Telomerase Activity in Pancreatic Juice Differentiates Ductal Carcinoma from Adenoma and Pancreatitis

Nobuhiro Suehara, Kazuhiro Mizumoto, Masao Tanaka, Hideaki Niiyama, Kazunori Yokohata, Yohei Tominaga, Hideo Shimura, Tsuyoshi Muta, and Naotaka Hamasaki

ABSTRACT
Telomerase activity was measured in pancreatic juice obtained by endoscopic retrograde pancreatectography from 34 patients (12 with ductal carcinoma, 12 with pancreatic adenoma, and 10 with pancreatitis). The activity in pancreatic juice was expressed as the number of cells of a human pancreatic cancer cell line, MIA PaCa-2, that exhibit an activity equal to that expressed in 1 μg of protein from pancreatic juice. A telomerase ladder was detected in the pancreatic juice obtained from a majority of the patients with ductal adenocarcinoma. The median value of relative telomerase activity in the carcinoma samples was 9.38 (25th percentile, 3.14; 75th percentile, 95.8), a value significantly higher than that derived from patients with either pancreatitis or pancreatic adenoma (P < 0.0001). When a threshold value of relative telomerase activity of 3.00 was used, 75% (9 of 12) of the samples obtained from patients with ductal carcinoma were positive. We conclude that telomerase activity in pancreatic juice differentiates adenocarcinoma from adenoma and pancreatitis and may serve as a useful diagnostic tool.

INTRODUCTION
Telomerase is a ribonucleoprotein enzyme that is responsible for cell immortality (1) and is known to be activated in germ-line cells (2) and in a variety of cancers (3–5). Using a modified sensitive PCR-based semiquantitative assay, we previously detected telomerase activity in 80% of a series of surgically resected human pancreatic ductal carcinomas (6, 7). Telomerase activity is not detectable in the normal pancreas or in those harboring pancreatitis. Although a few adenomas produced a weak telomerase ladder, the telomerase activity in pancreatic ductal carcinomas was much higher than that encountered in the adenomas. Another group also reported similar results concerning telomerase activity in pancreatic tissues (8).

Most pancreatic cancers are adenocarcinomas that originate from the epithelium of the pancreatic ducts; malignant cells can be found in the pancreatic juice. However, conventional cytological examination of pancreatic juice is not very efficient for the diagnosis of pancreatic cancer (9, 10). In attempts to detect pancreatic carcinomas at an early stage, we have measured the telomerase activity present in cells isolated from pancreatic juice obtained by ERP (11) from patients with pancreatitis, benign adenoma, or ductal carcinoma. Telomerase activity was detected exclusively in the pancreatic juice from patients with pancreatic ductal carcinoma.

MATERIALS AND METHODS

Sampling of Pancreatic Juice. Pancreatic juice was obtained by ERP at either the Kyushu University Hospital or its affiliated hospitals from 34 patients (12 with pancreatic ductal cancer, 12 with pancreatic adenoma, and 10 with chronic pancreatitis). The diagnoses were confirmed histologically after surgical resection in 9 of the patients with pancreatic carcinoma, in all 12 of the patients with adenoma, and in 2 of the 10 patients with chronic pancreatitis. In three patients, the pancreatic carcinoma could not be resected due to the presence of advanced lesions, liver metastases, or portal vein invasion. In eight patients with chronic pancreatitis, the diagnosis was made by clinical observation for over 6 months in an outpatient clinic. This study was conducted according to the Helsinki Declaration.

In all patients a 350-cm #6 French balloon catheter ( wedge pressure catheter JX-283; Arrow International, Inc., Reading, PA: Refs. 11 and 12) was inserted endoscopically into the pancreatic duct, and pancreatectography was performed. The endoscope was withdrawn over the catheter, and the patients were allowed to lie on their backs. Immediately after an i.v. injection of 1 unit/kg body weight of secretin (Eisai, Tokyo, Japan), pancreatic juice was collected for about 15 min through the balloon catheter. Three additional samples of pancreatic juice were collected from each patient at 5-min intervals. The first sample was discarded because of contamination of the pancreatic juice with the contrast medium (sodium diatrizoate). The second sample was used for cytolological examination. The third sample was used for a telomerase assay. The brushing technique described by Venu et al. (13) was used to collect cells for the cytological study and for the telomerase assay in some patients with a stenotic pancreatic duct.
Telomerase Activity in Pancreatic Juice

Epithelial cells were present, the samples were regarded as previously (6). The cell pellets were resuspended in CHAPS lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 2 mM EGTA, 0.1 mM 4-(2-aminophenyl)-benzenesulfonylfluoride hydrochloride, 5 mM 8-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and 1 µg/ml each of the protease inhibitors antipain, leupeptin, phosphoramidon, elastatinal, pepstatin A, and chymostatin [Peptide Institute, Osaka, Japan]) and then incubated at 20°C for 30 min on ice. The protein concentration of the extract was measured by Bradford assay (14), and a 0.6- or 6-µg extract was used for each telomerase assay. The CHAPS cell extracts were incubated at 20°C with 20 mM Tris-HCl (pH 8.3), 1 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 µM deoxynucleotide triphosphates, 0.3 µCi of [α-32P]dCTP, 2 units of Taq DNA polymerase (Promega, Madison, WI), and 0.1 µg of DNA polymerase (Promega, Madison, WI).

### Table 1 Relative values of telomerase activity in pancreatic juice obtained from patients with pancreatitis, adenomas, and carcinomas

<table>
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<tr>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>Age/sex</th>
<th>Staging*</th>
<th>Location</th>
<th>Pf¹</th>
<th>Tissue</th>
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<td>58/M</td>
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</tr>
</tbody>
</table>

### Adenomas

- Serous cystadenoma: 53/F, Ph, 0.87, 0.58, Class 1
- Mucinous cystadenoma: 54/M, Pb, 4.60, 0.56, Class 2
- Mucinous cystadenoma: 75/F, Pb, 0.49, 0.44, Class 2
- Mucinous cystadenoma: 60/F, Ph, 0.22, 0.68, Class 1
- Mucinous cystadenoma: 54/M, Pb, 0.13, NE, Class 2

### Carcinomas

- Intraductal papillary adenocarcinoma: 72/F, T₈, N₅, M₀, Ph, 14.1, 2750, Class 2
- Intraductal papillary adenocarcinoma: 59/M, T₂, N₅, M₀, Pt, 4.66, NE, Class 5
- Papillary adenocarcinoma: 80/M, T₂, N₅, M₀, Ph, 118, NE, Class 2
- Tubular adenocarcinoma: 50/M, T₈, N₅, M₀, Pb⁻¹, 1.21, 8.93, Class 1
- Tubular adenocarcinoma: 55/M, T₈, N₅, M₀, Ph, 18900, NE, Class 3
- Tubular adenocarcinoma: 72/F, T₈, N₅, M₀, Ph, 3.99, 57.5, Class 2
- Tubular adenocarcinoma: 40/M, T₂, N₅, M₀, Pb⁻¹, 2030, 2.35, Class 2
- Tubular adenocarcinoma: 74/F, T₂, N₅, M₀, Pb⁻¹, 3.58, 970, Class 2
- Tubular adenocarcinoma: 69/M, T₂, N₅, M₀, Ph, 1.03, 1.98, Class 1
- Carcinoma with liver metastasis: 67/F, T₈, N₅, M₀, Ph, 28.9, NE, Class 1
- Carcinoma with portal vein invasion: 77/M, T₈, N₅, M₀, Pb⁻¹, 2.99, NE, Class 1
- Carcinoma with liver metastasis: 59/F, T₈, N₅, M₀, Pb⁻¹, 29.3, NE, Class 1

The cytology (second) sample was immediately centrifuged at 400–700 × g for 5 min, and smears were submitted to Papanicolaou staining after fixation in 95% ethanol. If columnar epithelial cells were present, the samples were regarded as appropriate for the telomerase assay. The third sample was centrifuged at 1000 × g for 5 min at 4°C, and then stored at −80°C until assayed for telomerase.

### Assay of Telomerase Activity

Telomerase activity in the cell pellets of pancreatic juice was analyzed as described previously (6). The cell pellets were resuspended in CHAPS lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 2 mM EGTA, 0.1 mM 4-(2-aminophenyl)-benzenesulfonylfluoride hydrochloride, 5 mM 8-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and 1 µg/ml each of the protease inhibitors antipain, leupeptin, phosphoramidon, elastatinal, pepstatin A, and chymostatin [Peptide Institute, Osaka, Japan]) and then incubated for 30 min on ice. The protein concentration of the extract was measured by Bradford assay (14), and a 0.6- or 6-µg extract was used for each telomerase assay. The CHAPS cell extracts were incubated at 20°C with 20 mM Tris-HCl (pH 8.3), 1 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 µM deoxynucleotide triphosphates, 0.3 µCi of [α-32P]dCTP, 2 units of Taq DNA polymerase (Promega, Madison, WI), and 0.1 µg of DNA polymerase (Promega, Madison, WI).
Telomerase activity signals in pancreatic juice samples from patients with pancreatic ductal carcinoma, pancreatic adenoma, and pancreatitis. An aliquot of extract containing 6 μg of protein with (+) or without (−) RNase pretreatment (incubation with 1 μg RNase/assay for 30 min at 37°C) was used for each assay. Aliquots of extracts containing 1, 10, 10², and 10³ cells of a human pancreatic cancer cell line, MIA PaCa-2, having telomerase activity were used as a standard. Lysis buffer was used as a negative control. A 36-bp internal standard was used as an internal control. Telomerase activity was detected as a six-nucleotide repeat ladder after electrophoresis of the enzyme reaction products and autoradiography. Telomerase ladders were detected in the pancreatic juice from patients with carcinomas. A telomerase ladder was not detected in the pancreatic juice from patients with adenomas or pancreatitis, except in one patient with adenoma.

TS primer (5'-AATCCGTCAGCAGAGTT-3'). The samples were then heated at 90°C for 3 min to inactivate the telomerase enzyme. After this last step, 0.1 μg of CX primer (5'-CCCTTACCCCTTACCCTAA-3') was added, and the reaction mixture was subjected to 31 PCR cycles at 94°C for 30 s, at 50°C for 30 s, and at 72°C for 90 s (2 min for the final step). Half of the resultant PCR products (25 μl) were analyzed by electrophoresis in 0.5× Tris-borate EDTA buffer on 12% polyacrylamide nondenaturing gels. Telomerase activity was detected as an incremental six-nucleotide single-strand DNA ladder signal that was sensitive to RNase (Boehringer Mannheim, Mannheim, Germany). Measurement of telomerase activities in the human pancreatic cancer cell line MIA PaCa-2, provided by the Japanese Cancer Resources Bank (Tokyo, Japan), was always used as a standard in each assay. The intensity of the ladder was reduced in relation to the decrease in the number of cancer cells produced by dilution. Regression analysis was performed between logarithmic values of the cell number and telomerase intensity; the correlation coefficient always exceeded 0.9. The activity in pancreatic juice was expressed as the number of cancer cells that exhibit an activity equal to that expressed in 1 μg of protein from pancreatic juice. A 36-bp internal standard (Oncor Inc., Gaithersburg, MD) was used as an internal control. Signal intensity of all of the bands of the telomerase ladder was measured by NIH image, version 1.59 (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov). Measurement of Telomerase in Surgically Resected Tissues. The telomerase activity in specimens of pancreatitis, adenoma, and carcinoma obtained operatively was analyzed semiquantitatively as reported previously (6) and compared with that in pancreatic juice. Statistical Analysis. Because the values of relative telomerase activity in pancreatic juice after logarithmic transformation did not show a standard normal distribution, they were statistically analyzed using the Kruskal-Wallis test. The correlation between the values of relative telomerase activities in tissue and those in pancreatic juice was analyzed using Spearman’s rank correlation test.

RESULTS

To determine whether the digestive enzymes in pancreatic juice influence the telomerase assay, MIA PaCa-2 pancreatic carcinoma cells were first incubated in human pancreatic juice, from which cells were removed by centrifugation for 15 min at 37°C and then assayed semiquantitatively for telomerase activ-
Telomerase Activity in Pancreatic Juice

Fig. 2 Relative values of telomerase activity in pancreatic juice. Relative values of telomerase activity in pancreatic juice are expressed as the number of cells of a human pancreatic cancer cell line, MIA PaCa-2, that exhibit an activity equal to that expressed in 1 μg of protein from pancreatic juice, presented by a box-and-whisker plot analysis. The bottom and top edges of the box mark the 25th and the 75th percentiles. The center horizontal line is drawn at the sample median. The center vertical lines drawn from the boxes extend to the 10th or the 90th percentiles.

Fig. 3 Correlation between the relative telomerase activities of pancreatic tissue and pancreatic juice. Symbols represent carcinoma (■), adenoma (○), and pancreatitis (▲), respectively. Relative telomerase activities in pancreatic juice were well correlated with those in pancreatic tissue samples, and Spearman’s rank correlation was 0.728 (P = 0.0009).

The signal intensity of the telomerase ladder was not affected by incubation in pancreatic juice (data not shown).

Table 1 details the telomerase activity of the cells in pancreatic juice obtained from each of the patients in the study. Table 1 also gives the clinical profile of each patient. A telomerase ladder was detectable in most of the pancreatic juice samples obtained from the 12 patients with carcinoma (Fig. 1). Relative values of telomerase activity in carcinoma samples varied from 1.03 to 18,900, whereas the median value (25th percentile, 75th percentile) of the telomerase activities of carcinomas was 9.38 (3.14, 95.8). There was no significant difference between the locations of the carcinoma, that is, pancreatic head versus body and tail.

A telomerase ladder was very weak or not detected at all in the cell pellets of pancreatic juice obtained from patients with either pancreatic adenoma or pancreatitis (Fig. 1). The median (25th percentile, 75th percentile) telomerase activity in patients with either adenoma or pancreatitis was 0.49 (0.27, 0.84) and 0.42 (0.32, 0.65), respectively. Interestingly, the patient with an activity greater than 1.00 (patient 62) was diagnosed as having a mucinous cystadenoma with severe atypia. Fig. 2 shows that the relative telomerase activity in patients with pancreatic carcinoma was significantly higher than it was in those with adenoma or pancreatitis (P < 0.0001).

The relative telomerase activity in pancreatic juice correlated closely with that in the resected specimen obtained from each respective patient [Spearman’s rank correlation was 0.728 (P = 0.0009)], although the telomerase activity in pancreatic juice tended to be lower than that in the tissue (Fig. 3).

DISCUSSION

The present study documents that telomerase activity in pancreatic juice reflects the activity of the enzyme in tissue specimens. The data presented also document that telomerase activity in pancreatic juice can differentiate between the presence of pancreatic ductal carcinoma and either pancreatic adenoma or pancreatitis. Detection of telomerase activity in pancreatic juice may prove to be a potent tool for the diagnosis of pancreatic ductal carcinoma.

When the threshold of relative telomerase activity was set at 3.00, 75% (9 of 12) of the samples obtained from patients with pancreatic carcinoma were positive. Such a high positivity rate was obtained in the present study because, by using a PCR-based assay, very few cells were needed to detect telomerase activity. It is important to note that a telomerase ladder was not observed if epithelial cells were not present on cytological examination of pancreatic juice. Preliminary study failed to show telomerase activity in pancreatic juice obtained by conventional ERP without the use of a balloon catheter from the patients with ductal adenocarcinoma (data not shown). Thus, the collection of pancreatic juice using an indwelling balloon catheter in the pancreatic duct seems to be essential for obtaining an adequate number of duct epithelial cells for the telomerase assay.

Telomerase assay may allow the detection of even early pancreatic ductal carcinomas. In the present study, high telomerase activity (14.1 equivalent cells/μg protein) was observed in a patient (Fig. 1, number 5) that had a T1aN0M0 pancreatic cancer according to the tumor-node-metastasis classification system (15). The pancreatic cancer in this patient was only 5 mm in diameter. In this case, ERP revealed a localized, minimally stenotic segment in the main pancreatic duct, and samples collected at the stenosis by the brushing technique showed a clear telomerase ladder.

The telomerase activities in pancreatic juice from the patients with pancreatic ductal carcinomas were lower than those in the resected tissues. It is possible that the various digestive enzymes in pancreatic juice might disturb the telomerase assay by inhibiting Taq polymerase (16). However, incubation of a human pancreatic cancer cell line in pancreatic juice before the assay did not affect the telomerase activity. The CHAPS lysis
buffer contains abundant protease inhibitors that may minimize any effect of the digestive enzymes on the assay.

With one exception, a telomerase ladder was not detected in the pancreatic juice of patients with pancreatic adenoma. The tissue sample of the patient with telomerase activity in pancreatic juice was diagnosed histologically as mucinous cystadenoma with severe atypia. Interestingly, weak telomerase activity was detected in resected pancreatic adenomas; however, all of the relative values were less than 1.00 in these adenomas (7). Potentially malignant mucinous cystadenomas may contain immortalized cells. Nevertheless, the significance of the high telomerase activity in this patient with a mucinous cystadenoma must await further follow-up. In addition, there must be further study of telomerase activity in the pancreatic juice and tissues of more patients with adenomas.

Telomerase ladders were not detected in the pancreatic juice obtained from patients with pancreatitis. The absence of telomerase activity in pancreatitis was confirmed by analysis of the resected tissues (7). Differentiation of pancreatic ductal carcinoma from pancreatitis is clinically important, because some cases of chronic pancreatitis show pancreatographic findings similar to those in duc tal carcinoma. Before the present study, it was suspected that it might be possible to detect weak telomerase activity in pancreatic juice from patients with pancreatitis, because Piatyszek et al. (16) observed that peripheral blood mononuclear cells exhibit low levels of telomerase activity. Mononuclear cells may accumulate in the pancreatic juice of patients with chronic pancreatitis. In fact, up to $10^3$ mononuclear cells were detected cytologically in the pancreatic juice from all of the patients with chronic pancreatitis. However, no convincing telomerase activity could be detected in these patients, suggesting that the presence of mononuclear cells in pancreatic juice will not produce a false positive result.

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REFERENCES


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