# A Highly Sensitive and Quantitative Telomerase Activity Assay with Pancreatic Juice Is Useful for Diagnosis of Pancreatic Carcinoma without Problems due to Polymerase Chain Reaction Inhibitors

## *Analysis of 100 Samples of Pancreatic Juice from Consecutive Patients.*

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**BACKGROUND.** Early detection of pancreatic carcinoma is difficult even with current diagnostic tools. Novel biomarkers and detection techniques are urgently needed. Telomerase activity is a promising diagnostic marker. However, the conventional telomeric repeat amplification protocol (TRAP) assay is not suitable for clinical application because of its complexity, time-consuming nature, and the effects of polymerase chain reaction (PCR) inhibitors in samples leading to difficulties in quantification.

**METHODS.** The authors used a hybridization protection assay in combination with TRAP (TRAP/HPA) to investigate the effects of PCR inhibitors in pancreatic juice on quantification of telomerase activity. They analyzed 117 consecutive samples of pancreatic juice to determine the feasibility of TRAP/HPA for diagnosis of pancreatic carcinoma.

**RESULTS.** The authors found that TRAP/HPA was 1000-fold more sensitive than the conventional TRAP assay, and that the effects of PCR inhibitors could be avoided by diluting samples. In a large analysis of pancreatic juice samples with TRAP/HPA, 17 samples were excluded from the final analysis because of insufficient follow-up periods or inadequate treatment of the samples. Relative telomerase activity (RTA) in samples from patients with pancreatic carcinoma was significantly higher in comparison to samples from patients with pancreatitis and 13 (61.9%) of 21 samples from patients with pancreatic carcinoma showed high RTA (> 4 U). Meanwhile, high RTAs were observed in 4 of 35 (11.4%) samples from patients with intraductal papillary mucinous tumor and in 1 of 40 samples (2.5%) fom patients without malignant disease.

**CONCLUSIONS.** TRAP/HPA accurately evaluated weak telomerase activity in pancreatic juice samples without the problem due to PCR inhibitors. This large analysis of nonselected pancreatic juice samples suggested that TRAP/HPA is a promising approach for the diagnosis of pancreatic carcinoma. *Cancer* 2004;101: 2309–17. © *2004 American Cancer Society.*

**KEYWORDS: *telomerase, polymerase chain reaction inhibitor, hybridization protection assay, pancreatic carcinoma, pancreatic juice.***

Pancreatic carcinoma is one of the most aggressive malignant tumors and generally has an extremely poor prognosis.[1,2] Early detection of the disease is believed to be the only way to improve the prognosis because only a small percentage of patients are resectable at the time of diagnosis and have the possibility of being cured by


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radical resection.[3] However, preoperative diagnosis of pancreatic carcinoma remains difficult despite improvement in imaging techniques such as ultrasound, computed tomography scans, and magnetic resonance imaging scans. Endoscopic retrograde cholangiopancreatography (ERCP) combined with cytology of pancreatic juice is currently used as a less invasive diagnostic tool to assess malignancy before radical surgery.[4,5] However, the sensitivity of cytology varies from 30% to 80% even in the hands of experienced investigators.[6,7]

Telomerase is a ribonucleoprotein enzyme that catalyzes de novo synthesis of the GGTTAG telomeric DNA onto chromosome ends to stabilize the telomere length, and it has been implicated in cell immortality.[8] Telomerase activity has been detected in a variety of human cancers,[9–11] but it is not present in most normal somatic cells, with some exceptions, including germ cells and activated lymphocytes.[12] We and other investigators have reported the diagnostic utility of telomerase detection in pancreatic juice samples for patients with pancreatic carcinoma using a modified polymerase chain reaction (PCR)-based, semiquantitative assay (conventional telomeric repeat amplification protocol [TRAP] assay).[13–16] However, these reports included only a small amount of pancreatic juice samples. Analysis of a large amount of pancreatic juice samples has to be performed to determine the feasibility of a telomerase activity assay for diagnosis of pancreatic carcinoma before this assay can be used as a routine test in clinical settings. Also, several problems must be resolved before TRAP can be introduced as a routine test. One major problem is that the conventional TRAP assay requires radioisotopic labeling.

Recently, Hirose et al.[17] developed a hybridization protection assay (HPA) that, in combination with TRAP (TRAP/HPA), detects PCR-amplified telomerase products without radioisotopic labeling. This method is simple, rapid, sensitive, and useful for quantitative measurements of telomerase activity in even the smallest samples of various tissues.[17–19] Thus, telomerase activity assay with TRAP/HPA is useful for the analysis of a large amount of samples and may be advantageous clinically.

In the current study, we tested the sensitivity of TRAP/HPA and the effects of PCR inhibitors in pancreatic juice on measurements of telomerase activity. In addition, we collected samples of pancreatic juice obtained endoscopically from 117 nonselected consecutive patients with various pancreatic diseases. Our goals were to measure telomerase activity with TRAP/HPA and to test the feasibility of this diagnostic approach.

## MATERIALS AND METHODS

### Pancreatic Juice

Pancreatic juice samples were collected from 117 consecutive patients who underwent ERCP at the Kyushu University Hospital (Fukuoka, Japan) between February 15, 2001 and January 1, 2004, for suspected malignancy in the pancreas.[14] Briefly, a balloon catheter was inserted by endoscopy into the pancreatic duct. After pancreatography and an intravenous injection of 1 U/kg body weight of secretin (Eisai, Tokyo, Japan), ≥ 3 pancreatic juice samples were collected at 5-minute intervals from each patient through the balloon catheter. The first sample was discarded because of possible contamination of contrast medium. The second sample was subjected to cytologic examination by experienced cytologists according to standard criteria.[20] The third sample was centrifuged at 2000 × g for 5 minutes at 4 °C. The cell pellet was washed 3 times with ice-cold phosphate-buffered saline, centrifuged at 2000 × g for 5 minutes at 4 °C, and then stored at −80 °C until a telomerase assay was performed.

The diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens or the appearance of liver metastasis. The diagnosis of intraductal papillary mucinous tumor (IPMT), mucinous cystic tumor, and pancreatitis was made on the basis of the histologic examinations of resected specimens or on clinical observations with conventional diagnostic imaging for ≥ 6 months. Samples from 17 patients were excluded from analysis because of insufficient follow-up ($n$ = 15) and inadequate treatment of the samples ($n$ = 2). Therefore, after exclusion of these 17 samples, 100 pancreatic juice samples remained in the final analysis.

Informed consent was obtained from all patients, and the study was conducted according to the guidelines of the Helsinki Declaration.

### Telomeric Repeat Amplification Protocol Assay

The TRAP assay was performed with the TRAPeze kit (Intergen Co., Purchase, NY) according to the manufacturer's instructions, but with some modifications. In brief, cell pellets were resuspended in CHAPS lysis buffer (1 μg/mL each of the protease inhibitors antipain, leupeptin, phosphoramidon, elastatinal, pepstatin A, and chymostatin [Peptide Institute, Osaka, Japan]) and 0.5 U/mL RNase inhibitor (Wako Pure Chemical Co., Osaka, Japan) and then incubated for 30 minutes on ice. The protein concentration of the extract was measured by the Bradford assay,[21] and the indicated amount of protein extract was used for each TRAP assay. The 50-μL reaction mixture was subjected to 31 PCR cycles.

### Electrophoresis and Gel Staining with SYBR Green

Twenty-five microliters of TRAP assay products was analyzed by electrophoresis in 0.5 × Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer on 12% nondenaturing polyacrylamide gels and visualized with SYBR Green DNA stain (FMC Bioproducts, Rockland, ME). A 36-base pair internal TRAP assay standard (ITAS; Intergen) was used as an internal control to evaluate the effect of PCR inhibitors present in samples.

### Hybridization Protection Assay

A 5-μL aliquot of TRAP assay products was denatured for 5 minutes at 95 °C, after which 100 μL acridinium-ester–labeled probe (5'-CCCTAA CCCTAA CCCTAA CTCTGC TCGAC-3') with $3 \times 10^6$ relative light units (rlu) in hybridization buffer (0.1 M lithium succinate buffer, pH 4.7, 20% lithium lauryl sulfate, 1.2 M lithium chloride, 20 mM EDTA, and 20 mM ethyleneglycoltetraacetic acid) was added to each reaction tube and incubated for 20 minutes at 60 °C. Differential hydrolysis of the bound versus free probe was performed by adding 300 μL of hydrolysis buffer (0.6 M sodium tetraborate buffer, pH 8.5, 5% Triton X-100 [Sigma, St. Louis, MO]) and incubating at 60 °C for 10 minutes. Chemiluminescence was measured with a Leader I luminometer (Gen-Probe, Inc., San Diego, CA) and an automated reagent-injection method involving two detection reagents. Injection of 200 μL of detection reagent I (0.1% $H_2O_2$, volume/volume, 1 mM nitric acid) was followed after a 1-second delay by injection of 200 μL of detection reagent II (1 M NaOH). The resulting chemiluminescence was integrated for 2 seconds, and the reading was expressed in rlu. Measurement of telomerase activity in the human pancreatic carcinoma cell line MIA PaCa-2 (Japanese Cancer Resources Bank, Tokyo, Japan) was used as a positive control in each assay. One unit of relative telomerase activity (RTA) was defined as the activity equivalent to that in one MIA PaCa-2 cell.

### Statistical Analysis

Data were analyzed with the Kruskal–Wallis and Mann–Whitney *U* tests because a normal distribution was not obtained after logarithmic transformation. $P < 0.05$ was statistically significant.

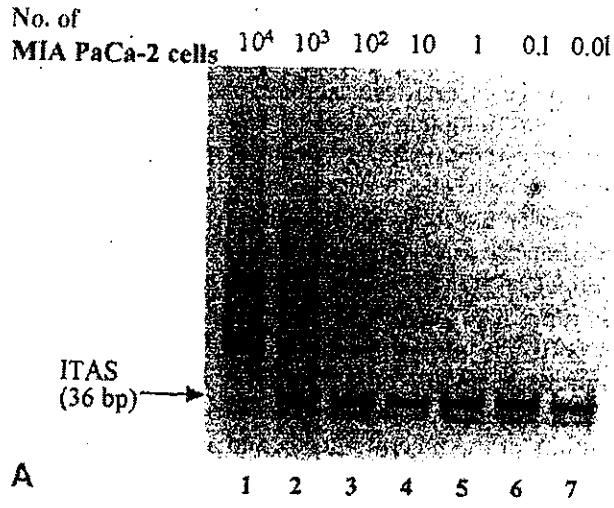
## RESULTS

### Sensitivity and Quantification of Telomerase Activity by Telomeric Repeat Amplification Protocol/Hybridization Protection Assay

We evaluated the sensitivity of conventional TRAP assay by staining TRAP assay products with nonisotopic SYBR Green, which is used widely in laboratories instead of $^{32}P$ because it is easy to handle and the fluorescence is relatively stable. DNA ladder bands indicative of telomerase activity were observed in extracts of ≥ 100 MIA PaCa-2 cells but not in extracts from ≤ 10 cells (Fig. 1A). As previously reported, the DNA ladder bands were detected in 10 MIA PaCa-2 cells when $^{32}P$ was used for labeling.[14] Therefore, conventional TRAP assay with SYBR Green appears to be ≥ 10 times less sensitive when compared with detection with $^{32}P$ and, consequently, it is not sensitive enough for detection of telomerase activity in pancreatic juice samples.[14] We then attempted to evaluate the utility of the recently developed TRAP/HPA assay for telomerase in pancreatic juice samples. We tested the sensitivity and linearity of this assay with telomerase-positive MIA PaCa-2 cells. Serial dilutions of protein extracts from these cells were tested by TRAP/HPA. As shown in Figure 1B, chemiluminescence intensity was linear between 0.1 and 100 cells equivalent of MIA PaCa-2 cells. Notably, TRAP/HPA was more than 1000-fold more sensitive than conventional TRAP assay with SYBR Green and more than 100-fold more sensitive than conventional TRAP assay with $^{32}P$ for detection of telomerase activity.

### Evaluation of the Effects of Proteases and RNases in Pancreatic Juice Samples on Measurement of Telomerase Activity

Several reports have suggested that the presence of proteases, RNases, and PCR inhibitors can affect the accuracy of TRAP assays because these molecules may affect primer extension or PCR amplification in the TRAP assay.[10] However, the presence and/or the effects of such factors in pancreatic juice samples are not clear. To evaluate the effects of proteases and RNases in pancreatic juice samples on detection of telomerase activity, human pancreatic carcinoma cells (MIA PaCa-2) were incubated in the acellular component of pancreatic juice samples for 15 minutes at room temperature. After centrifugation, the MIA PaCa-2 cell pellets were lysed in the presence or absence of protease inhibitors or/and RNase inhibitors and subjected to conventional TRAP assay with SYBR Green. Although the intensities of the DNA ladder bands were markedly decreased when cells were incubated with pancreatic juice in comparison to untreated control cells (Fig. 2), the addition of protease inhibitors and/or RNase inhibitors to the CHAPS lysis buffer ameliorated the effect of the pancreatic juice (Fig. 2, lanes 1, 2, 4–6), suggesting that the addition of a sufficient amount of protease and RNase inhibitors may improve detection of telomerase activity in pancreatic juice samples.



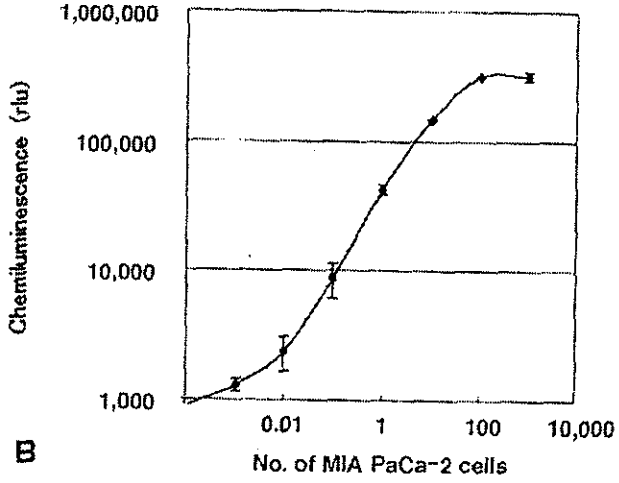




**FIGURE 1.** Detection of telomerase activity in serial dilutions of protein extracts from MIA PaCa-2 cells by conventional telomeric repeat amplification protocol (TRAP) assay and TRAP/ hybridization protection assay (HPA). MIA PaCa-2 cells were incubated in pancreatic juice samples and collected by centrifugation. Cell pellets were lysed and diluted to the indicated cell numbers per 2 μL. Cell extracts (2 μL) were then subjected to conventional TRAP with SYBR Green (A) or TRAP/HPA (B). (A) DNA ladder bands indicative of telomerase activity were observed in extracts of ≥ 100 MIA PaCa-2 cells but not in extracts from ≤ 10 cells. ITAS: internal TRAP assay standard; bp, base pair. (B) The chemiluminescence intensity was linear between 0.1 and 100 cells equivalent of MIA PaCa-2 cells. Bars: standard deviations; rlu: relative light unit. Data are expressed as the means of triplicate experiments.

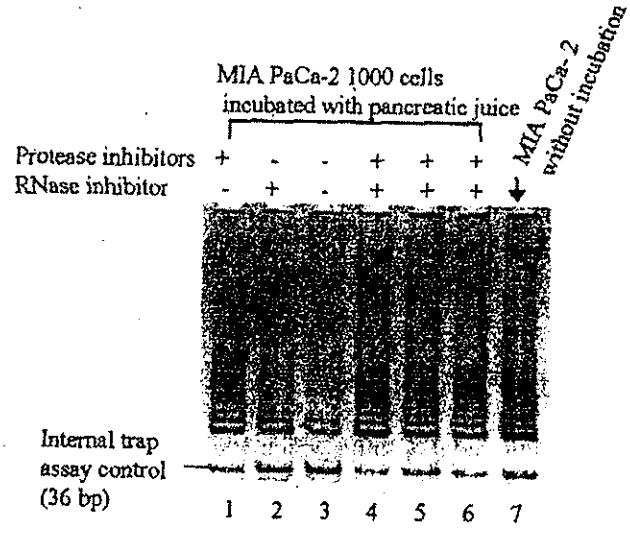



**FIGURE 2.** Effects of proteases and RNases on detection of telomerase activity by conventional telomeric repeat amplification protocol (TRAP) assay. A total of $5 \times 10^6$ MIA PaCa-2 cells were incubated in pancreatic juice samples for 15 minutes with or without protease and RNase inhibitors and collected by centrifugation. Cell pellets were lysed in CHAPS lysis buffer (1 μg/mL each of the protease inhibitors antipain, leupeptin, phosphoramidon, elastatinal, pepstatin A, and chymostatin) at a final concentration of 500 cells/μL. Two microliters of cell extract was used for conventional TRAP assay with SYBR Green. The intensities of the DNA ladder bands were markedly decreased when cells were incubated with pancreatic juice samples (lane 3) in comparison to untreated control cells (lane 7). The addition of protease inhibitors (lane 1), RNase inhibitors (lane 2), or both inhibitors (lanes 4–6) to the CHAPS lysis buffer ameliolated the effects of pancreatic juice.

### Evaluation of the Effects of Polymerase Chain Reaction Inhibitors in Pancreatic Juice Samples on Detection of Telomerase Activity

MIA PaCa-2 cells were incubated with protease and RNase inhibitors to evaluate the effect of PCR inhibitors in pancreatic juice samples on conventional TRAP assay. Amplification of the ITAS was examined in different dilutions of protein extracts from six samples of pancreatic juice. When 0.6 μg of protein extract was used in each reaction, ITAS bands of almost equal intensities were detected. However, when 6.0 μg of extract was used, the intensity of ITAS varied markedly among samples (Fig. 3).

We next evaluated the effects of PCR inhibitors in protein extracts from pancreatic juice samples on TRAP/HPA. The protein extracts from telomerase-negative pancreatic juice samples, which contained ≥ 6.0 μg/μL of protein, were diluted serially and then mixed with protein extracts from telomerase-positive MIA PaCa-2 cells. As shown in Figure 4A, for sample 45, the RTA of MIA PaCa-2 cells was stable when mixed with 0.6 or 1.0 μg of protein from

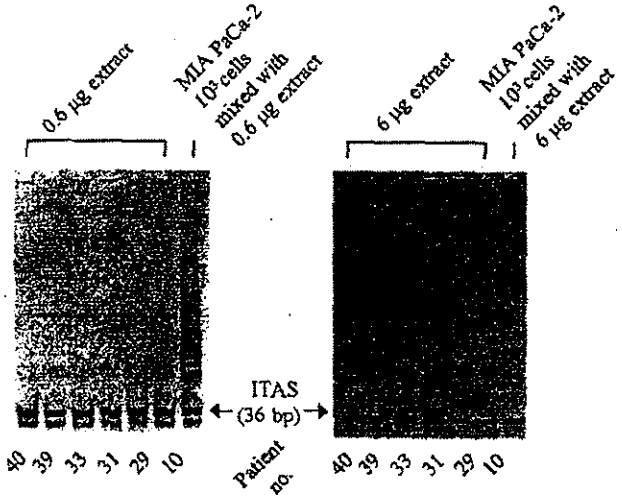
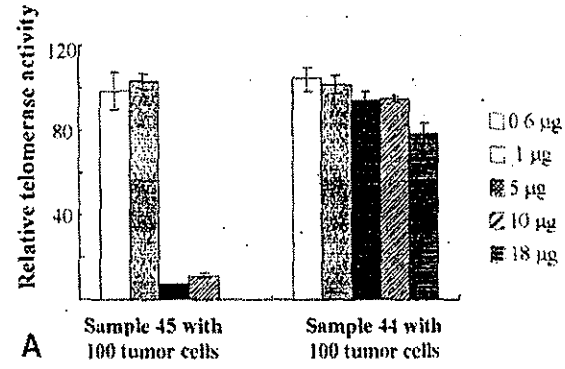



**FIGURE 3.** Effects of polymerase chain reaction inhibitors in pancreatic juice samples on conventional telomeric repeat amplification protocol (TRAP) assay. *Pancreatic juice samples from Patients 10, 29, 31, 33, 39, and 40 with protein concentrations of ≧ 6 μg per 2 μL protein extract were diluted to 0.3 μg/μL or 3 μg/μL and then subjected to conventional TRAP assay. The extract from 1000 MIA PaCa-2 cells was mixed with 0.6 μg or 6 μg extract of pancreatic juice from Patient 10 and then subjected to conventional TRAP assay. When 0.6 μg of extract was used in each reaction, internal TRAP assay standard (ITAS) bands of almost equal intensities were detected. However, when 6.0 μg of extract was used, the intensity of ITAS varied markedly among samples. bp: base pair.*



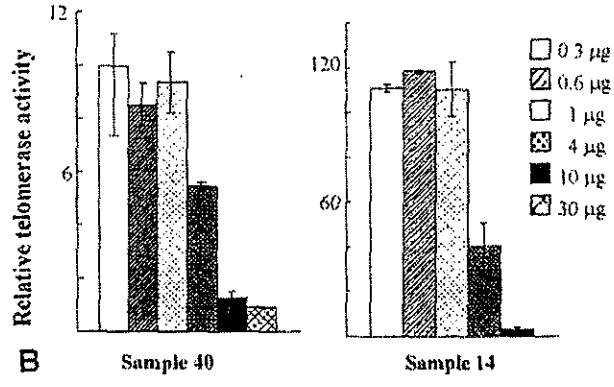




**FIGURE 4.** Effects of polymerase chain reaction inhibitors in pancreatic juice samples on telomeric repeat amplification protocol/hybridization protection assay (TRAP/HPA). (A) Protein extract from telomerase-negative pancreatic juice samples was diluted serially and then the indicated amount of protein was mixed with protein extract from telomerase-positive cancer cells (MIA PaCa-2 cells). The mixed proteins, including proteins derived from 100 cancer cells, were then subjected to TRAP/HPA. In sample 45, the relative telomerase activity was stable when mixed with 0.6 μμg or 1.0 μg of protein from pancreatic juice samples, but it decreased dramatically when mixed with 5 or 10 μg protein. In sample 44, however, only a slight decrease was observed when mixed with ≥ 1.0 μg protein. (B) The protein extracts from telomerase-positive pancreatic juice samples were diluted serially and subjected to TRAP/HPA. The values of telomerase activity per 0.6 μg extract protein were calculated. Data are expressed as means of triplicate experiments. Bars indicate standard deviations. The calculated telomerase activity was stable when the final amount of protein was ≤ 1.0 μg. In contrast, it decreased significantly when ≥ 1.0 μg of protein was used in the assay.

pancreatic juice samples, but telomerase activity decreased dramatically when cells were mixed with 5 or 10 μg protein from pancreatic juice samples. In sample 44, however, only a slight decrease in RTA was observed when cells were mixed with ≥ 1.0 μg protein. These data suggest that the effect of PCR inhibitors is different in each sample. However, the RTA was stable when ≤ 1.0 μg of protein extract was used regardless of the samples.

We also examined the effects of PCR inhibitors in telomerase-positive pancreatic juice samples from one patient with IPMT and from one patient with pancreatic carcinoma. After serial dilution, the protein extracts from these samples were subjected to TRAP/HPA. The calculated telomerase activity per 0.6 μg protein extract was stable when the final amount of protein was ≤ 1.0 μg (Fig. 4B). The calculated telomerase activity decreased significantly when ≥ 1 μg of protein was used in the assay. These results indicate that the effects of PCR inhibitors on amplification could be ameliorated when 0.6 μg extract is used in this assay. In subsequent experiments, we used 0.6 μg protein extracts from pancreatic juice samples in all telomerase assays.

### Analysis of 100 Pancreatic Juice Samples Obtained during Endoscopic Retrograde Cholangiopancreatography

To measure telomerase activity with TRAP/HPA, we collected pancreatic juice samples from 117 consecutive patients with different pancreatic diseases. Eventually, 17 samples from patients with nonmalignant disease were excluded from the current analysis because of insufficient follow-up periods or failure of the treatment of the samples. Therefore, 100 pancreatic juice samples were enrolled in the final analysis. These 100 samples were obtained from 21 patients with pancreatic carcinomas, 25 patients with chronic pancreatitis, 35 patients with IPMTs, 4 patients with malignancies other than pancreatic carcinoma, 9 patients with benign diseases other than pancreatitis and IPMT (3 endocrine tumors, 2 benign stenoses of pancreatic duct, 2 mucinous cystic tumors, and 2 patients with high serum levels of CA 19-9), and 6 patients with normal studies.

Of 100 pancreatic juice samples, 52 samples (which were collected in 2001 and 2002), were used for both conventional TRAP assay with SYBR Green and TRAP/HPA. Of these 52 samples, only 2 samples were positive in the conventional TRAP assay. These two samples included one carcinoma juice sample and one IPMT juice sample. Because the positive rate with the conventional TRAP assay was too low, 48 samples collected in 2003 were individually evaluated by TRAP/HPA.

All pancreatic juice samples from 13 patients with pancreatitis used for conventional TRAP assay were negative. In analysis of 25 pancreatitis juice samples with TRAP/HPA, the maximal RTA was 3.89 U, and the median value was 0.21 U. All these pancreatic juice samples showed low or modest RTA (< 4 U). It is possible that activated lymphocytes may contribute to the weak telomerase activities observed in pancreatitis samples.[22]

RTAs in carcinoma juice samples were significantly higher than those in pancreatitis samples ($P < 0.0001$; Fig. 5). Table 1 summarizes the clinicopathologic features, results of telomerase detection with conventional TRAP assay and SYBR Green, RTA measured by TRAP/HPA, and cytologic diagnosis from pancreatic carcinoma juice samples. TRAP/HPA yielded high RTA (> 4 U) in 13 (61.9%) of 21 pancreatic juice samples from patients with pancreatic carcinoma, and 1 was a case of early pancreatic carcinoma (Stage I, T1N0M0). Of eight samples with < 4 U RTA by TRAP/HPA, three showed complete obstruction of the main pancreatic duct (MPD). These data suggest that there may have been some difficulty in collecting a sufficient number of cancer cells through ERCP in these cases. Of the remaining five samples with low RTA (< 1 U), three were diagnosed as Class 4 or 5 by cytologic analysis. However, these samples contained a massive number of blood cells and showed high concentrations of protein extract (≧ 25 $\mu g/\mu L$). Therefore, a large dilution of extract protein was necessary to quantify telomerase activity in these samples, possibly reducing the detection of telomerase activity. It is also possible that these pancreatic carcinomas may have developed without the activation of telomerase.

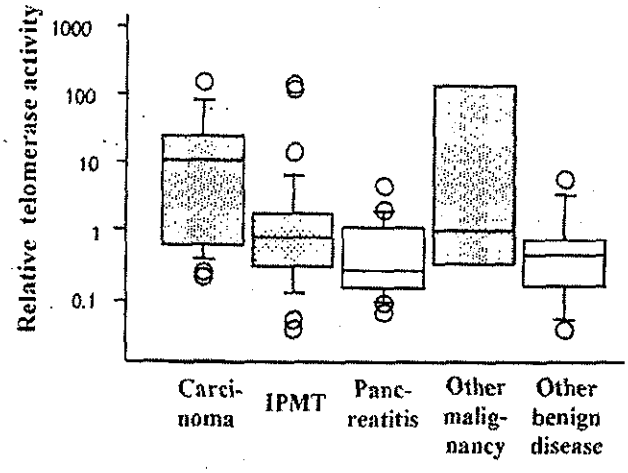



**FIGURE 5.** Relative telomerase activity (RTA) measured by telomeric repeat amplification protocol/hybridization protection assay (TRAP/HPA) in pancreatic juice samples from patients with various pancreatic disorders. RTA in pancreatic juice samples from patients with pancreatic carcinoma was significantly higher than that in samples from patients with intraductal papillary mucinous tumors or pancreatitis ($P = 0.0031$ and $P < 0.0001$, respectively).

The current study included samples from 35 patients with IPMTs (Tables 1 and 2). By TRAP/HPA, 4 IPMT samples showed high RTA (> 4 U) and 10 IPMT samples showed modest RTA (> 1 U and < 4 U), whereas the remaining 21 samples showed low RTA (< 1 U). In our study, most patients with IPMT had been followed for ≥ 6 months without surgical intervention because decisive findings for malignancy were not present. Only two patients with IPMT with modest telomerase activity, who had highly suggestive clinical findings for malignancy, underwent surgery. The pathologic findings showed severe atypia without malignancy for both patients.

We also measured telomerase activity in 15 samples excluded from the analysis due to insufficient follow-up. All these samples showed low telomerase activities.

## DISCUSSION

Telomerase detection with conventional TRAP assay has not been introduced widely into large analysis or

TABLE 1
RTA in Pancreatic Juice from Patients with Carcinoma.

| Patient no. | Final diagnosis | Age/gender | Classification[a] | Location | Gel staining (SYBR green) | TRAP/HPA RTA[b] | Cytology[c] | Complete obstruction of MPD[d] |
|---|---|---|---|---|---|---|---|---|
| 37 | Pancreatic carcinoma[e] | 53/F | T3N0M0 | Ph | + | 132.67 ± 15.63 | Class 3 | — |
| 38 | Pancreatic carcinoma[f] | 61/M | TxNxM1 | Ph | — | 21.42 ± 3.40 | Class 1 | — |
| 39 | Pancreatic carcinoma[f] | 67/M | T3N1M1 | Ph | — | 9.34 ± 0.47 | Class 5 | — |
| 40 | Pancreatic carcinoma[e] | 58/M | T3N1M0 | Ph | — | 6.29 ± 1.67 | Class 3 | — |
| 41 | Pancreatic carcinoma[f] | 67/M | TxNxM1 | Pb | — | 8.76 ± 1.69 | Class 3 | — |
| 42 | Pancreatic carcinoma[f] | 64/M | T4N1M1 | Pb | — | 19.00 ± 2.86 | Class 3 | — |
| 43 | Pancreatic carcinoma[e] | 78/M | T1N0M0 | Ph | — | 12.92 ± 3.03 | Class 3 | — |
| 44 | Pancreatic carcinoma[e] | 61/F | T3N0M0 | Pb | — | 0.45 ± 0.24 | Class 4 | — |
| 45 | Pancreatic carcinoma[e] | 58/M | T4N0M0 | Pb | — | 0.17 ± 0.14 | Class 5 | — |
| 46 | Pancreatic carcinoma[e] | 72/M | T2NxM0 | Pb | — | 0.51 ± 0.07 | Class 3 | + |
| 47 | Pancreatic carcinoma[f] | 43/F | TxNxM1 | Pb | — | 0.60 ± 0.17 | Class 2 | + |
| 53 | Pancreatic carcinoma[f] | 57/M | T4NxM1 | Pt | NE | 0.61 ± 0.22 | Class 2 | — |
| 54 | Pancreatic carcinoma[f] | 82/M | T4NxM1 | Pb | NE | 5.80 ± 1.30 | Class 3 | — |
| 55 | Pancreatic carcinoma[f] | 39/M | TxNxM1 | Pb | NE | 28.52 ± 3.03 | Class 1 | + |
| 56 | Pancreatic carcinoma[e] | 66/M | T4N0M0 | Ph | NE | 127.61 ± 11.66 | Class 3 | + |
| 57 | Pancreatic carcinoma[f] | 57/M | T4N3M1 | Pb | NE | 0.89 ± 0.06 | Class 2 | + |
| 58 | Pancreatic carcinoma[e] | 73/M | T4N1M0 | Pb | NE | 18.32 ± 4.81 | Class 3 | — |
| 59 | Pancreatic carcinoma[f] | 65/M | T3NxM1 | Ph | NE | 0.19 ± 0.03 | Class 5 | — |
| 60 | Pancreatic carcinoma[e] | 75/M | T4N1M0 | Pt | NE | 0.17 ± 0.02 | Class 3 | — |
| 61 | Pancreatic carcinoma[f] | 82/M | TxNxM1 | Pb | NE | 22.40 ± 2.86 | Class 5 | — |
| 62 | Pancreatic carcinoma[e] | 72/M | T3N0M0 | Ph | NE | 5.46 ± 0.69 | Class 4 | — |

RTA: relative telomerase activity; TRAP: telomeric repeat amplification protocol; HPA: hybridization protection assay; MPD: main pancreatic duct; F: female; Ph: head of the pancreas; —: negative; M: male; Pb: body of the pancreas; +: positive; Pt: tail of the pancreas; NE: not examined.

[a] Tumor classification was assessed according to the International Union Against Cancer TNM staging system.

[b] Relative telomerase activity equivalent to that in 1 MIA PaCa-2 cell was defined as 1 unit. RTA in 0.6 μg of protein extract from pancreatic juice was exhibited using unit. Values were expressed as the mean ± the standard deviation of triplicate experiments.

[c] Cytologic diagnosis was made according to the standard criteria as reported previously.[20]

[d] Endoscopic retrograde cholangiopancreatography findings.

[e] Resected and/or pathologically diagnosed.

[f] Diagnosed by liver metastasis.

TABLE 2
Comparison of Diagnosis of Pancreatic Carcinoma between TRAP/HPA and Cytology

| Disease | TRAP/HPA[a] No. (%) | Cytology[b] No. (%) | Combination[c] No. (%) |
|---|---|---|---|
| Pancreatic carcinoma | 13/21 (61.9) | 6/21 (28.6) | 16/21 (76.2) |
| IPMT | 4/35 (11.4) | 0/35 (0) | 4/35 (11.4) |
| Chronic pancreatitis | 0/25 (0) | 0/25 (0) | 0/25 (0) |
| Other malignancy[d] | 1/4 (25.0) | 0/4 (0) | 1/4 (25) |
| Normal findings and benign diseases[e] | 1/15 (6.7) | 0/15 (0) | 1/15 (6.7) |
| Total | 19/100 (19) | 6/100 (6) | 22/100 (22) |

TRAP: telomeric repeat amplification protocol; HPA: hybridization protection assay; IPMT: intraductal papillary mucinous tumor.

[a] Samples with > 4 U of relative telomerase activity were positive.

[b] Samples with Class 4 or 5 were positive.

[c] When one of either diagnostic tools was positive, the samples were considered to be positive.

[d] Malignancy other than pancreatic carcinoma.

[e] Benign diseases other than intraductal papillary mucinous tumor and chronic pancreatitis.

the clinical setting partly because of its complex, time-consuming, and labor-intensive nature. In the current study, we tested the sensitivity and the feasibility of a more sensitive, rapid, simple, quantitative, and reproducible telomerase activity assay, TRAP/HPA, in pancreatic juice samples. We found that, in comparison to the conventional TRAP assay, the sensitivity of TRAP/HPA was markedly higher in the detection of a small number of cancer cells and that even in the analysis of 100 patients, TRAP/HPA of the pancreatic juice samples showed high sensitivity and specificity as well as previous small analysis using the radioisotope. If we consider > 4 U of RTA as a positive finding for the diagnosis of cancer, the positivity rates for carcinoma and pancreatitis juice samples were 61.9% (13 of 21) and 0% (0 of 25), respectively (Table 2). To evaluate the specificity of this test, we selected 40 samples of nonmalignant tissue including 25 samples from patients with chronic pancreatitis and 15 samples from patients with normal findings. When < 4 U was considered negative, the specificity reached 97.5% (39 of

TABLE 3
Telomerase Activity in Pancreatic Juice Samples from Patients with Early-Stage Pancreatic Carcinoma

| Case no.[a] | Age/gender | Classification[b] | Location | Detection method | RTA[c] | Cytology[d] |
|---|---|---|---|---|---|---|
| 1 | 72/F | T1N0M0 | Ph | Conventional TRAP assay | 14.1 | Class 2 |
| 2 | 59/M | TisN0M0 | Pt | Conventional TRAP assay | 4.66 | Class 5 |
| 3 | 61/M | T1N0M0 | Ph | Conventional TRAP assay | 22.3 | Class 2 |
| 4 | 78/M | T1N0M0 | Ph | HPA TRAP | 13.7 | Class 3 |

RTA: relative telomerase activity; F: female; Ph: head of the pancreas; TRAP: telomeric repeat amplification protocol; M: male; Pt: tail of the pancreas; HPA: hybridization protection assay.
[a] Cases 1, 2, and 3 were described in previous reports.[14,23]
[b] Tumor classification was assessed according to the International Union Against Cancer TNM staging system.
[c] Relative telomerase activity equivalent to that in 1 MIA PaCa-2 cell was defined as 1 unit. RTA in 0.6 μg of protein extract from pancreatic juice was exhibited using unit. Values were expressed as the mean ± the standard deviation of triplicate experiments.
[d] Cytologic diagnosis was made according to the standard criteria as reported previously.[20]

40). In addition, TRAP/HPA is likely to be more advantageous than the conventional TRAP assay in terms of the clinical use because this assay provides rapid and reproducible results without the need for radioactive materials and requires only 4 hours to complete and only 1 tube with simple daily setup for the HPA step for detection. We have embarked on a project using this method prospectively in patients.

It is notable that three patients who showed telomerase activity of < 1 U by TRAP/HPA were diagnosed as having Class 4 or 5 disease by cytology. Thus, the combination of cytology and TRAP/HPA may improve the diagnostic yields (Table 2). Furthermore, complete obstruction of the MPD, which may be responsible for false-negative results, is a decisive ERCP finding for the diagnosis of pancreatic carcinoma. Therefore, ERCP findings also supported the diagnosis by TRAP/HPA and improved the diagnostic accuracy in the current study.

We also showed the remarkable effects of PCR inhibitors, proteases, and RNases in pancreatic juice samples on the quantification of telomerase activity. Pancreatic juice is often very rich in PCR inhibitors, which can potentially interfere with the TRAP assay. It has been reported that dilution of samples is the only way to avoid the effects of PCR inhibitors on telomerase assays.[18] In previous reports, however, a relatively large amount of protein extract (6 μg) was needed for semiquantification of positive telomerase DNA ladder bands determined by conventional TRAP assay, resulting in possible difficulties in quantification due to the presence of massive PCR inhibitors. In the current study, all samples with a large amount of protein extract were diluted to the concentration of 0.6 μg per 2 μL and 2 μL of protein extract was used for TRAP/HPA. Then, the use of a small amount of protein extract reduced the contamination of PCR inhibitors and permitted avoidance of the effects of PCR inhibitors in the pancreatic juice samples. In addition, the use of a large amount of protein extract limited the number of samples included in the previous analysis. In contrast, TRAP/HPA is sensitive enough to detect the low range of telomerase activity, which includes a threshold to distinguish carcinoma samples from benign samples, in a smaller amount (0.6 μg) of protein extract from pancreatic juice samples. Therefore, we included a large number of consecutive samples with a small amount of extract protein in the present analysis.

Detection of telomerase activity in pancreatic juice may be more suitable for detection of early-stage pancreatic carcinomas rather than for advanced-stage pancreatic carcinomas. Most pancreatic carcinomas develop and proliferate in the pancreatic duct in the early stage but obstruct the duct in the late stage, resulting in difficulties in collecting cancer cells. The current series included pancreatic juice samples from patients with early-stage pancreatic carcinoma. Including previous reports,[14,23] we detected high telomerase activities in all four patients with early-stage pancreatic carcinoma (Table 3).

We detected high RTA in four samples from patients with IPMT. Inoue et al.[24] reported that detection of telomerase activity in pancreatic juice samples reflects malignant potential in IPMTs. These results suggest that precancerous IPMT lesions might express telomerase activity and this expression of telomerase activity may be an early event in carcinogenesis in the adenoma-adenocarcinoma sequence of IPMT. To clarify the significance of telomerase activity in pancreatic IPMT, further studies with sensitive assays such as TRAP/HPA and follow-up observation long enough to determine the definitive diagnosis are needed.

In conclusion, we described the remarkable effects of PCR inhibitors and the feasibility of quantitative measurement of telomerase activity in pancreatic juice samples by TRAP/HPA for diagnosis of pancre-

atic carcinoma. Although further studies with prospective trials are needed to confirm our findings, we believe that TRAP/HPA with pancreatic juice samples is a promising approach for diagnosis of pancreatic carcinoma.

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Journal of HBP Surgery


# Familial pancreatic cancer: report of one Japanese family

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**Abstract**
Most familial pancreatic carcinomas have been reported from European countries and the United States, and there has been only one report from Japan. A 50-year-old Japanese woman presented with a pancreatic head mass and underwent pylorus-preserving pancreatoduodenectomy with portal vein resection. The histological diagnosis was well-differentiated adenocarcinoma of the head of the pancreas. Her mother died of pancreatic head carcinoma, which had been shown on computed tomography at the age of 70 years. One of her uncles on her father's side had had pancreatic tail carcinoma, and at the age of 59, had undergone distal pancreatectomy, splenectomy, wedge resection of the liver, and partial resection of the colon. The histological diagnosis was moderately differentiated tubular adenocarcinoma of the pancreas. He had had a subtotal gastrectomy for early gastric cancer (tubular adenocarcinoma limited to the mucosa) at the age of 53. He died of recurrence of the pancreatic tail carcinoma 3 months after the distal pancreatectomy had been performed. This communication reports a second Japanese family with familial pancreatic cancer, as shown by pancreatic carcinomas in two first-degree relatives and in one third-degree relative.

**Key words** Familial pancreatic cancer

## Introduction

The clinical course of patients with pancreatic carcinoma remains dismal, and improvements in the clinical course of patients with pancreatic cancer is desired. Familial pancreatic carcinoma has become a target to discover early pancreatic carcinoma in the United States. However, only one case has been reported in Japan.[1] This may be due to a really low incidence of familial pancreatic carcinoma, or it may be due to a lack of interest in this entity in Japan. This background urged us to review 103 patients with pancreatic cancer who had undergone surgical resection at our institution, and we found one family with possible familial pancreatic cancer. In this communication, we report familial pancreatic carcinomas occurring in two first-degree relatives and one third-degree relative.

*Offprint requests to:* K. Yamaguchi
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## Case report

A 50-year-old Japanese woman had obstructive jaundice (serum level of total bilirubin, 7.2 mg/dl (normal, <1.0 mg/dl) and visited us for surgical treatment. Her family history included pancreatic cancer in her mother and in one of her uncles on her father's side (Fig. 1).

Imagings showed a pancreatic head tumor, 3 cm in diameter (Fig. 2). The main pancreatic duct proximal to the tumor was dilated, with atrophy of the pancreatic parenchyma. Intrahepatic and extrahepatic bile ducts were dilated due to occlusion by the pancreatic tumor. Serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 were mildly elevated, to 4.3 ng/ml (<2.5 ng/ml) and 282.4 U/ml (<37 U/ml), respectively. Pylorus-preserving pancreatoduodenectomy with portal vein resection was done on March 10, 2000. After the operation, she underwent transarterial hepatic chemotherapy (with 5-fluorouracil [5-FU]) and external radiation (50 Gy). The histopathological diagnosis was well-differentiated adenocarcinoma of the pancreatic head (Fig. 3). One regional lymph node (no. 13) showed metastasis by the carcinoma. She has been well for 38 months after the operation, with no signs of recurrence.

## Family history

The patient's mother died of pancreatic head carcinoma, at the age of 71, in our hospital. Imagings

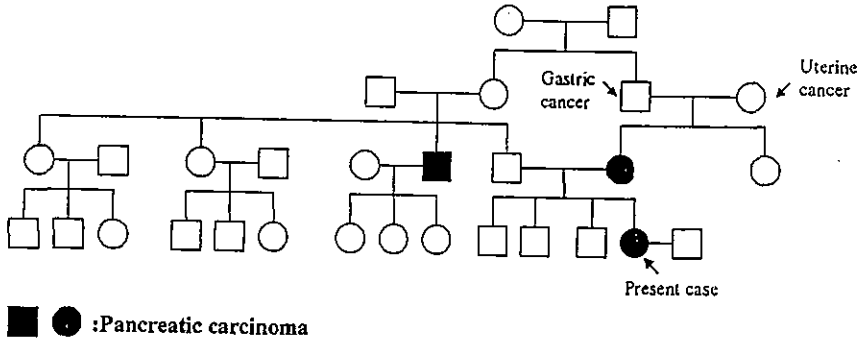


**Fig. 1.** Family tree shows that the present patient, her mother, and one of her uncles had a pancreatic carcinoma

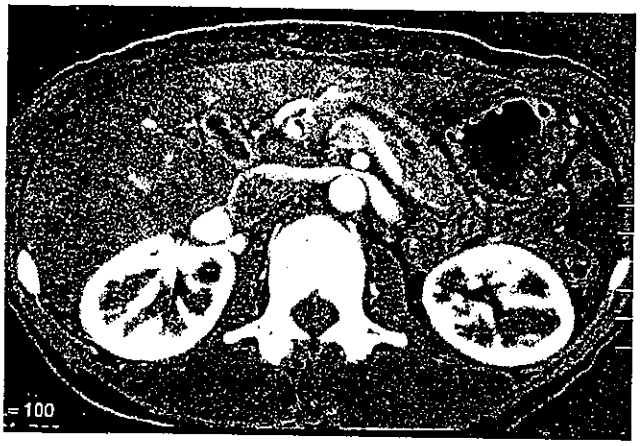


**Fig. 2.** Computed tomography in the patient shows a pancreatic head mass, 3 cm in diameter

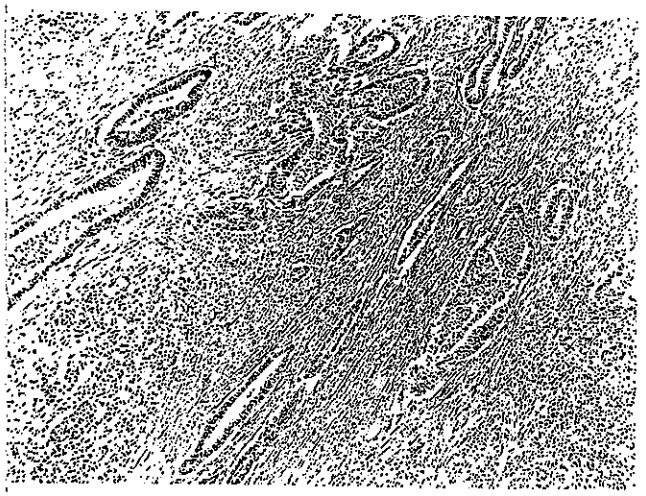

**Fig. 3.** Section of the resected specimen shows malignant glands invading the surrounding pancreas. H&E, ×85

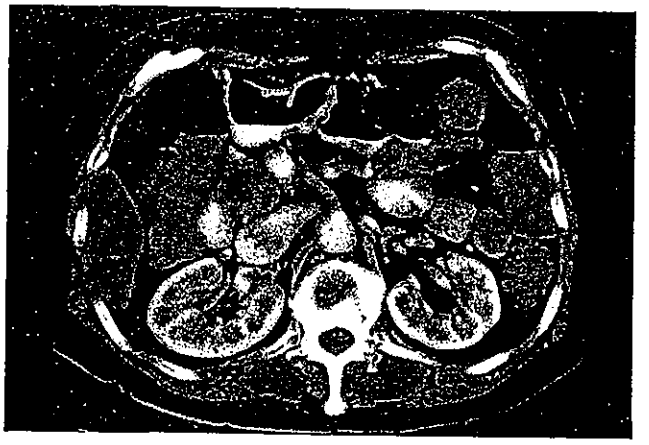

**Fig. 4.** Computed tomography in the patient's mother demonstrates a pancreatic head mass, 3 cm in diameter

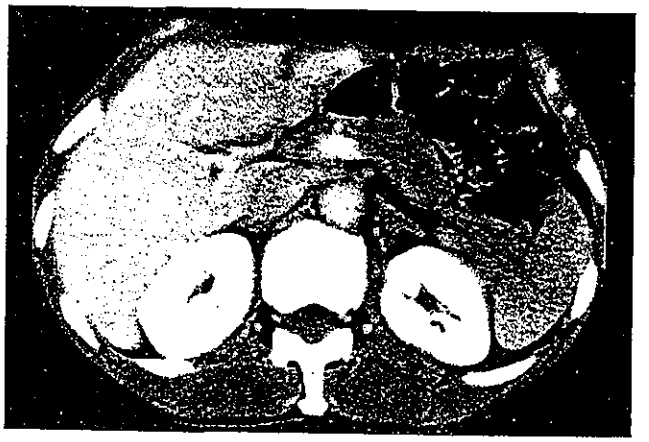

**Fig. 5.** Computed tomography in the patient's uncle displays a pancreatic body mass, 3 cm in diameter

3 months before her death showed a pancreatic head mass, 3 cm in diameter (Fig. 4). Unfortunately, neither surgical resection nor autopsy was done.

One of the patient's uncles on her father's side had undergone subtotal gastrectomy for early gastric carcinoma, at the age of 53 years. The histological diagnosis was tubular adenocarcinoma of the stomach limited to the mucosa. No lymph node metastasis was evident. At the age of 59, he had undergone distal pancreatectomy with combined resection of the transverse colon, for pancreatic tail carcinoma, at Kurume University Hospital. Figure 5 shows a computed tomography scan of the

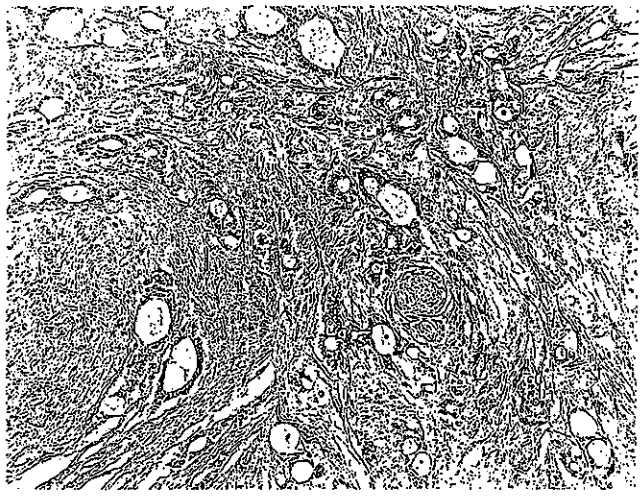

**Fig. 6.** Section of the resected specimen from the patient's uncle shows moderately differentiated adenocarcinoma of the pancreas. H&E, ×85

pancreatic mass. The histological diagnosis was moderately differentiated tubular adenocarcinoma of the pancreatic tail invading the colon, liver, splenic artery, and spleen (Fig. 6). Intraoperative radiation therapy (30 Gy) was done. He died of local and metastatic recurrences of the pancreatic cancer 3 months after the distal pancreatectomy had been performed.

## Discussion

Familial pancreatic cancer is defined as pancreatic carcinoma occurring in at least two family members who are first-degree relatives. In the present family, diagnosis of pancreatic duct carcinoma was proved by histology of the resected specimen in the patient and one of her uncles (third-degree relative). Pancreatic carcinoma in her mother (first-degree relative) was diagnosed by computed tomography, but histological diagnosis was not obtained. Strictly speaking, histological or cytological confirmation was mandatory for the diagnosis of pancreatic cancer in her mother. The incidence of pancreatic carcinoma is not high (about 1 per 10000 people) and the resectability of pancreatic cancer remains low, being about 10%–30%. Therefore, histological or cytological confirmation of pancreatic carcinoma from a resected specimen is sometimes difficult.

To our knowledge, there has been only one case report of familial pancreatic cancer in Japan.[1] According to the national registration of pancreatic cancer in Japan, the incidence of a family history (not limited to first-degree relatives) of pancreatic cancer is 3.7% (48/1296).[2] Studies over the past three decades in European countries and the United States of America have shown that 4%–16% of patients with pancreatic cancer had a family history of pancreatic cancer.[3–5] Therefore, it is possible that more cases of familial pancreatic cancer may exist in Japan, as well as in western countries. This prompted us to review our series of 103 patients with pancreatic cancer. Of these patients, 1 was found to be a possible familial pancreatic cancer.

Rieder et al.[4] reported that a shift towards a younger age at diagnosis in familial pancreatic cancer may indicate a genetic predisposition and/or changes in patterns of exogenous risk factors. In the present family, the patients with pancreatic cancer were a 50-year-old woman, a 59-year-old man, and a 71 year-old woman, all of whom were somewhat younger than the patients with usual pancreatic cancer in Japan, the incidence by decades being 21.7% (5053/23302) in the sixth decade, 34.3% (8003/23302; the peak), in the seventh decade, and 26.4% (6147/23302) in the eighth decade, according to the national registration of pancreatic cancer.[5]

Familial pancreatic cancer suggests a high possibility of occurrence of pancreatic cancer in members of the family. In families where three first-degree relatives had been diagnosed as having pancreatic cancer, the risk to the other individuals of developing pancreatic cancer rose to 57 times the baseline risk.[6] The risk and incidence of pancreatic cancer is extraordinarily high among first-degree relatives in a family with familial pancreatic cancer in which at least three first-degree relatives have already been diagnosed as having pancreatic cancer.[7] Rulyak et al. and Rulyak and Brentnall[9] proposed the endoscopic screening of carefully selected members of families with familial pancreatic cancer to increase patient life expectancy in a cost-effective manner. Klein et al.[3] from Johns Hopkins University, reported that individuals with a family history of pancreatic cancer in multiple first-degree relatives had a high risk of pancreatic cancer themselves, and the identification of such high-risk individuals would help clinicians to target screening programs and to develop preventive interventions with the hope of reducing the mortality of pancreatic cancer in these families. In the present family, screening of the patient's three brothers (first-degree relatives) was advised, but they were reluctant to undergo screening.

Inherited cancer syndromes include familial atypical multiple-mole melanoma, Peutz-Jeghers syndrome, hereditary breast-ovarian cancer, hereditary pancreatitis, and hereditary nonpolyposis colorectal cancer. These syndromes arise as a result of germline mutations in the *BRCA2*, *p16* (familial atypical multiple-mole melanoma), mismatch repair (hereditary nonpolyposis colorectal cancer), and *STK11* (Peutz-Jeghers syndrome) genes. Hahn et al.[10] examined *BRCA2* germline mutations in 26 European families with familial pancreatic cancer and concluded that 19% of the families had either a frameshift mutation or an unclassified variant of

*BRCA2*. Murphy et al.[11] also confirmed an increased risk of pancreatic cancer in individuals with *BRCA2* mutations, and they identified germline *BRCA2* mutations as the most common inherited genetic alteration. In our series of patients, we have not examined the genetic changes to date.

After reviewing 103 Japanese patients with pancreatic cancer who underwent a surgical resection, one family with possible familial pancreatic cancer was found. We would like to draw Japanese clinicians' attention to familial pancreatic cancer, because such families may be a target to screen for early pancreatic cancer.

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Review

# Novel strategic therapeutic approaches for prevention of local recurrence of pancreatic cancer after resection: trans-tissue, sustained local drug-delivery systems

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**Abstract**

Local recurrence and hepatic metastasis are still the major causes of death of patients who have undergone resection for pancreatic cancer. To decrease the incidence of local recurrence, we have proposed and devised several trans-tissue and local delivery systems, all of which could be applied immediately after surgery at the resected sites: (1) System I: a drug-loaded photocured gelatinous tissue-adhesive gel (bioactive substance reservoir) that enables the sustained release of a drug, protein, or gene-encoding adenovirus, (2) System II: an anti-cytokine antibody-fixed photocured gelatinous, tissue-adhesive gel (cytokine barrier) that prevents cytokine permeation into the resected tissue, (3) System III: a gene-modified cell sheet that enables the sustained release of a very costly protein produced by gene-transduced cells and (4) System IV: a percutaneous drug-delivery device that enables continuous drug infusion and easy removal from the body. This review article is a summary of our several years of efforts and attempts, which are composed of integrated disciplines including active biomaterials and genetic- and tissue-engineerings, to overcome the recurrence of pancreatic cancer. Here, we outline our proposed strategies and therapeutic devices/materials and discuss their potential therapeutic effectiveness, promises and challenges in the clinical settings.


*Keywords:* Local drug delivery; Pancreatic cancer; Local recurrence; Cytostasic therapy; Cytocidal therapy

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Contents



## 1. Introduction

Surgical resection is the first and most effective therapeutic choice for pancreatic cancer that is localized without distant metastases. Although extended surgery with portal vein resection has been attempted, the survival rate for patients who have undergone resection for pancreatic cancer is still very low (8–25%) [1–4]. One reason is that pancreatic cancer cells, which easily invade and develop into the extrapancreatic nerve plexus and lymph vessels, remain in the pancreatic bed (the retroperitoneal space) even after curative resection, and subsequently induce local recurrence at a high incidence [5–8]. Intraoperative radiotherapy as an adjuvant treatment has been used to try to reduce local recurrence, but its effectiveness is still under debate [9–11]. When detected clinically, local recurrence is mostly difficult to treat, because it occurs at a deep area in the body and has already established a tumor mass, which has physiological and environmental resistance to chemotherapy and radiotherapy [12,13]. The most critical timing of treatment for the prevention of local recurrence must be immediately after resection during surgery, prior to tumor mass formation derived from remnant cancer cells.

Regarding the environment of remnant cancer cells after surgery, surgical wounds induce inflammation and regeneration of tissues with an increased level of cytokines that provides favorable conditions for tumor recurrence [14–17]. That is, cytokines, which are produced and exist in a tumor-resected tissue and in the intraperitoneal space due to surgical trauma, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), accelerate cancer cell proliferation, migration, invasion and tumor angiogenesis, resulting in recurrence and metastasis of cancer (Fig. 1). This is a well-accepted scenario of recurrence of pancreatic cancer.

To suppress the progress of cancer cells, there must be two main strategies; one is cytostatic therapy using a cytokine antagonist or an anti-cytokine antibody, and the other is cytocidal therapy using anticancer drugs or radiotherapy (Fig. 1) [18]. The goal of cytostatic therapy is to suppress cancer cell proliferation, migration, invasion and tumor angiogenesis, thus generating to a state of dormancy, while cytocidal therapy is aimed at a measurable reduction in tumor bulk. Regardless of cytostatic or cytocidal drugs, most of these drugs have been applied systemically via oral administration or intravenous injection. However, a complete cure has rarely been seen, and some severe adverse effects have been reported [19].

To achieve the markedly elevated pharmacological effect while minimizing systemic administration-associated toxic adverse effect, we have proposed and devised several trans-tissue and local delivery systems as shown below, all of which are processed to be tightly adhered or fixed on the resected tissues during surgery:

(1) System I: a drug-loaded photocured gelatinous tissue-adhesive gel (bioactive substance reser-

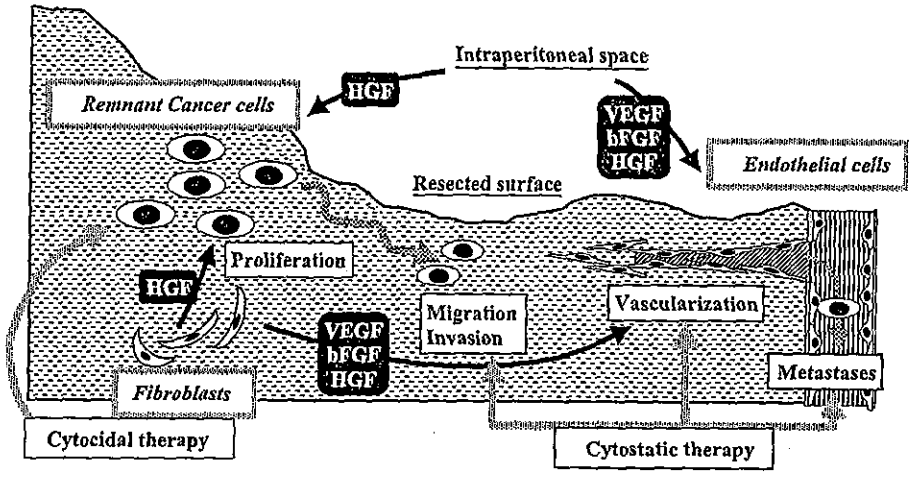


Fig. 1. Schematic of the progression of remnant cancer cells after surgery and relations with cytocidal and cytostatic therapies.

voir) that enables sustained release of a drug, protein, or gene-encoding adenovirus [20–22].

(2) System II: an anti-cytokine antibody-fixed photocured gelatinous, tissue-adhesive gel (cytokine barrier) that prevents cytokine permeation into the target tissue [23].

(3) System III: a gene-modified cell sheet that enables the sustained release of a very costly protein produced by gene-transduced cells [24].

(4) System IV: a percutaneous drug-delivery device that enables continuous drug infusion and easy detachment from the surgical site [25].

All the systems could be applicable for resected surfaces immediately after surgery (Fig. 2). The trans-tissue, local delivery systems devised have clear-cut advantages, including a high local concentration of drugs at the target tissue and a relatively low concentration at systemic organs, resulting in significant enhancement of the therapeutic effect of drugs and a marked reduction of systemic adverse effects.

In our studies [20–25], HGF antagonist, NK4, was used as a cytostatic drug. HGF plays an important role in tumor–stroma interaction and acts as a potent scattering factor by binding to c-Met

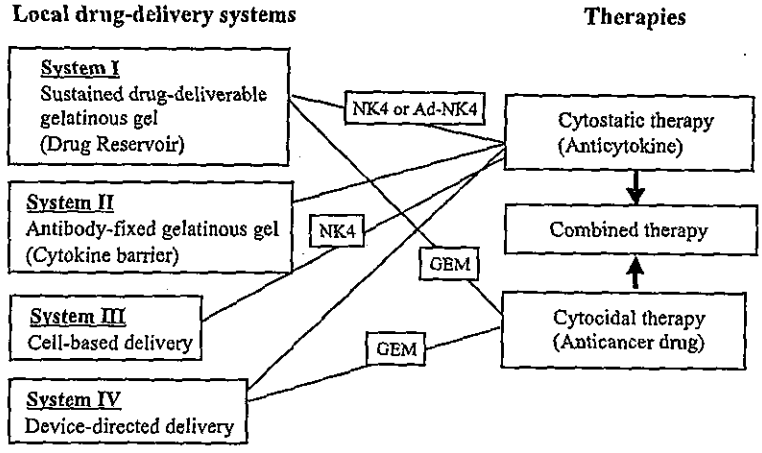


Fig. 2. Strategies for the prevention of local recurrence. NK4 is a cytostatic drug, which acts as a HGF antagonist and an angiogenic inhibitor. Gemcitabine (GEM) is a cytocidal drug, which acts as an antimetabolite [20–24].

receptor, which is frequently overexpressed in pancreatic cancers. NK4, composed of the N-terminal hairpin and four kringle domains of the α-subunit of HGF, binds to the c-Met receptor without tyrosine phosphorylation of c-Met, resulting in the inhibition of the mitogenic, motogenic and morphogenic activities of HGF [26–30]. Furthermore, NK4 acts as an angiogenic inhibitor, which inhibits the growth and migration of endothelial cells stimulated by VEGF, bFGF and HGF [31,32].

On the other hand, as a cytocidal drug, gemcitabine (2′ ,2′ -difluorodeoxycitidine, GEM) was used. GEM, which inhibits DNA synthesis by the inhibition of ribonucleotide reductase and by its incorporation into DNA [33,34], is currently the most effective chemotherapeutic drug for pancreatic cancer. Several investigators reported that the cytocidal efficacy of GEM, which is an antimetabolite, increases with the exposure time [35–38].

This review article is a summary of our several years of efforts and attempts to overcome the recurrence of pancreatic cancer, and we outline our proposed strategies and discuss their promises and challenges in the clinical setting.

## 2. System I: drug-loaded gelatinous gel [20–22]

This system was developed to provide an in situ-formed locally deliverable gelatinous matrix that could adhere to the resected tissues, from which bioactive substances including drugs, proteins or gene-encoding adenoviruses can be sustainably released into the target tissue. To meet the desired performance in this particular application, the system should have the following characteristic features: (1) rapid sol-to-gel transformation by visible light photoirradiation, (2) strong tissue adhesivity, (3) ease of drug immobilization, (4) biodegradability and biosorption (5) minimal or very mild informatory reaction. To this end, photopolymerizable gelatin, which is partially derivatized with styrene groups in gelatin molecule (ST-gelatin; Fig. 3A), was used as an in situ gelable drug-loadable matrix. ST-gelatin was originally prepared at our laboratory as a visible light-induced photocurable tissue-adhesive glue, which prevented bleeding from arteries, indicating that such a glue inherently has a high tissue adhesivity, causes minimal inflammatory reaction and exhibits little sign of toxicity [39].

A viscous buffer solution composed of ST-gelatin, water-soluble carboxylated camphorquinone (visible light-induced radical generator used in clinical dental applications for many years), and the drug of interest was coated on resected tissues and photoirradiated with a visible light lamp (used in clinical dental applications), resulting in gel formation within 3 min of irradiation (Fig. 3B). The viscosity, in situ gelation time, drug-release rate, adhesive strength, and biodegradability were easily controlled by material (degree of derivatization of styrene groups in a gelatin molecule), formulation (concentrations of ST-gelatin and camphorquinone), and operation (photointensity and irradiation time) variables [20]. The general trend is that higher concentration of ST-gelatin, longer

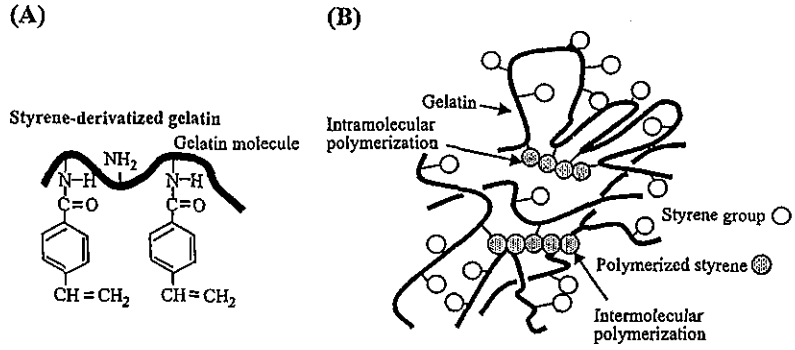


Fig. 3. Drug-loadable photocurable tissue-adhesive matrix. (A) Chemical structure of styrene-derivatized gelatin. (B) Photogelation by formation of cross-linked gelatin networks by the inter- and intra-molecular polymerizations of styrene groups in gelatin molecules.

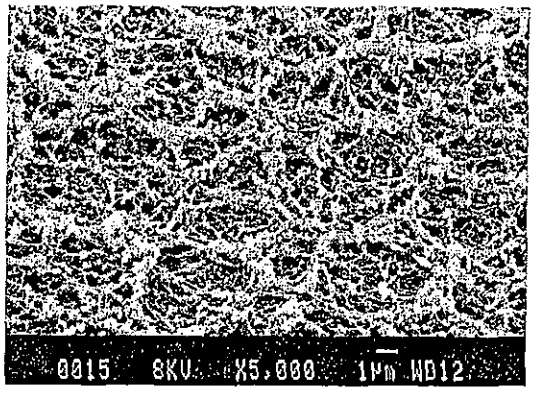


Fig. 4. Scanning electron micrograph of the beads-necklace type-surface of photocured ST-gelatin gel. Gelatin concentration: 30 wt.%. Bar: 1 μm [20].

photoirradiation time or higher photointensity produces a less water-swellable gel.

The formed gel consisted of supramolecular organization of polymerized gelatin with polymorphous nanostructures, which depends on the concentration of ST-gelatin and the degree of derivatization of styrene group in a gelatin molecule. The typical beaded necklace-type mesh appearance is shown in Fig. 4. The polymorphous nanostructured meshes or sheets of polymerized ST-gelatin are shown in Fig. 5. The general observation is that at low concentration of ST-gelatin, nanoparticles (diameter: approximately 100 nm) are adhered for each other to form continuous bead network with ample open-cell microvoids or channels (Fig. 5A1). Increased concentration of ST-gelatin produced beaded necklace-type mesh with a lesser degree of microvoids (Fig. 5A2). At high concentration, beads or necklace were fused to form a microporous continuous dense sheet (Fig. 5A3). In addition to the concentration of ST-gelatin, this tendency was enhanced with a lower degree of styrene derivatization (Fig. 5B). Such morphological features correlated well with the swellability of gel in water: Lower microvoid, lesser swellability. This in turn means that morphological features eventually determine the releasing profile of drug immobilized in a gel as shown below.

The typical time-dependent release profile of protein is a fast release in an early period, followed by reduced releasing characteristics (Fig. 6). The releasing rate of a proteinaceous drug was enhanced with a higher degree of swelling in water and with lower degree of derivatization of styrene in gelatin. The diffusion constants, calculated using Fick's second law, largely depended on the swellability of gels, which depends on the concentration of ST-gelatin (Fig. 7). The releasing period can be extended

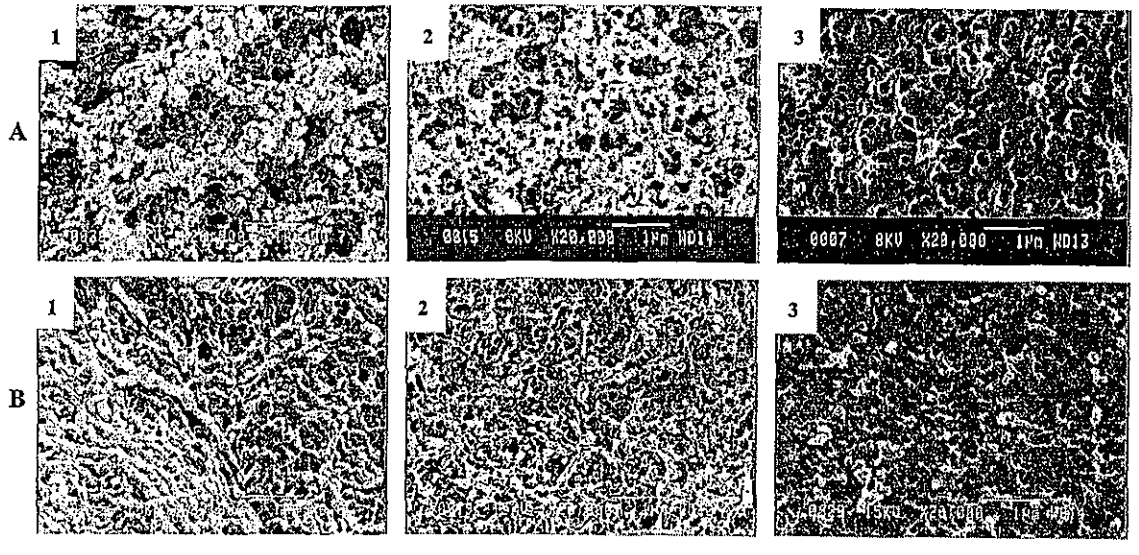


Fig. 5. Scanning electron micrographs of polymorphorous surfaces of photocured gelatins with variable concentration and variable styrene content in a gelatin molecule. Stryene content per molecule; 28.5 (A) and 22.4 (B). ST-gelatin content; 10 wt.% (1), 20 wt.% (2) and 40 wt.% (3). Bar: 1 μm.

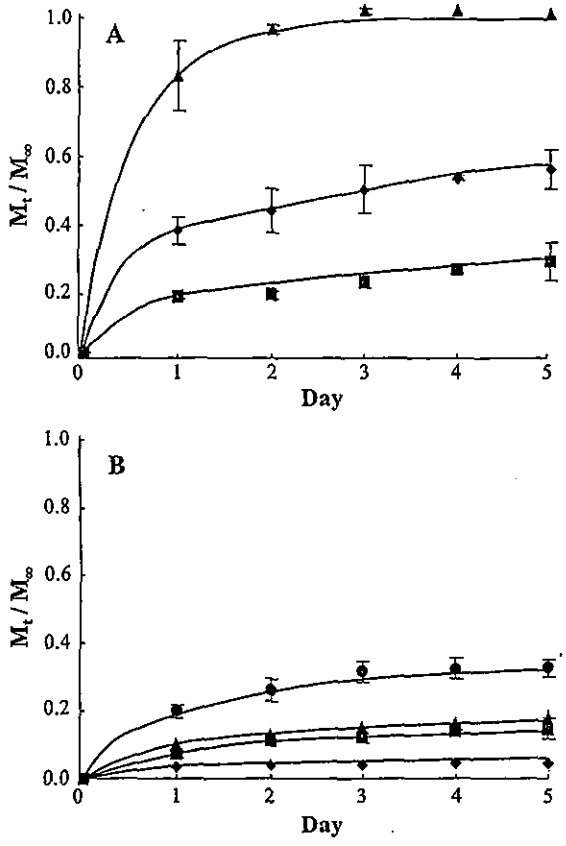


Fig. 6. Fraction release of rhodamine-albumin (r-Alb) as a function of time. Gel containing 10 wt.% (●), 20 wt.% (▲), 30 wt.% (■) and 50 wt.% (♦) of ST-gelatin of different contents of styrene per molecule (4.0 for A and 17.7 for B).

from days to several weeks, depending on the degree of photocuring or swellability of a formed gel. Fig. 8 shows the schematic of process of photogel formation and subsequent release of bioactive substances (A: drug, B: gene-encoding adenovirus) and experimental gel formation model using resected liver tissue (C). The followings are examples in System I loaded with bioactive substances.

### 2.1. NK4 [20]

The strategy for NK4 protein (MW; 64 000 g/mol) delivery is that an NK4-mixed ST-gelatin solution is photogelled on a target tissue where remnant cancer cells might remain, and NK4 released from the gel permeates gradually into the tissue, resulting in the suppression of cancer progression (Fig. 8A). The in vitro release profile of rhodamine-conjugated albumin (r-Alb, MW; 66 000 g/mol, used as a model protein of NK4) from ST-gelatin gel was characterized by an initial burst and subsequent gradual release over a prolonged period. In an in vivo experiment, 3 days after an r-Alb-loaded ST-gelatin solution was photogelled on the liver of a Wistar rat (Fig. 8C), r-Alb released from the gel remained in the liver from the surface to the deeper portions of the liver tissue. When NK4 is commercially manufactured at a low price, the prospective use of this system is very high. However, at present, NK4 is available only for experimental use in limited amounts so that it is doubtful that this system will be used in clinical situations in the near future.

### 2.2. NK4 gene-encoding adenovirus (Ad-NK4) [21]

Ad-NK4 is released from a photocured gelatinous gel formed on the tissue, and gradually permeates into the tissue, followed by transduction into various types of cells in the target tissue. Consequently, transduced cells produce NK4 (Fig. 8B). Although it is anticipated that the sustained release of an adenovirus might overcome the transient gene expression following adenoviral transduction, the amount of in vivo gene expression was substantially lower than that following simple injection of Ad-lacZ solution (Fig. 9A), probably because the adenoviral vector particles are too large to permeate the tissue via passive diffusion from the gel. In fact, our extensive study of gene transduction in rat's liver and muscle tissues showed

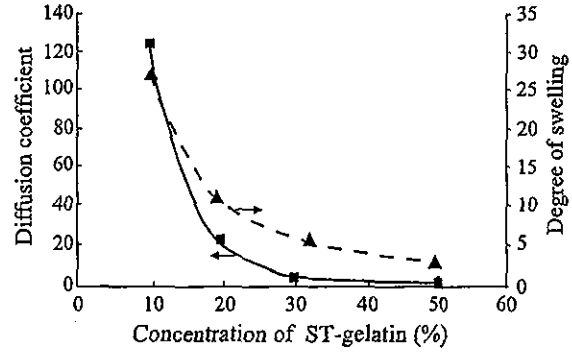


Fig. 7. Dependence of diffusion coefficient (■) and water swellability (▲) on concentration of ST-gelatin. Diffusion coefficient was obtained from the slope of the linear relation between $M_t/M_\infty$ and $t^{1/2}$.

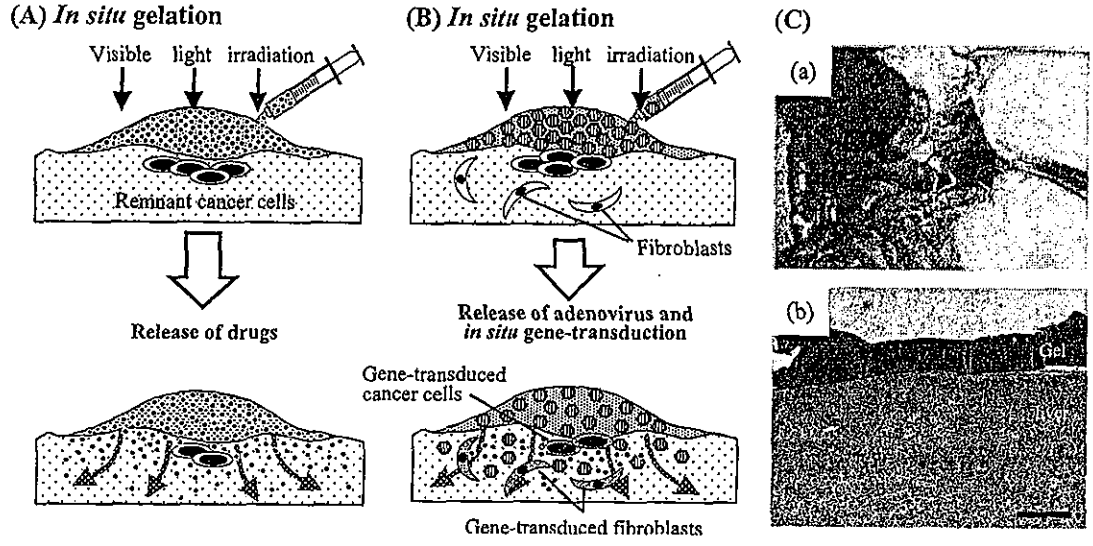


Fig. 8. Schematic of sustained drug-deliverable gelatinous gel (System I). (A) Local delivery of NK4 or Gemcitabine using photocurable gelatin. NK4- or Gemcitabine-loaded photocurable gelatin is coated on a tumor bed, and subsequently in situ photogelled under visible light irradiation. NK4 or Gemcitabine is released from the gel, and permeates into the tumor bed. (B) Local delivery of Ad-NK4 using photocurable gelatin. Ad-NK4-loaded photocurable gelatin is administered on a tumor bed, and subsequently in situ photogelled under visible light irradiation. Ad-NK4 is released from the gel, permeates into the tumor bed and transduces various cells around the tumor bed. Gene-transduced cells produce NK4. Irrespective of models, the inhibition of the progression of remnant cancer cells is expected. (C) In situ production of photocurable gelatin gel. (a) Procedure of visible-light irradiation of the aqueous gelatin solution on the liver surface. Gel formed on the liver surface. (b) Cross-sectional specimen (hematoxylin-and-eosin staining). Bar: 500 μm [20–22].

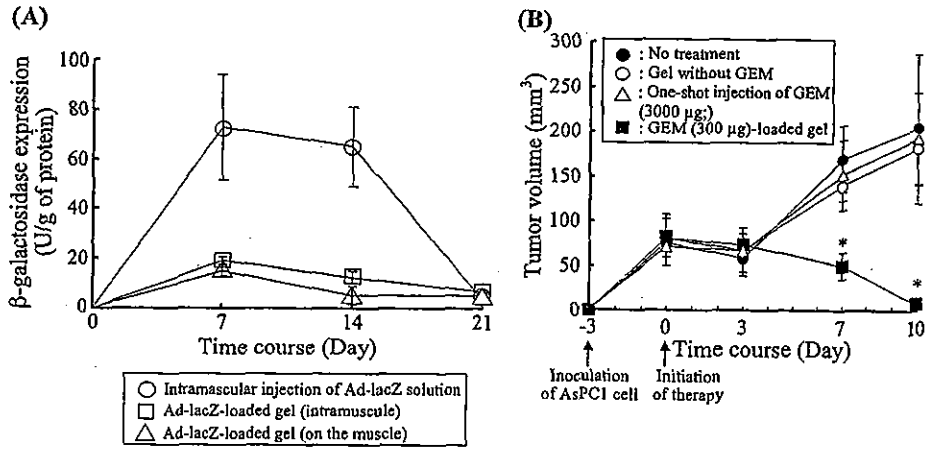


Fig. 9. (A) System I loaded with gene-encoding adenovirus. Time course of in vivo β-galactosidase expression in rat femoral muscle using Ad-lacZ-loaded gelatinous gel: One-shot intramuscular injection of Ad-lacZ solution ($1.25\times10^8$ PFU/500 μl, ○), intramuscular injection of Ad-lacZ-loaded gel ($1.25\times10^8$ PFU/500 μl, □), and covering on the muscle of Ad-lacZ-loaded gel ($1.25\times10^8$ PFU/500 μl, △). The expression with the gel was lower than that of one-shot injection. Values are shown as means±S.D. (three mice/each group). (B) System I loaded with GEM. Effect of GEM-loaded photocured gelatinous gel on tumor growth of pancreatic cell line AsPC-1: no treatment (●), gel without GEM (○), one-shot injection of GEM (3000 μg; △), GEM (300 μg)-loaded gel (■). Values are shown as means±S.D. (10 mice/each group). The tumor volume with GEM-loaded gel was compared with that with the others (*$P<0.01$) [22].