Quantitative Analysis of Human Telomerase Reverse Transcriptase in Pancreatic Cancer

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Abstract Although telomerase activity is a promising diagnostic marker, clinical introduction of this marker for cancer diagnosis is still problematic due to the lack of means of evaluating sample quality. Human telomerase reverse transcriptase (hTERT), one of the subunits of telomerase, is also a promising diagnostic marker. In the present study, we did large-scale analysis of 88 pancreatic juice samples to determine the feasibility of quantitative analysis of hTERT mRNA for diagnosis of pancreatic cancer. We found significant differences in hTERT expression among carcinoma-derived, intraductal papillary mucinous neoplasm (IPMN)-derived, and chronic pancreatitis–derived juice samples. Results showed that quantitative analyses of hTERT mRNAs are more useful in discriminating carcinoma from IPMN than from chronic pancreatitis. When the specificity was set at 100%, the sensitivity for differentiation between carcinoma and IPMN was 43.5%, whereas the sensitivity of cytologic examination was 22.0%. There were significant differences in hTERT expression among carcinoma cells, IPMN cells, and normal ductal cells isolated from pancreatic tissues by microdissection. Lymphocytes and hyperplastic epithelial cells isolated from tissues with the histologic appearance of pancreatitis showed various expression levels of hTERT. Our results suggest that quantitative analysis of hTERT mRNA in pancreatic juice is advantageous over cytologic analysis for differentiation between carcinoma and IPMN but probably not for differentiation between carcinoma and chronic pancreatitis.

Pancreatic cancer is the fifth leading cause of cancer death and has the lowest patient survival rate of any solid cancer (1, 2). Despite improvements in diagnostic imaging, diagnosis before surgery remains difficult due to the inaccessibility of the pancreas and surrounding organs. The vast majority of patients with pancreatic cancer suffer from a poor clinical outcome.

Endoscopic retrograde cholangiopancreatography (ERCP) is currently used as a diagnostic tool to distinguish pancreatic cancer from nonmalignant disorders (3, 4). However, chronic pancreatitis is one of these nonmalignant pancreatic diseases, and its ERCP features are often similar to those of pancreatic cancer. Therefore, several diagnostic strategies, employing cytology, DNA mutation markers, or aberrant expression of cancer-specific mRNA in pancreatic juice, have been reported for differential diagnosis between pancreatic cancer and chronic pancreatitis (5–7). Although these modalities have provided some benefits for diagnosis of pancreatic cancer, they have not yet been used worldwide.

Cystic lesions of the pancreas are being detected with increasing frequency due to application of diagnostic imaging technologies, such as computed tomography and magnetic resonance. Therefore, ERCP is being used with increasing frequency to further evaluate the cystic lesions identified by imaging studies. Intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm are the representative cystic neoplasms of the pancreas. Notably, since its first description by Ohhashi et al. in 1982 (8), IPMN has become an increasingly recognized cystic tumor with unique histopathologic features, including intraductal papillary growth, mucin hypersecretion, and resultant dilation of the pancreatic duct. IPMN is often associated with pancreatic cancer (9), occurring either as a separate lesion or as carcinoma derived from the IPMN. However, ERCP and other conventional diagnostic imaging modalities are not useful in evaluating the malignant potential of IPMN. This suggests that a diagnostic strategy to discriminate IPMN associated with cancer from benign IPMN is needed.

Telomerase activity is a promising diagnostic marker for pancreatic cancer (10, 11). We and other investigators have reported that detection of telomerase activity in pancreatic juice is useful for the diagnosis of pancreatic cancer (12–14). However, clinical introduction of this marker for cancer diagnosis is still problematic due to the lack of means of...
evaluating sample quality and the difficulties of quantitative measurement. Human telomerase reverse transcriptase (hTERT) is one of the subunits of telomerase, and its mRNA has been reported as a promising diagnostic marker (15–18). We have reported accurate quantitative analysis of hTERT mRNA in pancreatic juice obtained during ERCP (19). However, we were unable to show the utility of hTERT analysis of pancreatic juice for diagnosis of pancreatic cancer possibly due to the small number of samples.

In the present study, we did a large-scale analysis of 88 samples of pancreatic juice and determined the feasibility of quantitative analysis of hTERT mRNA to differentiate pancreatic cancer from IPMN or chronic pancreatitis. hTERT mRNA levels were significantly greater in carcinoma-derived juice samples than in pancreatitis-derived or IPMN-derived samples. The preoperative diagnostic utility of quantitative analysis of hTERT in pancreatic juice was evaluated by means of receiver operating characteristic (ROC) curves (20). To support the results of the analyses with pancreatic juice, we used microdissection to isolate normal ducts, IPMN cells, and invasive ductal carcinomas (IDC) and examined the levels of hTERT expression in the cells. In addition, we isolated lymphocytes and hyperplastic epithelial cells from pancreatic tissues with the histologic appearance of pancreatitis and investigated their hTERT expression levels.

Materials and Methods

Pancreatic juice and pancreatic tissues. Pancreatic juice samples were collected from 88 patients who had undergone ERCP for suspected malignancy of the pancreas at Kyushu University Hospital (Fukuoka, Japan) from January 1, 2002 to December 31, 2004 as described previously (12, 21). We used pellets of cellular material from pancreatic juice for preparation of RNAs. The diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens when available (12 cases), but when the case was inoperable a clinical diagnosis was made based on imaging findings (11 cases). Pancreatitis or IPMN was diagnosed based on histologic examination of resected specimens or clinical findings at the time of the initial diagnosis and during a follow-up of at least 6 months that included conventional diagnostic imaging. Tissue samples were obtained at the time of surgery at Kyushu University Hospital. Thirteen IDC or 8 IPMN tissue samples were obtained from the primary tumor of each resected pancreas, and 18 nonneoplastic tissues (normal pancreas, 8 samples; pancreatitis, 10 samples) were taken from peripheral tissues away from the tumor in each patient. The tissue samples were removed as soon as possible after resection and divided into at least two bulk tissue samples. A part of each sample was embedded in OCT compound (Sakura, Tokyo, Japan) and snap-frozen for analysis by microdissection. The remainder after resection and divided into at least two bulk tissue samples. A part of each patient. The tissue samples were removed as soon as possible after resection and divided into at least two bulk tissue samples. A part of each sample was embedded in OCT compound (Sakura, Tokyo, Japan) and snap-frozen for analysis by microdissection. The remaining was fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections for H&E staining. All tissues adjacent to the specimens were examined histologically, and the diagnosis was confirmed. Written informed consent was obtained from all patients, and the study was conducted according to the guidelines of the Helsinki declaration.

Quantitative analysis of hTERT mRNA levels by real-time PCR. Total RNA was extracted from cell pellets of pancreatic juice and cells isolated by microdissection techniques as described previously (22). We designed a real-time PCR protocol for the quantitative analysis of hTERT and β-actin mRNAs and did quantitative real-time PCR with a Quantitect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) using a LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany) as described previously (19). Briefly, 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 modified Taq polymerase followed by a 45-cycle amplification (95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 10 seconds) and 1 cycle (95°C for 0 second, 65°C for 15 seconds, and 0.1°C/s to 95°C) for melting analysis. Each sample was run twice. In addition, all samples showing >10% deviation in values were tested in a third run. The 10% deviation was calculated from concentrations after use of the calibration curve. mRNA expression of each sample was calculated from a standard curve constructed with the use of total RNA from the Capan-1 pancreatic cancer cell line. The range of thresholds cycles observed was 20 to 35 cycles for hTERT and 15 to 30 cycles for β-actin primers. For relative quantification, the expression of hTERT mRNA was normalized to that of β-actin mRNA.

Microdissection-based quantitative analysis of mRNA. The frozen tissue samples were cut into 8-μm-thick sections. One section was stained with H&E for histologic examination. IDC cells, IPMN cells, normal pancreatic ductal epithelial cells, hyperplastic epithelial cells, and lymphocytes were isolated selectively by means of laser microdissection and a pressure catapulting system (Palm Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer’s protocols. After microdissection, total RNA was extracted from the selected cells and subjected to real-time PCR for quantitative measurement of hTERT mRNA.

Statistical analyses. Data were analyzed by Mann-Whitney U test because normal distributions were not obtained. Statistical significance was defined as P < 0.05, but because we did multiple comparisons on our real-time PCR data in the analyses of pancreatic juice samples we conservatively used Bonferroni correction; thus, the adjusted significance level was P < 0.0167 in the analyses of pancreatic juice. The optimal cutoff points for each marker for discriminating between pancreatic carcinoma and other benign diseases were sought by constructing ROCs, which were generated by calculating the sensitivities and specificities of each marker at several predetermined cutoff points (23).

Results

Quantitative analysis of hTERT mRNA expression in pancreatic juice. We measured hTERT mRNA expression levels in 88 pancreatic juice samples, including 23 from pancreatic carcinomas, 29 from IPMN, and 36 from chronic pancreatitis. Relative expression of hTERT was significantly greater in carcinoma samples than in chronic pancreatitis or IPMN samples after Bonferroni correction (Fig. 1; P < 0.016 for carcinoma versus chronic pancreatitis and P < 0.0004 for carcinoma versus IPMN). The difference in hTERT mRNA expression between IPMN and chronic pancreatitis samples was not significant after Bonferroni correction was applied (Fig. 1; P = 0.0304).

ROC s for hTERT mRNA expression are presented in Fig. 2. The sensitivity of each marker was determined at several specificity levels. The area under the ROC was 0.778 for carcinoma versus IPMN [95% confidence interval (95% CI), 0.636-0.884] and 0.629 for carcinoma versus chronic pancreatitis (95% CI, 0.479-0.762). In particular, a significant difference between the areas for carcinoma versus IPMN and carcinoma versus chronic pancreatitis was observed (difference between areas, 0.149; 95% CI, 0.005-0.293; P = 0.042). These data reveal that the discriminability for carcinoma versus IPMN is greater than that for carcinoma versus chronic pancreatitis.

In this study, cytologic class 4 or 5 (24) was considered positive for a diagnosis of malignancy. The cytologic sensitivity for diagnosis of pancreatic cancer was only 22.0% (95% CI, 14.7-29.3), which was similar to that cited in previous reports (12), although the specificity was 100%. The ROC analyses
revealed that the sensitivity and specificity of differentiation between carcinoma and IPMN were 43.5% (95% CI, 23.2-65.5) and 100% (95% CI, 87.9-100), respectively, when the cutoff point was set at 36.4. Moreover, the sensitivity and specificity of differentiation between carcinoma and chronic pancreatitis were 21.7% (95% CI, 7.5-43.7) and 100% (95% CI, 90.2-100), respectively, when the cutoff point was set at 87.9. These data suggest that hTERT analysis may offer some advantage over cytologic analysis, especially for differentiation between carcinoma and IPMN.

Microdissection-based quantitative analysis of mRNA shows differential expression of hTERT mRNA in IDCs, nonmalignant IPMNs, lymphocytes, and normal ducts. To support the results of pancreatic juice analyses, the expression levels of hTERT mRNA in IPMN cells isolated from 8 resected IPMN tissues, which did not include malignant IPMN, and hyperplastic epithelial cells isolated from 10 tissues with the histologic appearance of pancreatitis were compared with those in normal ductal cells (n = 7) or IDC cells (n = 13). It has been reported that activated lymphocytes exhibit hTERT expression possibly leading to false-positive results in chronic pancreatitis samples. Therefore, we also isolated lymphocytes from 7 tissues with the histologic appearance of pancreatitis and investigated the expression levels of hTERT mRNA.

IDC cells showed a significantly higher level of hTERT mRNA expression than levels shown by normal duct cells, IPMN cells, hyperplastic cells, and lymphocytes (Fig. 3). Although IPMN cells isolated from several tissues with the histologic appearance of severe atypia exhibited a similar level to that of carcinoma cells, other IPMN cells with low-grade atypia had low hTERT expression. Hyperplastic epithelial cells isolated from tissue with the histologic appearance of pancreatitis had a level of hTERT mRNA expression similar to that of IPMN cells, although a few hyperplastic epithelial cells had a high level of hTERT mRNA expression, similar to that of IPMN cells with severe atypia (Fig. 3). Lymphocytes isolated from tissues with the histologic appearance of pancreatitis showed significantly lower levels of hTERT expression than IDC cells. However, lymphocytes expressed significantly higher levels of hTERT mRNA than normal duct cells. In addition, the median level of hTERT mRNA in lymphocytes was greater than the levels in IPMN cells and hyperplastic cells. Notably, lymphocytes from several tissues exhibited a high hTERT expression level approaching that of carcinoma cells.

Discussion

In the present study, pancreatic juice analyses showed that the power to discriminate between carcinoma and IPMN was significantly greater than that between carcinoma and chronic pancreatitis. In addition, we showed differential hTERT mRNA expression levels between normal ductal cells and carcinoma cells and relatively high hTERT mRNA expression levels in lymphocytes isolated from several resected tissues with the histologic appearance of pancreatitis.

Seki et al. (18) reported that detection of hTERT mRNA in pancreatic juice samples by nested PCR is useful for the diagnosis of pancreatic cancer. However, they also reported that several pancreatic juice samples from patients with chronic pancreatitis exhibited positive expression of hTERT mRNA. Our present results showed that the quantitative analysis of hTERT expression does not reduce the false-positive diagnosis rate in pancreatitis-derived juice. Several studies (25–27) have shown that activated lymphocytes exhibit telomerase activity. To investigate the cause of false-positive samples derived from patients with pancreatitis, we did microdissection and measured hTERT expression in lymphocytes and hyperplastic epithelium isolated from selected tissues with the histologic appearance of pancreatitis. These lymphocytes had relatively high levels of hTERT expression. In addition, hyperplastic epithelial cells isolated from 2 of 10 tissues with the histologic appearance of pancreatitis also exhibited relatively high levels of hTERT expression.
mRNA. These observations suggest that a subset of lymphocytes or hyperplastic epithelial cells expressing high levels of hTERT mRNA may cause false-positive diagnoses in patients with chronic pancreatitis. To avoid the effect of lymphocytes in pancreatic juice, it may be useful to isolate target epithelial cells from cytoclogic specimens of pancreatic juice by microdissection.

A trial is now in progress in our laboratory.

Inoue et al. (28) reported that detection of telomerase activity in pancreatic juice samples is useful for differentiation between nonmalignant and malignant IPMN. Most nonmalignant IPMN-derived juice samples in our study had low levels of hTERT mRNA, consistent with a previous report (28). However, several IPMN-derived juice samples exhibited high hTERT mRNA expression levels. One IPMN patient with pancreatic juice expressing a high level of hTERT mRNA underwent surgery, and histologic examination of the resected tissue showed severely atypical IPMN. Moreover, cells isolated from IPMN with histologically severe atypia had high hTERT mRNA expression. Our data indicate that precancerous IPMN cells expressing high levels of hTERT mRNA may have strong potential to progress to cancer.

It is possible that some of our pancreatic carcinoma samples were misclassified as IPMN or chronic pancreatitis samples. All patients who did not undergo resection were followed up for at least 6 months, but a longer observation period may be necessary to rule out the possibility of pancreatic carcinoma completely because a subset of pancreatic cancers, such as *in situ* malignant IPMN, show very slow growth.

In conclusion, the quantitative analysis of hTERT mRNA in pancreatic juice offers some advantage over cytologic analysis for differentiation between carcinoma and IPMN but probably not for differentiation between carcinoma and chronic pancreatitis.

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References


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