Diagnosis of Pancreatic Adenocarcinoma by Detection of Human Telomerase Reverse Transcriptase Messenger RNA in Pancreatic Juice with Sample Qualification

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ABSTRACT

Purpose: We evaluated the diagnostic efficacy of detection of human telomerase reverse transcriptase (hTERT) message, a catalytic domain of human telomerase, in endoscopic retrograde pancreatography (ERP)-derived pancreatic juice.

Experimental Design: Both hTERT and CD25 expression were detected by reverse transcription-PCR (RT-PCR) in 17 patients with pancreatic adenocarcinoma (PC), 12 patients with chronic pancreatitis (CP), and 7 patients with no ERP abnormality (N). In the same patients, β-actin expression was semiquantified by competitive RT-PCR. K-ras codon 12 mutations were concomitantly analyzed by enriched PCR-SSCP in 11 and 7 PC and CP cases, respectively.

Results: Expression of hTERT was detected in 88% of PC cases and 17% of CP cases but not in the normal control (N). Alterations in K-ras were detected in 73% of PC cases and 57% of CP cases, respectively. β-Actin mRNA was expressed in >3.0 × 10^3 copies/µl in all but two PC cases in which hTERT mRNA was not detected. CD25-positive and -negative peripheral lymphocytes were isolated from a normal volunteer using a fluorescent activating cell sorter. The hTERT message was detected in CD25-positive peripheral lymphocytes and in 18, 25, and 0% of the pancreatic juice samples from PC, CP, and N cases, respectively. All CP cases expressing hTERT message were also CD25 positive.

Conclusions: These results suggest that detection of hTERT mRNA in pancreatic juice is a powerful tool to discriminate PC from CP, particularly when the samples are qualified against β-actin mRNA levels and contaminating CD25-positive lymphocytes.

INTRODUCTION

PC is one of the most aggressive known cancers. The 5-year survival rate of PC is one of the lowest of all types of cancers (1). The poor prognosis results from both the difficulty of diagnosis in the early clinical stages and the highly metastatic and/or invasive nature of this tumor. It is believed that early diagnosis may drastically improve the survival rate. A common challenge for physicians is the differential diagnosis between PC and CP, which is assumed to be a risk factor for PC development (2), despite the availability of advanced endoscopic, radiological, and ultrasonographic techniques. This is particularly problematic when a solid tumor is forming. Although a cytological examination of the pancreatic juice collected under ERP facilitates the diagnosis, the sensitivity of this method varies from 30 to 80% (3, 4). Detection of molecular abnormalities commonly associated with PC is also applied in the clinical diagnosis, including K-ras and p53 gene mutations (5, 6). These mutations are also detected in noncancerous tissues, such as in adenoma and pancreatitis (7–10), and are therefore of limited value in the differential diagnosis of PC and CP.

Telomerase is an enzyme implicated in the de novo synthesis of GGTAG telomeric DNA onto chromosomal ends to stabilize telomeres, concomitant with immortality in cancer cells (11–13). Telomerase consists of three components, human telomerase RNA, an RNA template complementary to GGTAG (14), hTERT, a catalytic domain (15), and the other protein component, TP1 (16). Although hTR is expressed ubiquitously in both cancerous and noncancerous tissues (17), hTERT expression is rate limiting for telomerase activity (18). Detection of telomerase activity using a TRAP assay of pancreatic juice is more likely to diagnose PC than detection of K-ras codon 12 mutations (19–21). Several problems remain to be overcome.

3 The abbreviations used are: PC, pancreatic adenocarcinoma; CP, chronic pancreatitis; N, patient with no ERP abnormality; ERP, endoscopic retrograde pancreatography; PBL, peripheral blood lymphocyte; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformation polymorphism; TRAP, telomeric repeat amplification protocol.
before this technique can be introduced in clinics. These include
the use of radioisotopes and the possibility of false positives
attributable to contaminating lymphocytes, which can show
telomerase activity without malignant transformation (22, 23).
The present study aimed to determine whether the detection of
hTERT mRNA in pancreatic juice would be a useful diagnostic
tool for pancreatic cancer and attempted to decrease the risk of
false-positive and -negative diagnosis using this strategy.

MATERIALS AND METHODS

Pancreatic Juice. Thirty-six patients were referred to our
clinic for ERP. Informed consent was obtained from each pa-
tient, and the study protocol conformed to the ethical guidelines
of the 1975 Declaration of Helsinki, as reflected in a priori
approval by the institution’s human research committee. The
pancreatic juice was collected through a catheter placed selec-
tively into the pancreatic duct after an i.v. injection of secretin.
After discarding the first secretion, which included the contrast
medium, the pancreatic juice was collected in 2.5-ml serum
tubes, snap frozen in liquid nitrogen, and stored at −80°C until
use. Seventeen patients, cases 1 to 17, had PC proven by
histological examination of surgically resected specimens or
other clinical diagnostic modalities: ultrasonography, ERP in-
cluding cytopathological examination, dynamic computed tomog-
raphy, and magnetic resonance imaging. Twelve patients had a
clinical diagnosis of chronic pancreatitis void of cancer (cases
18–29). No abnormal findings were detected in 7 patients (cases
30–36).

RT-PCR. Approximately 2 ml of pancreatic juice were
diluted in 15 ml of phosphate buffer saline prepared with diethyl
pyrocarbonate-treated water. Cells were collected by centrifugation.
Total RNA was isolated using IsoGen (Wako, Osaka,
Japan) according to the manufacturer’s manual and resuspended in
20 μl of TE [10 mm Tris-HCl (pH 7.5), 0.1 mm EDTA].
cDNA synthesis was performed using 5.6-μl aliquots in a 20-μl
reaction mixture, which included random hexa-oligonucleotides
and RAV-2 reverse transcriptase (Takara, Kyoto, Japan).
hTERT cDNA was amplified by nested PCR. The first PCR
amplified a 499-bp (10–508 nucleotides) fragment, which included
exons 1 and 2, a region without alternative splicing (24).
One μl of a 250-fold dilution of the first PCR reaction was
subjected to a second PCR. Nested primers were used to amplify
a 429-bp fragment. A 491-bp α chain of interleukin 2 receptor
cDNA was amplified in a 10-μl reaction mixture using primers
5’-AATGCACAAGCTGCACTCCTC (sense) and 5’-GCC-
CAGCTGACTGGTAC (antisense). To quantify the amount of
mRNA recovered, β-actin cDNA was co-amplified with a constant
amount of competitor DNA, which could be
amplified with the same primers but produced 100-bp shorter
products. PCR was terminated at 30 cycles, at which the products
were linearly generated. After separation through 6% poly-
acrylamide gel, the products were visualized by ethidium bromide
staining. The image was digitally captured with a DC40
digital camera (Eastman Kodak, Rochester, NY), and intensity
ratios of native and competitor DNA products were calculated
using ScionImage (Scion, Frederick, MD). The number of
cDNAs was calculated by transforming the ratio along the
standard curve, which was deduced from the dilution experi-
ments using known amounts of native and competitor DNAs.

Enriched PCR-SSCP. Mutations in K-ras exon 1 codon
12 were analyzed by a two-step PCR method with restriction
enzyme digestion, followed by nonradioisotopic SSCP analysis
(25). Briefly, a 157-bp fragment containing K-ras codon 12 was
amplified from genomic DNA extracted from frozen pancreatic
juice. The PCR product was digested with BstNI overnight at
60°C. The 250-fold dilution of the digested PCR product was
amplified with nested primers to generate a 135-bp fragment.
Aliquots of the second PCR reaction products were diluted with
a loading buffer consisting of 90% deionized formamide, 20 mm
EDTA, and 0.05% bromphenol blue and xylene cyanol. After
denaturation at 80°C for 5 min, the samples were electrophore-
sed through 15% polyacrylamide gels. The fragments were
visualized under UV irradiation after staining with GelStar
(Takara).

Separation of CD25-positive and -negative Peripheral
Lymphocytes. Peripheral blood was obtained from a healthy
human volunteer with approval to investigate hTERT expres-
sion. PBLs were collected by Ficoll-Isopaque (1.077) gradient
centrifugation. After lysing the erythrocytes with ammonium
chloride solution (155 mm NH₄Cl + 10 mm KHCO₃, 1 mm
EDTA, 0.2 mm Tris-HCl, pH 7.6), the isolated PBLs were washed
twice and suspended in RPMI 1640 supplemented with 10% FCS.
The surface phenotype of the cells was identified using
phycoerythrin-conjugated anti-CD25 monoclonal antibody
(B1.49.9; Immunotech, Westbrook, CT). CD25 and interleukin
2 receptor α are nomenclatures for the same molecule. The
fractions of CD25⁺ PBLs and CD25⁻ PBLs were sorted by FACStar II plus (Becton Dickinson, Lincoln Park, NJ). Purity
was >98%.

TRAP Assay. Telomerase activity in the sorted lympho-
cytes was analyzed by the TRAP, which included an internal
control (TRAPEze; Intergen Co., Purchase, NY). Amplified
products were electrophoresed through 12% polyacrylamide
gels in TBE (0.09 m Tris-borate, 0.02 m EDTA) and visualized
using GelStar (Takara).

RESULTS

hTERT Expression and Detection of K-ras Codon 12
Mutations in Pancreatic Juice. The expression of hTERT in
pancreatic juice was measured using reverse transcription cou-
pled with nested PCR (see “Materials and Methods”). Message
was detected in 15 of 17 patients with PC (Table 1) but in only
2 of 12 patients with CP devoid of cancer. No message was
detected in any of the 7 cases with no pancreatic abnormalities
detected by ERP (N; Table 2). The sensitivity, specificity, and
overall accuracy of the diagnosis of PC based on the presence
of hTERT message in pancreatic juices were 88, 83, and 86%
respectively. Because there was not enough pancreatic juice
collected in all of the cases for analysis of both hTERT message
and K-ras codon 12 mutations, K-ras analysis was carried out in
only 11 and 7 cases of PC and CP, respectively. Alterations of
K-ras codon 12 were detected in 8 of 11 PC cases using the
enriched PCR-SSCP method (Table 1). Mutations were also
detected in 4 of 7 CP cases (Table 2). The sensitivity, specific-
ity, and overall accuracy of the diagnosis of PC based on the

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Evaluation of Sample Quality in hTERT Detection

3.0

in every 1

findings suggest that a sample in which

in the presence of clinical evidence for advanced PC. These

16 and 17, in which hTERT message could not be detected, even

was quantified by competitive RT-PCR (Fig. 1). This level

evaluation, the amount of

mutations were also hTERT positive.

ras
73, 43, and 61%, respectively. All cancer cases with K-

alterations in K-ras codon 12 detected in pancreatic juices were

73, 43, and 61%, respectively. All cancer cases with K-ras

mutations were also hTERT positive.

Quantification of β-Actin mRNA in Pancreatic Juice.

To define the minimum amount of mRNA suitable for hTERT

evaluation, the amount of β-actin message in pancreatic juice

was quantified by competitive RT-PCR (Fig. 1). This level

varied between samples from \(<3.0 \times 10^3\) to \(>3.0 \times 10^3\) copies

in every 1 \(\mu\)l of the reverse-transcribed products. More than

\(3.0 \times 10^3\) copies/\(\mu\)l were recovered in all but 2 PC cases, cases

16 and 17, in which hTERT message could not be detected, even

in the presence of clinical evidence for advanced PC. These

findings suggest that a sample in which \(<3.0 \times 10^3 \text{ copies/}\mu\)l

of β-actin mRNA are detected should be discarded without

evaluation. We therefore evaluated only those CP cases in which

any evidence of PC, and 6.7

Table 1  Detection of several messages and K-ras mutations in pancreatic juice from pancreatic cancer cases

| Case | Age | Gender | hTERT | K-ras | β-actin (copy) | CD25 | Diagno
|------|-----|--------|-------|-------|---------------|------|--------
| 1    | 63  | M      | P     | N     | 3.4 \(\times\) 10^{2} | N    | CP     |
| 2    | 68  | F      | P     | P     | 1.5 \(\times\) 10^{3} | P    | CP     |
| 3    | 69  | M      | P     | P     | 1.3 \(\times\) 10^{3} | N    | CP     |
| 4    | 61  | M      | P     | ND    | 3.1 \(\times\) 10^{3} | N    | CP     |
| 5    | 67  | M      | P     | P     | 3.0 \(\times\) 10^{3} | N    | CP     |
| 6    | 67  | M      | P     | P     | 1.9 \(\times\) 10^{3} | P    | CP     |
| 7    | 70  | F      | P     | P     | 3.0 \(\times\) 10^{3} | N    | CP     |
| 8    | 65  | M      | P     | ND    | 2.5 \(\times\) 10^{3} | N    | CP     |
| 9    | 76  | M      | P     | P     | 3.2 \(\times\) 10^{3} | N    | CP     |
| 10   | 66  | F      | P     | ND    | 1.6 \(\times\) 10^{3} | N    | CP     |
| 11   | 70  | F      | P     | N     | 2.3 \(\times\) 10^{3} | N    | CP     |
| 12   | 74  | M      | P     | N     | 3.0 \(\times\) 10^{3} | P    | CP     |
| 13   | 60  | M      | P     | ND    | 6.6 \(\times\) 10^{3} | N    | CP     |
| 14   | 62  | F      | P     | N     | 1.8 \(\times\) 10^{3} | N    | CP     |
| 15   | 79  | F      | P     | P     | 8.5 \(\times\) 10^{3} | N    | CP     |
| 16   | 74  | M      | N     | ND    | 3.0 \(\times\) 10^{3} | N    | CP     |
| 17   | 56  | M      | N     | ND    | 3.0 \(\times\) 10^{3} | N    | CP     |

* P, positive; N, negative; ND, not determined.

Table 2  Detection of several messages and K-ras mutations in pancreatic juice from cases without pancreatic cancer

<table>
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<th>Case</th>
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<th>CD25</th>
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<td>N</td>
<td>GBS</td>
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* P, positive; N, negative; ND, not determined; NR, no remarkable abnormality; CBDS, common bile duct stone; GBCP, cholesterol polyp of the gallbladder; GBS, gallbladder stone.

Fig. 1  Semiquantitative RT-PCR with human β-actin cDNA. A, serial dilutions of cDNA from pancreatic juice were amplified with 100 copies of competitor DNA, which has exactly the same sequence as β-actin cDNA except for removal of a 100-bp fragment between the primers. A, β-actin cDNAs were coamplified from 30 to 3000 copies with 100 copies of the competitor for 30 cycles and separated through 6% polyacrylamide gels. ○ and ●, positions of the β-actin cDNA and competitor products, respectively. B, the ratio between the β-actin cDNA and competitor DNA products was logarithmically plotted against a known amount of β-actin cDNA subjected to the PCR. A linear standard curve was obtained from 30 to 950 copies of β-actin cDNA. The function of this line was expressed as \(F(x) = 66.1x^{1.33}\). C, representative electrophoresis patterns for the semiquantification of β-actin cDNA. In cases 16 and 17, Lanes 1 and 3, respectively, the band intensity of β-actin cDNA (○) was ~2.5-fold weaker than that of the 100 copies of the competitor DNA (●). Because coamplification of 30 copies of β-actin cDNA and 100 copies of the competitor DNA produced the bands with a 0.59:1 intensity ratio, the amount of β-actin cDNA amplified in cases 16 and 17 was <30 copies. In contrast, in case 4 (Lane 2), the intensity of the β-actin cDNA band was 3.2-fold higher than that of the competitor DNA. Lane M, 100-bp ladder including spike at 1000 bp. Lanes 4 and 5 revealed independent amplification of the competitor DNA or β-actin cDNA, respectively.
hTERT Message in CD25-positive Peripheral Lymphocytes. The pancreatic juice samples in which hTERT message was detected without any evidence of PC were collected immediately after acute aggravation of chronic pancreatitis. We therefore assumed that the activated lymphocytes in the pancreatic juice may account for the hTERT message in these cases. To evaluate this possibility, we first analyzed whether hTERT message was preferentially detected in activated lymphocytes circulating in the peripheral blood stream. The peripheral lymphocytes from a normal volunteer were separated into CD25-positive and -negative groups by a fluorescence activated cell sorter, using an antibody against the α chain of the interleukin 2 receptor (Fig. 2). Telomerase activity was not detected in either group of lymphocytes using a nonisotopic TRAP assay on half of the recovered cells (5 × 10^5 cells). However, analysis of the remaining cells detected hTERT message in CD25-positive lymphocytes but not in the negative cells.

CD25 mRNA in Pancreatic Juice. CD25 message was detected in 3 of 17 PC cases, 3 of 12 CP cases, and in none of the 7 normal controls. Intriguingly, this message was detected in both of the 2 cases expressing hTERT message in the absence of PC (Fig. 3). Taken together with the quantification of β-actin message, the detection of hTERT mRNA in pancreatic juice to diagnose PC should be limited in its application to samples from which sufficient mRNA is recovered and which have a negligible level of activated lymphocyte contamination. Finally, the overall accuracy of this strategy reached 100% in the present study, when there were 28 sample cases in total: 12, 9, and 7 cases with PC, CP and N, respectively.

**DISCUSSION**

Detection of telomerase activity in pancreatic juice has been proposed as a useful tool in the diagnosis of PC (19–21). Unfortunately, several problems exist when applying this technique clinically. The method necessitates the use of radioisotopes. We tried unsuccessfully to detect TRAP products from the pancreatic juice of 10 patients with definite PC on polyacrylamide gel with GelStar staining, which provides a 10-fold higher sensitivity than ethidium bromide (data not shown). It was clear that the sensitivity of GelStar is insufficient because telomerase activity could not be detected, even in 5 × 10^5 CD25-positive lymphocytes in which hTERT message was detected (Fig. 2). Radioisotopic analysis of normal adult peripheral mononuclear cells detected a low level of telomerase activity in as low as 10^6 cells (26). Although TRAP products were detected...
in pancreatic juice by staining with SYBR Green, the bands were too weak to use as a diagnostic indicator in clinics (21).

The second problem is the difficulty in evaluating sample suitability for telomerase analysis. Confidence in the sample reliability is a requirement for clinical use in diagnosis in addition to the higher sensitivity and specificity. The reliability depends not only on the methodology itself but also on sample quality. For the diagnosis of PC using pancreatic juice, it appears that insufficient recovery and/or degradation of the ductal cells (27), because the ductal cells are collected under remote operation of ERP and in a suspension of pancreatic juice containing active digestive enzymes. To determine the quality and quantity of each sample with respect to recovery of mRNA, we quantified the β-actin message as an internal control. This analysis revealed that samples expressing <3.0 × 10^3 copies/µl of β-actin message in the reverse-transcribed products were not suitable for evaluation of hTERT. In such samples, hTERT message could not be detected, even in patients with advanced PC. This study, therefore, indicated that sample quality assessment is easier for detection of hTERT message than it is for TRAP analysis.

PC is quite resistant to all anticancer drugs currently available (28). Surgical resection is deemed as the only effective method to treat PC. This approach, however, is sometimes quite radical and drastically reduces the quality of the patient's life (29). In this context, the risk of a false-positive diagnosis should be minimized at all costs. It has been reported that weak telomerase activity may be detected in CP devoid of cancer (19). It was suspected that an increased number of infiltrating lymphocytes might be the cause of this activity. An up-regulation of telomerase activity without transformation was reported with presentation of antigen to T cells. In addition, the samples showing hTERT mRNA expression in the absence of cancer cells were obtained from patients with acute aggravation of CP. Furthermore, hTERT message was detected in CD25-positive but not in CD25-negative peripheral lymphocytes from a normal volunteer. Thus, we propose that removal of samples exhibiting CD25 expression from the evaluation would decrease the number of false positives. This is supported by the findings in this study that all hTERT-positive pancreatic juice samples without cancer expressed CD25 mRNA. Together, these observations strongly suggest that detection of hTERT message in pancreatic juice expressing CD25 is not suitable for cancer diagnosis by detection of hTERT message. It may be difficult to assess this type of contamination using protein in pancreatic juice.

It was reported by several others that the detection of K-ras mutations in exon 1 codon 12 is useful in the diagnosis of PC (30, 31). This mutation is, however, represented with substantial frequency in hyperplastic foci and pancreatic juice from patients with chronic pancreatitis in the absence of cancer (7–8). In fact, a K-ras mutation was detected in 4 of 7 CP cases in this study. Consequently, the specificity of detection of K-ras mutations fell to 43%. This value is consistent with that published in the literature. The detection of K-ras mutations in pancreatic juice is therefore lower in both sensitivity and specificity than the detection of hTERT message. If the samples were controlled for CD25 expression, hTERT mRNA was not detected in any of the patients with CP without cancer. To maximize the accurate and efficient diagnosis of PC, we recommend the detection of hTERT mRNA, particularly after evaluation of the sample with respect to mRNA recovery and contamination of activated lymphocytes.

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