. Qin X, Zhang H, Zhou X, Wang C, Zhang H, Zhang X, Ye L. Proliferation and migration mediated by Dkk-1/Wnt/β-catenin cascade in a model of hepatocellular carcinoma cells. *Transl Res* 2007;150:281–94.
10. Wirths O, Waha A, Weggen S, Schirmacher P, Kuhne T, Goodyer CG, Albrecht S, Von Schweinitz D, Pietsch T. Overexpression of human Dickkopf-1, an antagonist of wingless/WNT signaling, in human hepatoblastomas and Wilms' tumors. *Lab Invest* 2003;83:429–34.
11. Koch A, Waha A, Hartmann W, Hrychyk A, Schuller U, Waha A, Wharton KA, Jr, Fuchs SY, von Schweinitz D, Pietsch T. Elevated expression of Wnt antagonists is a common event in hepatoblastomas. *Clin Cancer Res* 2005;11:4295–304.
12. Patil MA, Chua MS, Pan KH, Lin R, Lih CJ, Cheung ST, Ho C, Li R, Fan ST, Cohen SN, Chen X, So S. An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. *Oncogene* 2005;24:3737–47.
13. Chamorro MN, Schwartz DR, Vonica A, Brivanlou AH, Cho KR, Varmus HE. FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development. *EMBO J* 2005;24: 73–84.
14. Yamabuki T, Takano A, Hayama S, Ishikawa N, Kato T, Miyamoto M, Ito T, Ito H, Miyagi Y, Nakayama H, Fujita M, Hosokawa M, et al. Dikkopf-1 as a novel serologic and prognostic biomarker for lung and esophageal carcinomas. *Cancer Res* 2007;67:2517–25.
15. Voorzanger-Rousselot N, Goehrig D, Journe F, Doriath V, Body JJ, Clezardin P, Garnero P. Increased Dickkopf-1 expression in breast cancer bone metastases. *Br J Cancer* 2007;97:964–70.
16. Hall CL, Kang S, MacDougald OA, Keller ET. Role of Wnts in prostate cancer bone metastases. *J Cell Biochem* 2006;97:661–72.
17. Eloubeidi MA, Desmond RA, Wilcox CM, Wilson RJ, Manchikalapati P, Fouad MM, Eltoum I, Vickers SM. Prognostic factors for survival in pancreatic cancer: a population-based study. *Am J Surg* 2006; 192:322–29.
18. Iwamura T, Katsuki T, Ide K. Establishment and characterization of a human pancreatic cancer cell line (SUIT-2) producing carcinoembryonic antigen and carbohydrate antigen 19-9. *Jpn J Cancer Res* 1987; 78:54–62.
19. Kitamura N, Iwamura T, Taniguchi S, Yamanari H, Kawano MA, Hollingsworth K, Setoguchi T. High collagenolytic activity in spontaneously highly metastatic variants derived from a human pancreatic cancer cell line (SUIT-2) in nude mice. *Clin Exp Metastasis* 2000; 18:561–71.
20. Fedi P, Bafico A, Nieto Soria A, Burgess WH, Miki T, Bottaro DP, Kraus MH, Aaronson SA. Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling. *J Biol Chem* 1999;274:19465–72.
21. Fukushima T, Tezuka T, Shimomura T, Nakano S, Kataoka H. Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24. *J Biol Chem* 2007; 282:18634–44.
22. Iwamura T, Katsuki T. Kinetics of carcinoembryonic antigen and carbohydrate antigen 19-9 production in a human pancreatic cancer cell line (SUIT-2). *Gastroenterol Jpn* 1987;22:640–6.
23. Cheng H, Fukushima T, Takahashi N, Tanaka H, Kataoka H. Hepatocyte growth factor activator inhibitor type 1 regulates epithelial to mesenchymal transition through membrane-bound serine proteinases. *Cancer Res* 2009;69:1828–35.
24. Klaus A, Birchmeier W. Wnt signaling and its impact on development and cancer. *Nat Rev Cancer* 2008;7:387–98.
25. Sato H, Suzuki H, Toyota M, Nojima M, Maruyama R, Sasaki S, Takagi H, Sogabe Y, Sasaki Y, Idogawa M, Sonoda T, Mori M, et al. Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. *Carcinogenesis* 2007;28:2459–66.
26. Qiao L, Xu ZL, Zhao TJ, Ye LH, Zhang XD. Dkk-1 secreted by mesenchymal stem cells inhibits growth of breast cancer cells via depression of Wnt signalling. *Cancer Lett* 2008;269:67–77.
27. Zhu Y, Sun Z, Han Q, Liao L, Wang J, Bian C, Li J, Yan X, Liu Y, Shao C, Zhao RC. Human mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1. *Leukemia* 2009;23:925–33.
28. Crnogorac-Jurcevic T, Efthimiou E, Nielsen T, Loader J, Terris B, Stamp G, Baron A, Scarpa A, Lemoine NR. Expression profiling of microdissected pancreatic adenocarcinomas. *Oncogene* 2002;21: 4587–94.
29. Terris B, Blaveri E, Crnogorac-Jurcevic T, Jones M, Missiaglia E, Ruszniewski P, Sauvanet A, Lemoine NR. Characterization of gene expression profiles in intraductal papillary-mucinous tumors of the pancreas. *Am J Pathol* 2002;160:1745–54.
30. Logsdon CD, Simeone DM, Binkley C, Arumugam T, Greenson JK, Giordano TJ, Misek DE, Kuick R, Hanash S. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Res* 2003; 63:2649–57.
31. Grutzmann R, Foerder M, Alldinger I, Staub E, Brummendorf T, Ropcke S, Li X, Kristiansen G, Jesnowski R, Sipos B, Lohr M, Luttges J, et al. Gene expression profiles

of microdissected pancreatic ductal adenocarcinoma. *Virchows Arch* 2003;443: 508–17.
32. Missiaglia E, Blaveri E, Terris B, Wang YH, Costello E, Neoptolemos JP, Crnogorac-Jurcevic T, Lemoine NR. Analysis of gene expression in cancer cell lines identifies candidate markers for pancreatic tumorigenesis and metastasis. *Int J Cancer* 2004;112:100–12.
33. Grutzmann R, Boriss H, Ammerpohl O, Luttges J, Kalthoff H, Schackert HK, Kloppel G, Saeger HD, Pilarsky C. Meta-analysis of microarray data on pancreatic cancer defines a set of commonly dysregulated genes. *Oncogene* 2005;24:5079–88.
34. Harada T, Chelala C, Crnogorac-Jurcevic T, Lemoine NR. Genome-wide analysis of pancreatic cancer using microarray-based techniques. *Pancreatology* 2008;9:13–24.
35. Murtaugh LC. The what, where, when and how of Wnt/β-catenin signaling in pancreas development. *Organogenesis* 2008; 4:81–6.
36. Pasca di Magliano M, Biankin AV, Heiser PW, Cano DA, Gutierrez PJ, Deramaudt T, Segara D, Dawson AC, Kench JG, Henshall SM, Sutherland RL, Dlugosz A, et al. Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. *PLoS One* 2007;2: e1155.
37. Pilarsky C, Ammerpohl O, Sipos B, Dahl E, Hartmann A, Wellmann A, Braunschweig T, Lohr M, Jesnowski R, Friess H, Wente MN, Kristiansen G, et al. Activation of Wnt signalling in stroma from pancreatic cancer identified by gene expression profiling. *J Cell Mol Med* 2008; 12:2823–35.
38. Heiser PW, Cano DA, Landsman L, Kim GE, Kench JG, Klimstra DS, Taketo MM, Biankin AV, Hebrok M. Stabilization of β-catenin induces pancreas tumor formation. *Gastroenterology* 2008;135:1288–300.
39. Mikheev AM, Mikheeva SA, Liu B, Cohen P, Zarbl H. A functional genomics approach for the identification of putative tumor suppressor genes: Dickkopf-1 as suppressor of HeLa cells transformation. *Carcinogenesis* 2004;25:47–59.
40. Endo Y, Beauchamp E, Woods D, Taylor WG, Toretsky JA, Uren A, Rubin JS. Wnt-3a and Dickkopf-1 stimulate neurite outgrowth in Ewing tumor cells via a Frizzled3- and c-Jun N-terminal kinase-dependent mechanism. *Mol Cell Biol* 2008;28:2368–79.

Hepatocellular carcinomas can develop in simple fatty livers in the setting of oxidative stress

Sir,

It is doubtless that non-alcoholic fatty liver disease (NAFLD) has become a critical public health issue in most developed countries.[1] In addition to its close association with metabolic disorders and cardiovascular events, NAFLD itself can progress to life-threatening liver diseases, cirrhosis and hepatocellular carcinoma (HCC).[1,2] Surprisingly, recent clinical observations have revealed that HCC can develop in non-cirrhotic, but steatotic livers without significant fibrosis.[3,4] In the reports, authors emphasised the importance of underlying metabolic disorders and oxidative stress in hepatocarcinogenesis in such NAFLD cases.

We herein report a case of NAFLD complicated by HCC, in which the potential contribution of oxidative stress to hepatocarcinogenesis in NAFLD has been further strongly suggested.

In May 2006, a 72-year-old obese Japanese man was admitted to the National Hospital Organization Osaka National Hospital, Japan, because of a hepatic nodule. The patient had a 5 year medical history of hypertension and fatty liver. He underwent an operation for abdominal aortic aneurysm in April 2004, and had since been an outpatient. Follow-up abdominal ultrasonography showed, in addition to hepatic steatosis, a nodular lesion approximately 2 cm in diameter in the left lobe of the liver. The hepatic nodule had been growing larger, and findings of computed tomography (CT) strongly suggested that it was HCC (Fig. 1).

On admission, he appeared to be healthy except for obesity, and the laboratory test results showed normal serum levels of transaminases and negativity for hepatitis B and C viruses. After the examinations, under the tentative diagnosis of HCC, a surgical operation was performed to remove the left lobe of the liver. The cut surface of the liver sample was smooth and yellowish-brown, and demonstrated the clearly circumscribed nodule 5 cm in diameter (Fig. 2). Histological examination revealed that the nodular lesion was well-differentiated HCC (Fig. 3A), and the background hepatic disorder was simple steatosis without inflammation and fibrosis (Fig. 3B). Routine pathological examination couldn't detect any causative factors in hepatocarcinogenesis, such as iron overload. However, an immunohistochemical analysis revealed that as well as tumour cells, a few non-tumourous hepatocytes showed immunoreactivity for anti-oxidised phosphatidylcholine (oxPC; Fig. 3A,B insets), a marker of

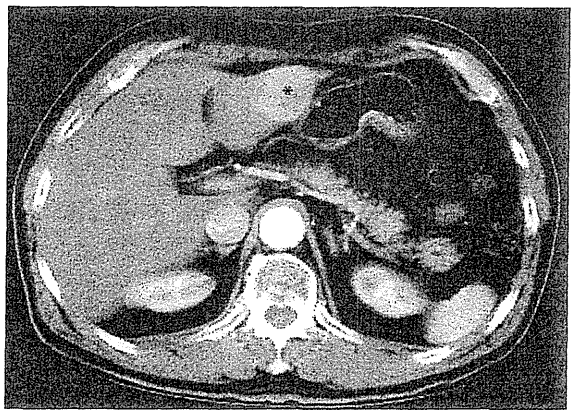

Fig. 1 Findings of computed tomography. A hypervascular nodule is seen in the left lobe of the liver (asterisk).

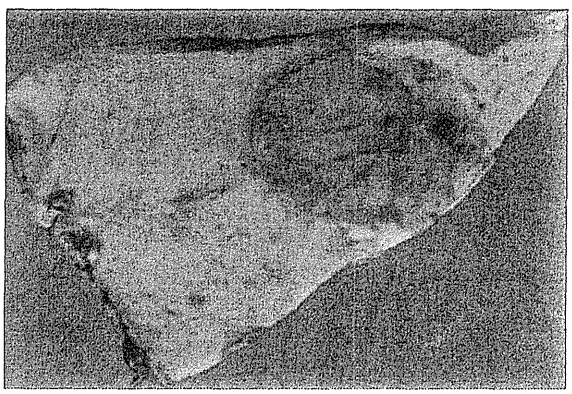

Fig. 2 The cut-surface of the liver sample. A well-circumscribed nodular lesion 5 cm in diameter is present.

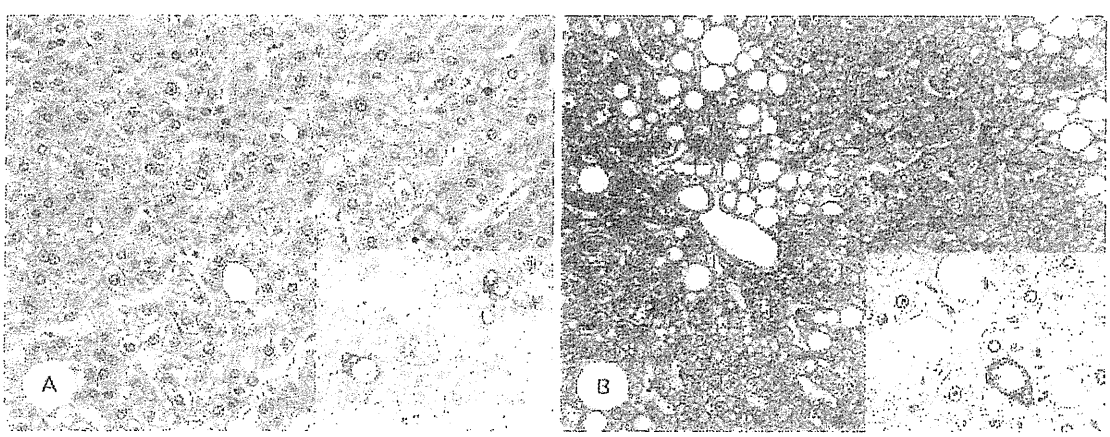

Fig. 3 Microscopic findings of the liver sample. (A) The hepatic nodule consists of well-differentiated HCC (H&E). (B) A background liver condition is simple steatosis without fibrosis (Azan-Mallory stain for collagen). Insets: steatotic liver cells of both tumourous and non-tumourous portions are positive for oxPC (immunoperoxidase with anti-oxPC).

oxidative cellular injury.[5] The immunoreactivity was found restrictively in steatotic cells in both tumourous and non-tumourous portions. It suggested that the steatosis-related oxidative hepatocellular injury had persisted and had played a certain role in the development of HCC. His postoperative course was uneventful and he was discharged. Follow-up examinations have been done every 3 months, resulting in no evidence of tumour recurrence. When last seen, in December 2009, he was well.

Like the present case, HCC can develop in non-inflammatory, non-fibrotic, but steatotic livers. A previous case report of HCC arising in the absence of advanced NAFLD emphasised the potential roles for metabolic factors (diabetes, hypertension, obesity and dyslipidaemia) and oxidative stress in hepatocarcinogenesis.[4] Oxidative stress is generally recognised as an important factor in hepatocarcinogenesis.[2,6] Our immunohistochemical results showed oxPC-positive steatotic hepatocytes in the background liver tissue of HCC. OxPC, one of the lipid peroxides, is a highly specific marker of oxidative damage. In our previous observation, its immunoreactivity was seen mainly in steatotic or degenerated hepatocytes in NAFLD, and only in some Kupffer cells in normal liver tissues.[5] Hence, the present result suggested that the fatty liver had chronically been exposed to oxidative stress, which was probably initiated prior to hepatocarcinogenesis. To our knowledge, this is the first direct evidence of oxidative hepatocellular injury occurring in simple fatty livers complicated by HCC. Excess fat accumulation itself, even in the absence of inflammation, can be a source of oxidative stress that induces hepatocarcinogenesis. In conclusion, simple steatosis-related oxidative stress should be considered as one of the risk factors of HCC.

Yoshihiro Ikura*
Eiji Mita†
Shoji Nakamori‡

**Department of Pathology, Osaka City University Graduate School of Medicine, Osaka, †Departments of Gastroenterology, and ‡Surgery, National Hospital Organization, Osaka National Hospital, Osaka, Japan*

Contact Dr Y. Ikura.
E-mail: ikura@med.osaka-cu.ac.jp

1. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; 37: 1202–19.
2. Caldwell SH, Crespo DM, Kang HS, Al-Osaimi AM. Obesity and hepatocellular carcinoma. *Gastroenterology* 2004; 127(Suppl 1): S97–103.
3. Paradis V, Zalinski S, Chelbi E, Guedj N, Degos F, Vilgrain V, *et al.* Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. *Hepatology* 2009; 49: 851–9.
4. Guzman G, Brunt EM, Petrovic LM, Chejfec G, Layden TJ, Cotler SJ. Does nonalcoholic fatty liver disease predispose patients to hepatocellular carcinoma in the absence of cirrhosis? *Arch Pathol Lab Med* 2008; 132: 1761–6.
5. Ikura Y, Ohsawa M, Suekane T, *et al.* Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. *Hepatology* 2006; 43: 506–14.
6. Sasaki Y. Does oxidative stress participate in the development of hepatocellular carcinoma? *J Gastroenterol* 2006; 41: 1135–48.

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Benign prostatic glands as a tissue component of testicular teratoma: an uncommon histological finding

Sir,

The occurrence of benign prostatic tissue as a component of testicular teratoma has only been described as a single case report.[1] This may be due to its rare occurrence, as well as the failure of the diagnostic pathologist to recognise glands as prostatic on routine sections and to subsequently confirm this by immunohistochemistry. In our laboratory we utilise Solufix (Tissugen, Australia), a glutaraldehyde-based tissue fixative for routine histology. This agent preserves the spermine content of prostatic secretory granules (PSG) which stain intensely with eosin on routine stains allowing easy recognition of prostatic epithelium.[2]

We present two cases of benign prostatic glandular tissue occurring in two patients with primary testicular tumours; a mature teratoma in a 35-year-old male and a mixed germ cell tumour (30% mature teratoma) in a 39-year-old male. Each patient underwent routine inguinal orchidectomy for a clinically detected testicular mass.

On microscopy, benign gland structures were recognised which were lined by two cell types and bore some resemblance to prostatic acini. These glands were highlighted by their intense eosinophilic PSG (Fig. 1 and 2), and were distributed either singularly or as clusters separated by bland stroma. Lining epithelium showed variable morphology ranging from bland epithelial cells consistent with benign prostatic glands (Fig. 1) to epithelial cells with larger nuclei and prominent nucleoli (Fig. 2), consistent with prostatic intraepithelial neoplasia (PIN). The prostatic nature of the glands was subsequently confirmed by strong diffuse cytoplasmic reactivity with immunohistochemistry for PSA (Dako, USA; Fig. 1 inset) and prostatic acid phosphatase (Dako; not shown). Basal cells were confirmed with immunostaining for high molecular weight cytokeratin 34βE12 (Dako; Fig. 2 inset).

Recognition of the first case prompted a histological review of all testicular teratomas over a 10 year period which included 20 testicular tumours comprising pure teratomas ($n = 10$) and mixed germ cell tumours with a mature teratoma component of $>10\%$ ($n = 10$). This review identified the second case,

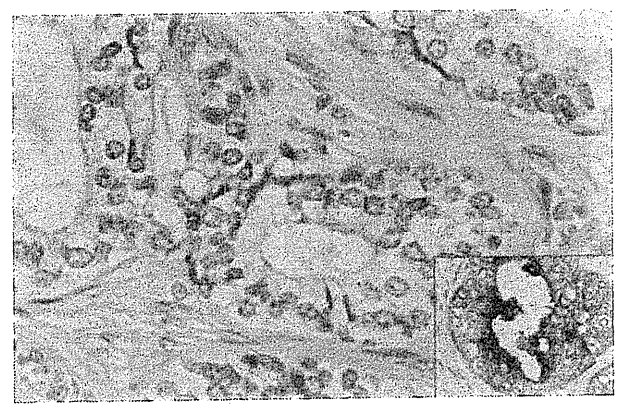

Fig. 1 Benign prostatic glands within a testicular germ cell tumour surrounded by bland stroma. Small eosinophilic granules (prostate secretory granules) are seen in the apical cytoplasm confirming prostatic epithelial differentiation. Inset: These cells are strongly labelled with PSA immunostaining.

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Expression and the role of 3′-phosphoadenosine 5′-phosphosulfate transporters in human colorectal carcinoma

Shin Kamiyama[2,3,11*], Tomomi Ichimiya[2,*], Yuzuru Ikehara[4], Tomofumi Takase[2], Izumi Fujimoto[2], Takeshi Suda[2], Shoji Nakamori[5], Mitsuru Nakamura[6], Fumiaki Nakayama[7], Tatsuro Irimura[8], Hayao Nakanishi[9], Masahiko Watanabe[10,12], Hisashi Narimatsu[4], and Shoko Nishihara[1,2]

[2]Laboratory of Cell Biology, Department of Bioinformatics, Soka University, 1–236 Tangi-cho, Hachioji, Tokyo 192-8577; [3]Research Association for Biotechnology, 3-9, Nishi-Shinbashi 2-chome, Minato-ku, Tokyo 105-0003; [4]Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568; [5]Department of Surgery, Osaka National Hospital, National Hospital Organization, 2-1-14 Houenzaka, Chuo-ku, Osaka 540-0006; [6]East Hospital, Kitasato University, 2-1-1 Asamizodai, Sagamihara, Kanagawa 228-8520; [7]Department of Radiation Emergency Medicine, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555; [8]Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033; [9]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681; and [10]Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, 160-8582 Tokyo, Japan

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Sulfation represents an essential modification for various molecules and regulates many biological processes. The sulfation of glycans requires a specific transporter for 3′-phosphoadenosine 5′-phosphosulfate (PAPS) on the Golgi apparatus. This study investigated the expression of PAPS transporter genes in colorectal carcinomas and the significance of Golgi-specific sulfation in the proliferation of colorectal carcinoma cells. The relative amount of *PAPST1* transcripts was found to be higher than those of *PAPST2* in colorectal cancerous tissues. Immunohistochemically, the enhanced expression of PAPST1 was observed in fibroblasts in the vicinity of invasive cancer cells, whereas the expression of PAPST2 was decreased in the epithelial cells. RNA interference of either of the two PAPS transporter genes reduced the extent of sulfation of cellular proteins and cellular proliferation of DLD-1 human colorectal carcinoma cells. Silencing the PAPS transporter genes reduced fibroblast growth factor signaling in DLD-1 cells. These findings indicate that PAPS transporters play a role in the proliferation of colorectal carcinoma cells themselves and take part in a desmoplastic reaction to support cancer growth by controlling their sulfation status.

Keywords: colorectal carcinoma/heparan sulfate/PAPST1/PAPS transporter/sulfation

Introduction

In malignant transformation, specific carbohydrate antigens are expressed on glycoproteins or glycolipids on the surface of cancer cells. Appearance of these carbohydrate epitopes is associated with alterations in the expression of several glycosyltransferases. These carbohydrate epitopes play important roles in the progression and metastasis of cancer cells and are used as typical tumor markers for the diagnosis of various human cancers. In addition, it has been reported that certain nucleotide sugar transporters are involved in the expression of carbohydrate epitopes in cancer. Nucleotide sugar transporters are proteins that are localized on membranes of the endoplasmic reticulum or the Golgi apparatus. These proteins provide substrates for glycosyltransferases in the lumen. Kumamoto et al. (2001) reported that the expression of uridine diphosphate (UDP)-galactose transporter (solute carrier family 35, member A2; SCL35A2) is increased in human colon carcinoma and is responsible for the synthesis of the Thomsen–Friedenreich antigen and sialyl Lewis A (Le^a) and X (Le^x) epitopes. Moriwaki et al. (2007) reported that guanidine diphosphate (GDP)-fucose transporter (SLC35C1) expression is upregulated in hepatocellular carcinoma and plays a role in an increased rate of fucosylation. These reports suggest that the expression of nucleotide sugar transporters is a key factor for regulating the synthesis of carbohydrate epitopes in cancer cells.

Another essential modification is sulfation which is frequently found on the glycans of proteoglycans, glycolipids, and glycoproteins, and on tyrosine residues of proteins. Sulfation is catalyzed by various sulfotransferases, and it modifies the properties of molecules by imparting a negative charge. Heparan sulfate (HS) and chondroitin sulfate (CS) proteoglycans are well-characterized macromolecules that have highly sulfated glycosaminoglycan (GAG) chains and play significant roles in many biological processes. Inhibition of

*The first two authors contributed equally to this article.

[11]Present address: Department of Health and Nutrition, Faculty of Human Life Studies, University of Niigata Prefecture, 471 Ebigase, Higashi-ku, Niigata 950-8680, Japan
[12]Present address: Department of Surgery, School of Medicine, Kitasato University, 1-15-1 Kitasato Sagamihara, Kanagawa 228-8555, Japan
[1]To whom correspondence should be addressed: Tel: +81-42-691-8140; Fax: +81-42-691-8140; e-mail: shoko@soka.ac.jp

sulfation using chlorate, an inhibitor of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) sulfurylase, reduces the signaling of various growth factors such as the fibroblast growth factor (FGF) (Rapraeger et al. 1991; Yayon et al. 1991) and Wnt (Reichsman et al. 1996). Analysis of *Drosophila* mutants with defects in sulfotransferases revealed the importance of sulfation of GAGs on growth factor signaling during development (Lin and Perrimon 1999; Lin et al. 1999; Kamimura et al. 2001, 2006).

On the other hand, it has been reported that some sulfated structures alter the expression of molecules associated with the progression of cancer. Sulfated sialyl Le^x epitopes have been identified as ligands for L-selectin (Mitsuoka et al. 1998), which plays a crucial role in the leukocyte homing process in high endothelial venules. It is known that the risk of malignancy in colorectal cancer is associated with an increase in sialylation (Nakamori et al. 1993; Matsushita et al. 1995; Nakayama et al. 1995) and a decrease in sulfation of carbohydrate epitopes (Yamori et al. 1989; Irimura et al. 1991; Matsushita et al. 1995; Izawa et al. 2000). Immunohistochemical studies have revealed that the goblet cells of human colonic epithelia of Lewis-positive individuals show a strong signal for sulfated mucins (Tsuiji et al. 1998b). The expression of sulfomucins is much lower in colon adenocarcinomas than in the normal mucosa due to the decreased expression of specific sulfotransferases (Seko et al. 2002b).

Two PAPS transporter genes have been previously identified in both humans and *Drosophila* (Kamiyama et al. 2003, 2006; Goda et al. 2006). Lüders et al. (2003) independently reported that *slalom* is a PAPS transporter gene in *Drosophila*. These PAPS transporters are required for the sulfation of cellular proteins and normal development in *Drosophila* (Kamiyama et al. 2003; Lüders et al. 2003; Goda et al. 2006). Additionally, both human PAPST1 (SLC35B2) and PAPST2 (SLC35B3) were found to be involved in the sulfation of the 6-sulfolactosamine epitope in a human colorectal carcinoma cell line (Kamiyama et al. 2006). Huopaniemi et al. (2004) reported that the CMP-sialic acid transporter (SLC35A1), the GDP-fucose transporter (SLC35C1) and the PAPS transporter (SLC35B2) are involved in coordinated transcriptional regulation during induction of sialyl sulfo-Le^x glycan biosynthesis during acute inflammation. These studies indicate that PAPS transporters regulate the sulfation process in addition to sulfotransferases; however, studies have not been conducted on the expression status of PAPS transporter genes in cancer. Therefore, in the present study, we investigated the expression of PAPS transporter genes in colorectal carcinomas and their role in the regulation of sulfation in colorectal carcinoma cells. The significance of Golgi-specific sulfation in proliferation of colorectal carcinoma cells is also discussed.

Results

Expression status of PAPS transporter genes in colorectal carcinomas

Previously, we identified two human PAPS transporter genes: *PAPST1* and *PAPST2* (Kamiyama et al. 2003, 2006). In normal colon tissue, *PAPST1* was expressed at substantially lower level than *PAPST2* (Kamiyama et al. 2006). In the present study, the expression of these PAPS transporter genes in human colorectal carcinomas was investigated. Initially, the expression levels of these transcripts in colorectal carcinoma cell lines were quantified using real-time polymerase chain reaction (PCR). The relative expression level of *PAPST1* transcripts was higher than that of *PAPST2* in all of the 22 colorectal carcinoma cell lines tested (Figure 1A).

Subsequently, the expression levels of *PAPST1* and *PAPST2* were determined in human colorectal tissues. Figure 1B indicates the expression levels of *PAPST1* and *PAPST2* transcripts in cancerous and noncancerous colorectal tissues obtained from seven specimens. Consistent with the result from the cell lines, the relative amount of *PAPST1* transcripts was higher than that of *PAPST2* in colorectal cancerous tissues. Meanwhile, a considerable amount of *PAPST1* transcripts was detected in noncancerous colorectal tissues. In the previous study, we used only one sample for the determination of *PAPST1* and *PAPST2* expression in human colon tissue (Kamiyama et al. 2006). Therefore, the difference might be due to the individual and/or position-specific differences. The expression of *PAPST2* was found to be decreased in colorectal cancerous tissues.

The expression of *PAPST1* mRNA in colorectal carcinoma was confirmed through *in situ* hybridization. As shown in Figure 2A, *PAPST1* mRNA was detected in both adenocarcinoma cells and the stromal cells. To further characterize the distribution of PAPST1 in colorectal tissues, we prepared an antibody against a C-terminal peptide of mouse PAPST1. The immunoreactivity of the anti-PAPST1 antibody to human PAPST1 protein was confirmed by western blot analysis against c-myc-tagged human PAPST1 protein, which was expressed in HEK293 cells (Figure 2C). The anti-PAPST1 antibody specifically recognized both endogenous (Figure 2D, left and middle lanes) and c-myc-tagged human PAPST1 protein (Figure 2D, right lane). Immunohistochemical analysis identified that the predominant expression of PAPST1 is on epithelial cells rather than on stromal cells in both noncancerous (Figure 2E and E′) and cancerous (Figure 2F and F′) colorectal tissues. The expression levels of PAPST1 protein in epithelial cells were similar in cancerous and noncancerous tissues, whereas the observed levels of stromal cells were region-specific and dispersed (Figure 2G, G′ and I, arrows). Moreover, enhanced expression of PAPST1 was also observed in fibroblasts in the vicinity of invasive cancer cells (Figure 2I, asterisks). This suggests that the desmoplastic reaction is associated with elevated levels of PAPST1 in cancerous tissue. PAPST1 protein was detected in all cancerous and noncancerous sections tested in the perinuclear region (Golgi apparatus) of the cells (Figure 2G, G′ and H, arrowheads).

On the other hand, PAPST2 protein was predominantly detected in epithelial cells in noncancerous colorectal tissues (Figure 2J). However, the expression of PAPST2 protein in epithelial cells was faintly detectable in cancerous colorectal tissues (Figure 2K). Strong expression of PAPST2 protein was observed in cells of hematopoietic lineage in both noncancerous and cancerous tissues (Figure 2J and K, diamond arrows). These results indicate that colorectal cancerous tissue increases the expression of PAPST1 in fibroblasts in the vicinity of the desmoplastic reaction and decreases the expression of PAPST2 in epithelial carcinoma cells.