Quantitative Assessment of Telomerase Activity and Human Telomerase Reverse Transcriptase Messenger RNA Levels in Pancreatic Juice Samples for the Diagnosis of Pancreatic Cancer

Kenoki Ohuchida, Kazuhiro Mizumoto, Yasuhiro Ogura, Nami Ishikawa, Eishi Nagai, Koji Yamaguchi, and Masao Tanaka
Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ABSTRACT
Measurement of telomerase activity is a promising diagnostic tool for pancreatic cancer. Detection of mRNA for human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is also a diagnostic candidate. In the present study, we developed a telomeric repeat amplification protocol assay with real-time PCR and a protocol for quantification of hTERT mRNA with real-time PCR. To evaluate the feasibility of these methods for diagnosis of pancreatic cancer, we measured telomerase activity and hTERT expression in pancreatic cancer cell lines, pancreatic tissues, and pancreatic juice samples from patients with different pancreatic diseases. There were significant correlations between telomerase activity and hTERT expression in cell lines, tissues, and juice samples. The levels of telomerase activity and hTERT expression were significantly higher in tumoral tissues than in nontumoral tissues. In pancreatic juice specimens, some carcinoma samples showed remarkably high expression of hTERT. However, there were no significant differences in hTERT expression between patients with carcinoma and those with benign diseases, although significant differences in telomerase activity were observed. Our present results suggest that the combined assessment of hTERT and telomerase activities in pancreatic juice provides a potent diagnostic method for pancreatic cancer.

INTRODUCTION
Pancreatic cancer is the fifth leading cause of cancer death and has the lowest survival rate of any solid cancer (1, 2). The vast majority of patients with pancreatic cancer experience a poor clinical course because of the limited efficiency of conventional therapeutic approaches, including surgical resection, chemotherapy, and radiotherapy (3). Early diagnosis is crucial if the prognosis is to be improved. However, this remains difficult due to the inaccessibility of the pancreas and the highly malignant nature of the disease despite improvements in diagnostic imaging. Thus, novel biomarkers and detection techniques are urgently needed.

Telomerase activity is a promising diagnostic marker for pancreatic cancer (4, 5). We and other investigators have reported that detection of telomerase activity in pancreatic juice is useful in the diagnosis of pancreatic cancer (6–8). In these studies, a modified PCR-based semiquantitative assay [conventional telomeric repeat amplification protocol (TRAP) assay] with radioisotopes was used for analysis of telomerase activity. However, this conventional TRAP assay is not suitable for clinical application because of its complex and time-consuming nature.

Some investigators have reported that detection or quantification of human telomerase reverse transcriptase (hTERT) is useful in cancer diagnosis and that it eliminates the need for complicated procedures (9–12). However, quantification of hTERT in pancreatic juice has not been reported, possibly due to the very small amount of RNA that is extracted with this method.

In the present study, we quantitatively measured expression of hTERT mRNA in pancreatic juice by real-time PCR and developed a new TRAP assay with real-time PCR (real-time TRAP) to measure telomerase activity in clinical samples rapidly and simply. This method was 1,000 times more sensitive than conventional TRAP assay with SYBR Green. hTERT expression and telomerase activity were significantly increased in pancreatic cancer and were well correlated in both tissue samples and pancreatic juice. The combined assessment of hTERT expression and telomerase activity in pancreatic juice is a potent method for diagnosis of pancreatic cancer.

MATERIALS AND METHODS
The following pancreatic carcinoma cell lines were used: ASPC-1, BxPC-3, KP-1N, KP-2, KP-3, Panc-1, Suit-2 (from Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan), MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan), NOR-P1 (established in our laboratory), Capan-1, Capan-2, CFPAC-1, H48N, HS766T, and SW1990 (American Type Culture Collection, Manassas, VA). Normal human fibroblasts (MRC5, RIKEN Cell Bank, Tsukuba, Japan) were used as a normal control. Cells were maintained in DMEM (Sigma Chemical Co., St. Louis, MA) supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin.
(100 units/mL) at 37°C in a humidified atmosphere with 10% CO₂. Tissue samples were obtained at the time of surgery at Kyushu University Hospital (Fukuoka, Japan). Twenty-nine tumoral tissue samples were obtained from cancerous lesions of resected pancreas with primary pancreatic ductal carcinoma, and 29 normal tissue samples were taken from peripheral soft tissue away from the tumor. The tissue samples were removed as soon as possible after resection and stored at −80°C until use. Tissue adjacent to the specimen was examined histologically and the diagnosis was confirmed.

Pancreatic juice samples were collected from a series of 86 patients who underwent endoscopic retrograde cholangiopancreatography for suspected malignancy of the pancreas at Kyushu University Hospital between February 15, 2001 and January 23, 2004 (6). For each patient, a balloon catheter was inserted by endoscopy into the pancreatic duct. After pancreatography and i.v. injection of secretin, at least three pancreatic juice samples were collected serially through the catheter at 5-minute intervals. The first sample was discarded because of possible contamination with contrast medium. The second sample was subjected to cytologic examination by an experienced cytologist according to standard criteria. The remaining samples were centrifuged at 2,000 × g for 5 minutes at 4°C. The cell pellets were washed thrice in ice-cold PBS at 2,000 × g for 5 minutes at 4°C and then stored at −80°C until use.

Diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens or the presence of liver metastasis. The diagnosis of intraductal papillary mucinous neoplasm (IPMN), pancreatitis, or cholangiothiasis was made on the basis of histologic examination of resected specimens or clinical observation with conventional diagnostic imaging for at least 6 months. Written informed consent was obtained from all patients, and the study was conducted according to the Helsinki Declaration.

**Telomeric Repeat Amplification Protocol Assay with Real-time PCR.** Cell pellets from cells lines or pancreatic juice samples were suspended in 3-[3-cholamidopropyl]dimethylamino-1-propanesulfonate (CHAPS) lysis buffer. Frozen pancreatic tissue samples were washed once in ice-cold PBS and homogenized in CHAPS lysis buffer with 1 μg/mL each of inhibitors, antipain, leupeptin, phosphoramidon, elastatin, pepstatin A, and chymostatin (Peptide Institute, Osaka, Japan) and 0.5 units/mL RNase inhibitor (Wako Pure Chemical Co., Osaka, Japan) and then incubated for 30 minutes on ice. Protein concentrations were measured by Bradford assay. The presence of PCR inhibitors is an inherent problem in TRAP analysis. Recently, we (13) and other investigators (14, 15) reported that problems due to PCR inhibitors could be avoided by using a small amount of protein extract (less than 1 μg) in this highly sensitive telomerase activity assay. Therefore, 0.6 μg of protein extract from tissue and pancreatic juice samples and 0.02 μg of protein extract from cell line pellets were used for each real-time TRAP assay. Real-time TRAP was done in a LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany). The total volume of the reaction mixture was 20 μL and contained 2 μL of 10× LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 2 mmol/L MgCl₂, 5 pmol TS primers (5’-AATCCGTC-GAGCAGAGTT-3’), 1.25 pmol ACX primer (5’-GCCG-GG(CTTACG)CTAACCC-3’; ref. 16), and 2 μL of protein extract. The reaction mixture was first incubated at 25°C for 20 minutes to allow the telomerase in the protein extracts to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then initiated at 95°C for 10 minutes to activate the modified Taq polymerase, followed by 40 cycles of 95°C for 10 seconds, 50°C for 5 seconds, and 72°C for 4 seconds and one cycle of 55°C for 15 seconds and 0.1°C/s to 95°C. SYBR Green bound to the new amplicons and generated fluorescent signals that were collected and analyzed with Detector software (LC Run Version 3.39, Roche Diagnostics) during the late extension step of each cycle. All samples were run in triplicate, and lysis buffer was used as a negative control. Measurement of telomerase activity in the human pancreatic cancer cell line MIA PaCa-2 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.

**Quantitative Assessment of Human Telomerase Reverse Transcriptase Levels by Real-time PCR.** hTERT mRNA levels were quantified by real-time, one-step reverse transcription-PCR with a LightCycler Telomerase hTERT Quantification Kit (Roche Diagnostics) according to the instructions of the manufacturer but with minor modifications. Briefly, total RNA was prepared from pellets of cultured cells or pancreatic juice or snap-frozen tissue samples according to the standard acid guanidinium thiocyanate-phenol-chloroform protocol (17) with or without glycogen (Funakoshi, Tokyo, Japan). Total RNA (0.2-0.5 μg) from pellets of cultured cells was analyzed for both hTERT and porphobilinogen deaminase in separate reactions. Expression of hTERT was calculated on a standard curve constructed from the standards supplied with the kit. For quantification, hTERT values were normalized to those of porphobilinogen deaminase and expressed as the ratio of hTERT mRNA copy numbers to porphobilinogen deaminase mRNA copy number. We developed another protocol with real-time PCR for quantitative analysis of hTERT in snap-frozen tissue and pancreatic juice samples. We designed new primers (hTERT forward, gcggagacaggtggagact; hTERT reverse, agctggagtagtagtctgctca; GAPDH forward, caatgaccccttcattgacc; GAPDH reverse, gatgcgctctgctgaagga; β-actin forward, aatcctgcaaccacctcct; β-actin reverse, gggtggatggtctgctaa), did BLAST searches to ensure the gene specificity of these primers, and used a Quantitect SYBR Green reverse transcription-PCR Kit (Qiagen, Tokyo, Japan) according to the instructions of the manufacturer. Briefly, the total volume of the reaction mixture was 20 μL and contained 10 μL SYBR Green buffer, 0.2 μL RT Mix, 1 μL each primer (10 μmol/L), and 1 μL total RNA (0.01 μg/μL). The reaction mixture was first incubated at 50°C for 15 minutes to allow for reverse transcription. PCR was then initiated at 95°C for 10 minutes to activate the modified Taq polymerase followed by 45 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 10 seconds and one cycle of 65°C for 15 seconds and 0.1°C/s to 95°C. All samples were run in triplicate, and hTERT and GAPDH and/or β-actin expressions were calculated on a standard curve constructed with total RNA from the Capan-1 pancreatic cancer cell line. For quantification in pancreatic tissue samples, hTERT expression was based on the
amount of total RNA extracted from Capan-1 cells equivalent to that of 10 ng of total RNA extracted from tissues. For quantification in pancreatic juice samples, hTERT expression was normalized against that of β-actin and expressed as the ratio of expression of hTERT mRNA to that of β-actin mRNA.

**Conventional Telomeric Repeat Amplification Protocol Assay.** Conventional TRAP assays were done with a TRAP Assay Kit (Intergen, Purchase, NY) according to the instructions of the manufacturer but with some modifications. Briefly, cell pellets were resuspended in CHAPS lysis buffer with protease inhibitors and RNAse inhibitors. Fifty microliters of reaction mixture containing 2 μL of protein extract were incubated at room temperature for 30 minutes and then subjected to 31 PCR cycles. Twenty-five microliters of TRAP product were analyzed by electrophoresis in 0.5% Tris-borate-EDTA buffer on 12% polyacrylamide nondenaturing gels and visualized with SYBR Green DNA stain (FMC Bioproducts, Rockland, ME).

**Inhibition of Telomerase Activity by siRNA Targeting of Human Telomerase Reverse Transcriptase.** Inhibition of hTERT expression was achieved by RNA interference with an siRNA mixture targeting hTERT (Dharmacon, Lafayette, CO). To verify the specificity of the knockdown effect, we used control siRNA provided by Qiagen. Panc-1 cells were transfected with various amounts of siRNA mixture with Nucleofector (Amaxa GmbH, Köln, Germany) according to the instructions of the manufacturer. Cells were harvested at various times after transfection. Total RNA and protein were extracted from each sample, and hTERT expression and RTA were evaluated by real-time PCR and real-time TRAP.

**Statistical Analysis.** Data were analyzed by Mann-Whiney U test and Spearman rank correlation test because normal distributions were not obtained. \( P < 0.05 \) was considered statistically significant. For siRNA experiments, values were expressed as mean ± SD. Comparison of values between groups was done using Student’s \( t \) test; statistical significance was set at \( P < 0.01 \).

**RESULTS**

**Sensitivity of Telomeric Repeat Amplification Protocol Assay with Real-time PCR and Quantification of Telomerase Activity.** To evaluate the utility of real-time TRAP, we tested the sensitivity and linearity of this assay with telomerase-positive MIA PaCa-2 cells. Serial dilutions of protein extracts from cell pellets were used. As shown in Fig. 1A, linearity of fluorescence intensities was observed with the equivalent of 0.1 to 100 MIA PaCa-2 cells. Notably, detection of telomerase activity by real-time TRAP was 1,000 times more sensitive than that of conventional TRAP with SYBR Green (Fig. 1B). As shown in 6 and 7 of Fig. 1A, fluorescence signals were occasionally detected in negative controls or in samples with the equivalent of less than 0.1 cell due to primer-dimer artifacts. Gel electrophoresis (Fig. 1A) revealed that the primer-dimer artifact observed after 46 cycles of PCR is a 50-bp product, which is the same size as the first ladder band of telomerase products. These data are consistent with data reported by Kim and Wu (16). Primer-dimer formation is an inherent problem in TRAP assays with or without modifications and may limit the sensitivity of TRAP assay. However, signals due to primer-dimers usually appeared at a late stage of amplification (40-45 cycles), which is out of the quantitative and linear range.

We also investigated the effect of PCR inhibitors in all tissues and juice samples using gel electrophoresis with the
internal TRAP assay standard, which was used to evaluate the effect of PCR inhibitors. When 0.6 μg of extract protein was used for TRAP assay, we observed no effect of PCR inhibitors, which is consistent with previously reported data (13–15). For the present studies, we used less than 0.6 μg of extract protein without internal TRAP assay standard.

Measurement of Telomerase Activity and Human Telomerase Reverse Transcriptase mRNA in Pancreatic Carcinoma Cell Lines. We measured telomerase activity by real-time TRAP and hTERT levels in pancreatic cancer cell lines and a human normal fibroblast cell line (MRC5). Telomerase activity and hTERT expression were detected in all pancreatic carcinoma cell lines but not in normal human fibroblasts, and these values were significantly correlated (Fig. 2; \( P = 0.0383 \)).

Quantitative Analysis of Relative Telomerase Activity and Human Telomerase Reverse Transcriptase expression in Pancreatic Juice Samples. We measured RTA in pancreatic juice samples from a total of 86 patients with different pancreatic diseases (pancreatic carcinoma, \( n = 21 \); pancreatitis and cholelithiasis, \( n = 28 \); IPMN, \( n = 37 \)) and hTERT expression in 35 pancreatic juice samples.

The amount of total RNA extracted from pancreatic juice samples was too small to measure quantitatively because few cells are present. Therefore, reference gene was necessary for quantification of hTERT expression. We tested two major housekeeping genes (\( GAPDH \) and \( \beta\)-actin) as reference genes in pancreatic tissue samples. As previously reported (18, 19), expression of both the \( GAPDH \) and \( \beta\)-actin genes was

![Fig. 2](image)

Significant correlation between RTA and hTERT expression in pancreatic cell lines. Protein extracts (0.02 μg) and total RNA (0.2 μg) extracted from 15 pancreatic cell lines were used for real-time TRAP or analysis of hTERT expression with real-time PCR (LightCycler TeloTAGGG hTERT Quantification Kit, Roche), respectively: a, KP-2; b, NOR-P1; c, Panc-1; d, Suit-2; e, KP-3; f, BxPC-3; g, MRC5; h, SW1990; i, CFPAC; j, ASPC-1; k, MIA PaCa-2; l, H48N; m, Capan-1; n, KP-1N; o, Capan-2; p, HS766T.

![Fig. 3](image)

Relative telomerase activity and hTERT expression in tumoral and nontumoral tissues. Protein extracts (0.6 μg) and total RNA (10 ng) extracted from snap-frozen tissues were used for real-time TRAP and analysis of hTERT expression with our real-time PCR protocol. Expression of hTERT is expressed as the amount of total RNA extracted from Capan-1 cells equivalent to that of 10 ng of total RNA extracted from tissues. Both RTA and hTERT expressions in tumoral tissues were significantly higher than those in nontumoral tissues (A). There was significant correlation between RTA and hTERT expression in pancreatic tissues (B; \( P = 0.045 \)).
significantly higher in tumoral tissues than in nontumoral tissues (Fig. 4A, $P = 0.013$ for GAPDH, $P = 0.023$ for $\beta$-actin). The ratio of hTERT expression to $\beta$-actin expression was significantly higher in tumoral tissues than in nontumoral tissues ($P = 0.0037$), whereas the ratio of hTERT expression to GAPDH expression was not significantly different (Fig. 4B, $P = 0.1852$). Therefore, $\beta$-actin was used as an internal control for quantification of hTERT mRNA in pancreatic juice samples.

As shown in Fig. 5A, RTA values were significantly higher in carcinoma juice samples than in pancreatitis or cholelithiasis samples ($P < 0.0001$). Relative hTERT expression in carcinoma samples was modestly but not significantly ($P = 0.2251$) higher
than in pancreatitis and cholelithiasis pancreatic juice samples (Fig. 5B). However, in carcinoma samples, there was significant correlation between RTA and hTERT expression levels (Fig. 5C, \( P = 0.0436 \)). Values of telomerase activity, hTERT expression, and cytology for pancreatic carcinoma are summarized in Table 1. Thirteen of 21 (62%) carcinoma samples showed high RTA values (>10 units); one was a case of early pancreatic cancer. Only two of 28 (7.1%) pancreatitis and cholelithiasis samples showed more than 10 units of RTA. The pancreatitis sample with the highest RTA value (52.72 units) was from a patient with severe acute pancreatitis. It is possible that activated lymphocytes induced the activation of telomerase in this case.

The present study included 37 IPMN samples. Most of these patients were followed up without resection. Eight of 37 IPMN samples showed high RTA values (>10 units). Only two patients with modestly high RTA values underwent subsequent surgery. Histologic examination of samples from these two patients showed severe atypia with no malignancy. There were significant differences in RTA values between pancreatic carcinoma and IPMN samples (Fig. 5d, \( P = 0.0005 \)). In contrast, there were no significant differences in hTERT expression between pancreatic carcinoma and IPMN samples (Fig. 5b, \( P = 0.0877 \)).

Cytologic classification of Class 4 or 5 was defined as positive for diagnosis of malignancy. The cytologic sensitivity for diagnosis of pancreatic cancer was only 28.6%, which is similar to that in previous reports (6).

**Effect of siRNA Targeting of Human Telomerase Reverse Transcriptase Expression on Telomerase Activity.** Despite a significant correlation between telomerase activity and hTERT expression, hTERT expression in pancreatic juice samples was less sensitive than telomerase activity for diagnosis. To investigate the difference between these sensitivities, we investigated changes in telomerase activity in response to inhibition of hTERT expression with siRNA. One-hundred picomoles of hTERT-targeting siRNA mixture were used to inhibit hTERT expression. Inhibition of hTERT mRNA peaked 24 to 72 hours after transfection and the inhibition rate was 47.0% to 52.9%, whereas inhibition of telomerase activity peaked 48 to 96 hours after transfection and the inhibition rate was 4.7% to 5.3% (Fig. 6). These data show that a modest change in hTERT expression can induce remarkable changes in telomerase activity, indicating that detection of differences in telomerase activity between carcinoma and nonmalignant specimens may be more advantageous for clinical diagnosis than assessment of hTERT expression.

**DISCUSSION**

Detection of mRNA for hTERT, a catalytic subunit of telomerase, may aid in the diagnosis of malignancies including pancreatic cancer (10–12). Seki et al. (12) reported that hTERT mRNA is frequently detectable by nonquantitative nested PCR in pancreatic juice samples from pancreatic cancer patients. In the present study, we did quantitative analysis of hTERT expression.

### Table 1  Relative telomerase activity by real-time TRAP in pancreatic juice from patients with carcinoma

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Diagnosis *</th>
<th>Age/Sex</th>
<th>Classification*</th>
<th>Location</th>
<th>RTA (unit)$</th>
<th>Grade$</th>
<th>hTERT expression$</th>
<th>Cytology$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carcinoma</td>
<td>53/F</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>218.10</td>
<td>5</td>
<td>5.98</td>
<td>Class 3</td>
</tr>
<tr>
<td>2</td>
<td>Carcinoma</td>
<td>61/M</td>
<td>T1N1M1</td>
<td>Pb</td>
<td>21.84</td>
<td>5</td>
<td>69.63</td>
<td>Class 1</td>
</tr>
<tr>
<td>3</td>
<td>Carcinoma</td>
<td>67/M</td>
<td>T1N0M1</td>
<td>Ph</td>
<td>56.86</td>
<td>5</td>
<td>NE</td>
<td>Class 5</td>
</tr>
<tr>
<td>4</td>
<td>Carcinoma</td>
<td>58/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>14.67</td>
<td>3</td>
<td>NE</td>
<td>Class 3</td>
</tr>
<tr>
<td>5</td>
<td>Carcinoma</td>
<td>67/M</td>
<td>T1N1M1</td>
<td>Ph</td>
<td>46.07</td>
<td>5</td>
<td>NE</td>
<td>Class 3</td>
</tr>
<tr>
<td>6</td>
<td>Carcinoma</td>
<td>44/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>48.88</td>
<td>4</td>
<td>NE</td>
<td>Class 3</td>
</tr>
<tr>
<td>7</td>
<td>Carcinoma</td>
<td>78/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>23.89</td>
<td>4</td>
<td>NE</td>
<td>Class 4</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma</td>
<td>61/F</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>13.99</td>
<td>3</td>
<td>NE</td>
<td>Class 5</td>
</tr>
<tr>
<td>9</td>
<td>Carcinoma</td>
<td>58/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>14.26</td>
<td>3</td>
<td>NE</td>
<td>Class 3</td>
</tr>
<tr>
<td>10</td>
<td>Carcinoma</td>
<td>72/M</td>
<td>T1N1M0</td>
<td>Ph</td>
<td>12.94</td>
<td>3</td>
<td>NE</td>
<td>Class 2</td>
</tr>
<tr>
<td>11</td>
<td>Carcinoma</td>
<td>43/F</td>
<td>T1N1M1</td>
<td>Ph</td>
<td>8.38</td>
<td>2</td>
<td>29.22</td>
<td>Class 2</td>
</tr>
<tr>
<td>12</td>
<td>Carcinoma</td>
<td>57/M</td>
<td>T1N1M1</td>
<td>Pt</td>
<td>9.04</td>
<td>2</td>
<td>148.72</td>
<td>Class 3</td>
</tr>
<tr>
<td>13</td>
<td>Carcinoma</td>
<td>82/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>17.78</td>
<td>3</td>
<td>11.48</td>
<td>Class 1</td>
</tr>
<tr>
<td>14</td>
<td>Carcinoma</td>
<td>59/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>280.4</td>
<td>5</td>
<td>478.52</td>
<td>Class 3</td>
</tr>
<tr>
<td>15</td>
<td>Carcinoma</td>
<td>66/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>8.15</td>
<td>2</td>
<td>4.83</td>
<td>Class 2</td>
</tr>
<tr>
<td>16</td>
<td>Carcinoma</td>
<td>57/M</td>
<td>T1N1M0</td>
<td>Ph</td>
<td>19.83</td>
<td>3</td>
<td>29.76</td>
<td>Class 3</td>
</tr>
<tr>
<td>17</td>
<td>Carcinoma</td>
<td>73/M</td>
<td>T1N1M1</td>
<td>Ph</td>
<td>3.50</td>
<td>2</td>
<td>0.24</td>
<td>Class 5</td>
</tr>
<tr>
<td>18</td>
<td>Carcinoma</td>
<td>65/M</td>
<td>T1N1M1</td>
<td>Ph</td>
<td>210.5</td>
<td>5</td>
<td>313.35</td>
<td>Class 3</td>
</tr>
<tr>
<td>19</td>
<td>Carcinoma</td>
<td>75/M</td>
<td>T1N0M0</td>
<td>Pt</td>
<td>29.69</td>
<td>4</td>
<td>4.49</td>
<td>Class 5</td>
</tr>
<tr>
<td>20</td>
<td>Carcinoma</td>
<td>82/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>8.85</td>
<td>2</td>
<td>52.07</td>
<td>Class 4</td>
</tr>
<tr>
<td>21</td>
<td>Carcinoma</td>
<td>72/M</td>
<td>T1N0M0</td>
<td>Ph</td>
<td>8.85</td>
<td>2</td>
<td>52.07</td>
<td>Class 4</td>
</tr>
</tbody>
</table>

Abbreviations: Ph, head of pancreas; Pb, body of pancreas; Pt, tail of pancreas; NE, not examined.

* Tumor classification was assessed according to Union Internationale Contre le Cancer tumor-node-metastasis staging.

RTA equivalent to that in one MIA PaCa-2 cell was defined as 1 unit. RTA in 0.64g of protein extract from pancreatic juice was exhibit using unit.

hTERT expression was normalized to y-actin expression.

Cytologic diagnosis was made according to the standard criteria as reported previously (6).

**Diagnosed by liver metastasis.

Resected and/or pathologically diagnosed.
mRNA in pancreatic juice samples as well as pancreatic cell lines and tissues. We found significant correlation between telomerase activity and hTERT mRNA expression in pancreatic cancer cell lines, pancreatic tissues, and pancreatic carcinoma juice samples. However, in pancreatic juice samples, the diagnostic significance of hTERT expression seems to be much lower than that of telomerase activity. Experiments with hTERT siRNA may reveal the reason for this discrepancy. These data showed that an almost 10-fold change in telomerase activity could be induced by only a 2-fold change in hTERT expression in immortalized cells. There is the possibility of technical artifact. Because the concentration of RNA extracted from pancreatic juice samples is too small to measure quantitatively, we had to use a reference gene to normalize the expression of hTERT. The β-actin gene was a better reference gene than GAPDH gene for quantitative analysis of hTERT mRNA in pancreatic tissues (Fig. 4B). However, we attempted to quantify hTERT expression using pancreatic tissue samples; expression of β-actin mRNA was significantly higher in tumoral tissues than in nontumoral tissues (Fig. 4A). Therefore, β-actin may not be a good reference gene for pancreatic juice samples. To improve the clinical application of analysis of hTERT expression, identification of better reference genes or more sensitive measurements for extracted total RNA is needed.

In the present study, we also showed that quantitative analysis of telomerase activity in pancreatic juice with real-time TRAP is useful for diagnosis of pancreatic cancer. We recently developed another sensitive assay for telomerase activity that we termed hybridization protection assay (HPA) combined with TRAP (TRAP/HPA; ref. 20). The sensitivity of TRAP/HPA is comparable to that of real-time TRAP (13). Both of these assays permit the evaluation of telomerase activity in very small amounts of protein extract from pancreatic juice thereby avoiding the negative effects of PCR inhibitors that are abundant in pancreatic juice (13–15). Real-time TRAP is similar in sensitivity and superior in terms of simplicity and detection time to TRAP/HPA; therefore, real-time TRAP may be the more useful clinically for detection of telomerase activity in the diagnosis of pancreatic cancer.

In conclusion, we found that telomerase activity and expression of hTERT mRNA correlated significantly in pancreatic cancer cells, tissues, and carcinoma juice samples. Both values were significantly higher in pancreatic cancer tissues than in normal tissues, suggesting that they are useful markers for diagnosis of pancreatic cancer. In the present results, quantitative assessment of telomerase activity seems to be more informative than quantitative assessment of hTERT expression for diagnosis of pancreatic cancer. However, reverse transcription-PCR–based quantitative analysis of mRNA allows introduction of a quality control, which is lacking in the telomerase assay. In addition, technical issues, such as low RNA input or selection of reference genes, can be addressed, which may further improve this assay. We believe that real-time TRAP with pancreatic juice samples is a promising approach for the diagnosis of pancreatic cancer and that quantitative assessment of hTERT mRNA expression in pancreatic juice with real-time PCR is also a potential diagnostic tool although some technical improvements are needed.

ACKNOWLEDGMENTS

We are grateful to M. Ohta (Department of Clinical Pathology, Kyushu University) for skillful cytologic examinations and analyses.

REFERENCES

Quantitative Assessment of Telomerase Activity and Human Telomerase Reverse Transcriptase Messenger RNA Levels in Pancreatic Juice Samples for the Diagnosis of Pancreatic Cancer

Kenoki Ohuchida, Kazuhiro Mizumoto, Yasuhiro Ogura, et al.