Telomerase Elevation in Pancreatic Ductal Carcinoma Compared to Nonmalignant Pathological States

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ABSTRACT

Telomerase activity was measured in surgically resected tissues of 20 human pancreatic ductal carcinomas, 12 adenomas, 5 pancreatitis tissues, 14 normal pancreatic ducts, and 13 normal pancreatic tissues (primarily made up of acinar cells) using a PCR-based telomerase assay. Relative telomerase activity was expressed as the equivalent telomerase intensity of the number of cells of a human pancreatic cancer cell line, MIA PaCa-2, per microgram of protein in the tissue samples. The median value (25th percentile, 75th percentile) of relative telomerase activity in pancreatic carcinomas was 13.2 GB (3.58, 244), which was significantly higher relative to normal tissues, normal ducts, pancreatitis tissues, and adenomas (P < 0.0001). When the cutoff value of relative telomerase activity was set at 1.00 and 3.00, the positivity rates of telomerase activity in pancreatic carcinomas were 13.2 and 3.00, respectively. Some of the adenoma samples displayed a weak telomerase activity. However, when semiquantitatively analyzed, the relative telomerase activity of all adenoma tissues was less than 1.00 equivalent cells per microgram protein of the tissues, which was equivalent to the values encountered in normal ducts. Thus, these results indicate that telomerase expression may occur at a late stage of pancreatic ductal carcinogenesis. Therefore, telomerase may be a specific marker for distinguishing pancreatic cancer from pancreatitis and adenomas.

INTRODUCTION

Telomerase is an enzyme ribonucleoprotein responsible for cell immortality, and it synthesizes a six-nucleotide sequence designated as TTAGGG of telomeric DNA onto the chromosomal ends in germ-line cells and in immortal cells (1, 2).

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Although germ-line cells expressing telomerase activity maintain telomeric repeats, somatic cells lose telomeres progressively as the cells undergo cell division (3, 4), which may lead to cellular senescence due to the repression of telomerase activity (5, 6). Cancer cells appear to attain immortality with the reactivation of telomerase (7).

The conventional assay for telomerase activity requires a large quantity of cells or tissue (8), which in itself is a limitation in the investigation of the role of telomerase in carcinogenesis. Recently, a highly sensitive PCR-based assay was developed to detect telomerase activity in tissue extracts derived from a very small number of immortal cells (7). By this method, telomerase activity was detected in approximately 85–90% of various carcinoma tissues such as lung cancer (9), colorectal cancer (10), hepatocellular carcinoma (11), gastric cancer (12), and breast cancer (13). In addition, in cultured cells, 98 of 100 immortal cell populations and none of 22 mortal cell populations expressed telomerase activity (7). Thus, it is apparent that telomerase is expressed in normal somatic cells and tissues but is reactivated in most immortal cells and human cancers. The results of these studies suggest that telomerase may ultimately be required to maintain cell proliferation indefinitely. Therefore, detection of telomerase activity in clinically available specimens may be of value in the diagnosis of malignant tumors.

Pancreatic cancer is one of the most aggressive malignant tumors and generally has an extremely poor prognosis (14, 15). Diagnosis of pancreatic cancer in the early stage may contribute to drastic improvement of the prognosis. Human pancreatic ductal carcinomas display variable but consistent genetic changes, including mutations of K-ras (16, 17), p53 (18), APC (19), and p16 (20). If such genetic changes can be detected using a small number of cells, they can serve as a clinical marker to the diagnosis of cancer. K-ras mutations have been detected in the pancreatic juice obtained by endoscopic retrograde pancreatography in 55 to 100% of patients with pancreatic cancer (21–24). However, K-ras mutations are present in noncancerous tissues such as ductal hyperplasia (25) and adenoma (26, 27) and, therefore, are of limited value as a clue to the diagnosis of pancreatic cancer.

We reported previously that telomerase is highly activated in human pancreatic ductal carcinoma when investigated by a semiquantitative modified PCR-based assay (28). Apparently, telomerase is a new prevalent marker for human pancreatic ductal carcinoma. However, it is not known at which step of pancreatic carcinogenesis telomerase is reactivated. If telomerase is activated exclusively in cancer and repressed in preneoplastic lesions of pancreatitis or adenoma, telomerase could well be a specific marker of pancreatic cancer. In the present study, relative values of telomerase activity were measured in surgically resected tissues of pancreatic carcinoma, adenoma, pancreatitis, normal pancreatic ducts, and normal pancreatic tissues to evaluate the putative role of telomerase in the diagnosis of pancreatic cancer.
Table 1  Relative values of telomerase activity in patients with pancreatic adenomas and carcinomas

<table>
<thead>
<tr>
<th>Case</th>
<th>Histology</th>
<th>Age</th>
<th>Sex</th>
<th>Staging</th>
<th>Differentiation</th>
<th>Location</th>
<th>Relative telomerase activity</th>
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<tbody>
<tr>
<td>25</td>
<td>Serous cystadenoma</td>
<td>82</td>
<td>F</td>
<td>Pt</td>
<td>T_{1,0}N_{0}M_{0}</td>
<td>Ph</td>
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<td>35</td>
<td>Intraductal papillary adenocarcinoma</td>
<td>72</td>
<td>F</td>
<td>T_{1,0}N_{0}M_{0}</td>
<td>Ph</td>
<td>196</td>
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<tr>
<td>59</td>
<td>Tubular adenocarcinoma</td>
<td>77</td>
<td>M</td>
<td>T_{1,0}N_{0}M_{0}</td>
<td>Well</td>
<td>518,000</td>
<td></td>
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<tr>
<td>65</td>
<td>Tubular adenocarcinoma</td>
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<td>F</td>
<td>T_{1,0}N_{0}M_{0}</td>
<td>Well</td>
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<td>Well</td>
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<td>Ph</td>
<td>5.19</td>
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<td>12</td>
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<td>Ph</td>
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MATERIALS AND METHODS

Cell Lines and Tissue Samples. The human pancreatic cancer cell line MIA PaCa-2, generously provided by Japanese Cancer Resources Bank (Tokyo, Japan), was cultured in DMEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with penicillin (1000 units/ml), streptomycin sulfate (100 μg/ml), and 10% heat-inactivated fetal bovine serum.

Tissue samples were obtained at the time of surgery either at Kyushu University Hospital (Fukuoka, Japan) or at affiliated hospitals. Specimens were from 20 primary pancreatic ductal carcinomas, including 2 intraductal papillary adenocarcinomas, 16 tubular adenocarcinomas (7 well, 6 moderately, and 3 poorly differentiated adenocarcinomas), a mucinous cystadenocarcinoma, and an anaplastic adenocarcinoma; 12 pancreatic adenomas, including 4 serous cystadenomas, 2 mucinous cystadenomas, and 6 intraductal papillary adenomas; 5 pancreatitis tissues; 14 normal main pancreatic ducts; and 13 normal pancreatic tissues (primarily made up of acinar cells). They were removed as soon as possible after resection and were stored at −80°C until use. Normal pancreatic ducts were taken from the main pancreatic duct in patients with pancreatitis and from the main ducts apart from the tumor in patients with neoplasms.

Normal pancreatic tissue samples were collected from a peripheral soft part of the pancreas in the same manner. The tissue samples in pancreatitis were primarily from a hard portion of the pancreatic mass including the stenotic ducts. The samples of serous cystadenomas or mucinous cystadenomas were obtained from the cyst wall. In patients with intraductal papillary adenomas, the affected ducts were opened, and a sample was taken from a macroscopically distinct tumor. The tissues of pancreatic carcinoma were excised from a hard portion of the tumors. All tissues adjacent to the specimens were histologically examined, and the diagnosis was confirmed. In cases of unresectable pancreatic carcinomas such as were found in patients 12, 69, 93, and 121 (Table 1), needle biopsies were performed at laparotomy, and a portion of the same sample was diagnosed histologically. The definitions of stage grouping, histological classification, and lymphatic metastasis of pancreatic cancers were made according to the UICC-TNM classification (29).

The abbreviation used is: UICC-TNM, International Union against Cancer, Tumor-Node-Metastasis.
Telomerase Assay. Telomerase activity was measured as described previously (28). Briefly, the cell pellets from MIA PaCa-2 were suspended with CHAPS lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and protease inhibitors. The frozen pancreatic tissues were washed once in ice-cold PBS and also homogenized with CHAPS lysis buffer in microtubes with matching pestles. The supernatants (CHAPS cell extract) were stored at −80°C until use. Protein concentration of the tissue extract was measured by Bradford assay (30), and 6 or 0.6 μg extract was used for each telomerase assay. Telomerase activity was assayed by the modification of the PCR-based telomerase assay as described by Kim et al. (7) and Tahara et al. (31). The CHAPS cell extracts were incubated with reaction buffer containing 50 μM deoxynucleotide triphosphates, 0.3 μCi of [α-32P]dCTP, 2 units of Taq DNA polymerase (Promega Corp., Madison, WI), and 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTF-3′). During this step, 0.1 μg of CX primer (5′-CCCTTACCTTACCTTACCTTACCTA-3′) was added, and the reaction mixture was subjected to 31 PCR cycles. Telomerase activity was detected as a 6-base ladder signal that disappeared with RNase (Boehringer-Manheim Corp., Manheim, Germany) pretreatment, indicating that the reaction was specific for telomerase activity (see Fig. 1). MIA PaCa-2 extracts equivalent to 1, 10, 10², and 10³ cells were always measured as a standard per assay, and relative values of telomerase activity were expressed as the equivalent telomerase intensity of the number of MIA PaCa-2 cells per microgram protein in each sample as described previously (28). Signal intensity of the 6-base ladder was measured by NIH image, version 1.59 (NTIS, Springfield, VA). A 36-bp internal standard (Oncor, Inc., Gaithersburg, MD) was used as an internal control. Logarithmic values of the relative telomerase activity were statistically analyzed by one-way ANOVA and Fisher’s test because they showed a normal distribution.

RESULTS

Efficiency of the PCR reaction was confirmed with the detection of a 36-bp internal standard (Fig. 1). Telomerase activity in the human pancreatic cancer cell line MIA PaCa-2 was detected as a six-nucleotide repeat ladder, and the signal intensity was reduced according to the decrease in the number of cancer cells by dilution. Regression analysis was performed between logarithmic values of the cell numbers and telomerase intensity, and the correlation coefficient always exceeded 0.9.

A definite telomerase ladder was not observed in all normal pancreatic tissue and duct specimens (Fig. 1a), and the median values (25th percentile, 75th percentile) of relative telomerase activity expressed by the equivalent telomerase intensity of the number of MIA PaCa-2 cells were 0.25 (0.20, 0.45) and 0.31 (0.14, 0.50), respectively. In all pancreatitis sample extracts, the telomerase ladder was also undetectable, and the median value (25th percentile, 75th percentile) of relative telomerase activity was 0.17 (0.09, 0.46). All relative values of telomerase activities of normal pancreas, normal ducts, and pancreatitis were under 1.00 equivalent cells per microgram of tissue protein.

A telomerase ladder was indistinct in most pancreatic adenomas. However, some of the adenomas presented weak telomerase ladder signals, which would have been regarded as positive had they been assessed by simple qualitative telomerase analysis. Semiquantitatively analyzed, relative telomerase activities of each adenoma are listed in Table 1. All values of adenomas were under 1.00 equivalent cells per microgram of tissue protein. The median value (25th percentile, 75th percentile) in adenomas was 0.44 (0.30, 0.68), which was slightly higher than that encountered in normal tissues and ducts but not significantly different from these two groups. Mucinous cystadenomas and intraductal adenomas are generally believed to have a greater malignant potential than serous cystadenomas. However, telomerase activity was not significantly different between these two groups (Table 2).

A telomerase ladder was clearly detected in almost all pancreatic ductal carcinomas (Fig. 1a). All of the values of relative telomerase activity in carcinomas were over 1.00 equivalent cells per microgram of tissue protein and varied from 1.98 to 518.000. The median value (25th percentile, 75th percentile) of relative telomerase activities of ductal carcinomas was 13.2 (3.58, 244), which was significantly higher than that encountered in normal tissues, normal ducts, pancreatitis, and adenomas (P < 0.0001). Relative values of telomerase activity of all samples are summarized in Fig. 2 by means of a box-and-whisker plot analysis, which clearly demonstrates exclusive activation of telomerase in pancreatic ductal carcinomas. With regard to the histological differentiation in pancreatic ductal carcinomas, there were no significant differences among intraductal papillary, well, moderately, and poorly differentiated adenocarcinomas. However, relative telomerase activity in the group of intraductal papillary and well-differentiated adenocarcinomas was significant when compared to the group of moderately to poorly differentiated adenocarcinomas (Table 2; P < 0.05). In terms of staging of the disease, the median value of telomerase activity in pancreatic ductal carcinomas in the T1-T2 group tended to be higher than that in the T3-T4 group, although the probability value of the difference was 0.0544. There was no significant difference between the locations of the carcinomas, i.e., pancreatic head versus tail.

Telomerase activity was measured mixing carcinoma tissues with normal ducts to evaluate the effect of normal cells on the telomerase ladder. The intensity of the telomerase ladder of carcinoma was reduced to some extent, consistent with the inclusion of normal ducts (Fig. 1b).

DISCUSSION

The present study has revealed that telomerase was definitely activated in almost all pancreatic ductal carcinomas. In addition to the repression of telomerase in normal pancreatic tissues, normal ducts that are precursor cells of ductal carcinomas did not show any telomerase ladder. When the cutoff value of relative telomerase activity was set at 1.00 and 3.00, the positivity rate of pancreatic ductal carcinomas was 100 and 80%, respectively. Furthermore, relative values of telomerase activity in pancreatitis or adenomas were comparable to those of normal ducts. These results suggest that the possibility that telomerase could be a new prevalent marker for pancreatic ductal carcinoma.
Relative values of telomerase activity in pancreatic ductal carcinomas varied from 1.98 to 518,000. The wide variation of the results does not mean instability of the assay per se. Radioactivity of $^{32}$P is unstable and, as such, difficult to maintain at the same level in each analysis. However, determination of telomerase activity in a human pancreatic cancer cell line can standardize the results of each assay. Nearly the same activity value was obtained when one sample was analyzed several times. Pancreatic cancer tissues include variable numbers of cancer cells along with abundant stromas with fibroblasts, which may vary from sample to sample. The study involving mixing of carcinoma tissues and normal ducts revealed that normal cells could reduce the intensity of the telomerase ladder of carcinomas (Fig. 1b). In addition to the difference in the number of carcinoma cells, the difference in the proportion of malignant cells to normal cells in the tissue could be one of the possible explanations for the wide variation in the telomerase activity in pancreatic ductal carcinomas.

Adenoma-carcinoma sequence can be applied in pancreatic carcinogenesis as indicated in hamster models of pancreatic carcinogenesis (32). Clinically, mucinous cystadenoma and intraductal papillary adenoma are presumed to have malignant potential. Some adenomas showed a clear 6-base ladder, but the intensity of the signal was much weaker when compared to any of pancreatic ductal carcinomas. Recently, it was reported that weak telomerase activity was detected in precancerous lesions, for example, in 23% of gastric intestinal metaplasias and 50% of gastric adenomas (31). In colorectal lesions, telomerase activity was detected in 0% (10) or 100% (31) in adenomas by qualitative analysis. There is still a possibility that weak telomerase activity may also be detected in pancreatic adenomas as more patients with adenoma are evaluated. However, the notable point is that there is a clear difference in telomerase activity expressed between adenomas and carcinomas.

Of practical clinical importance is the distinction between chronic pancreatitis and pancreatic cancer. It has been pointed out that mass-forming pancreatitis masquerades as pancreatic cancer (33). A preoperative diagnostic tool is sorely needed to
Table 2  Relative telomerase activity in patients with normal pancreatic ducts, adenomas, and carcinomas

<table>
<thead>
<tr>
<th>Lesion</th>
<th>No. of samples</th>
<th>Relative telomerase activitya Median (25th percentile, 75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ducts</td>
<td>14</td>
<td>0.31 (0.14, 0.50)</td>
</tr>
<tr>
<td>Adenomas</td>
<td>12</td>
<td>0.44 (0.30, 0.68)</td>
</tr>
<tr>
<td>Serous cystadenomas</td>
<td>4</td>
<td>0.35 (0.22, 0.55)</td>
</tr>
<tr>
<td>Mucinous cystadenomas and intraductal papillary adenomas</td>
<td>8</td>
<td>0.50 (0.31, 0.75)</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>20</td>
<td>13.2 (3.58, 244)</td>
</tr>
</tbody>
</table>

  Differentiation

  - intraductal papillary and well-differentiated adenocarcinomas: 9 samples, median 196 (13.5, 1520)
  - moderately and poorly differentiated adenocarcinomas: 9 samples, median 3.83 (2.26, 21.8)

  T classification

  - T1-T2: 9 samples, median 196 (4.35, 1860)
  - T3-T4: 11 samples, median 5.27 (3.35, 21.7)

  Location

  - Head: 15 samples, median 5.27 (3.50, 260)
  - Body-tail: 5 samples, median 17.5 (5.64, 514)

  a Relative values of telomerase activity are expressed as the equivalent telomerase intensity of the number of cells of MIA PaCa-2 per microgram of protein in the tissue samples.

  b Significantly different from groups of normal ducts and adenomas (P < 0.0001).

  c Significantly different from a group of intraductal papillary and well-differentiated adenocarcinomas (P < 0.05).

  d Tumor stage is assessed according to UICC-TNM staging (29).

Fig. 2 Relative values of telomerase activity. Relative values of telomerase activity are expressed as the equivalent telomerase intensity of the number of cells of MIA PaCa-2 per microgram protein of samples. The results are expressed by means of a box-and-whisker plot analysis. The bottom and top edges of the box are located at the sample 25th and 75th percentiles, respectively. The center horizontal line is drawn at the sample median. The center vertical lines from the box extend to a distance of the 10th or 90th percentiles. The relative values of telomerase activity in carcinoma samples are significantly higher than those in other tissue samples (P < 0.0001).

discriminate pancreatic cancer from chronic pancreatitis. The present study demonstrated that telomerase was exclusively activated in the tissues of pancreatic carcinomas while being completely repressed in chronic pancreatitis. The future study of telomerase activity on cell samples in pancreatic juice obtained preoperatively by endoscopic retrograde pancreatectomy is warranted.

In regard to the staging of pancreatic cancer, relative telomerase activity tended to be higher in the T1-T2 group than in the T3-T4 group. In the T1-T2 group, the unique composition of the far-advanced pancreatic carcinoma, consisting of a large amount of fibrous stroma, necrotic tissue, and mucin in some cases, along with a relatively small number of cancer cells, may account for the lower telomerase activity. Telomerase activity was significantly higher in the group of intraductal papillary and well-differentiated adenocarcinomas when compared to the group of moderately to poorly differentiated adenocarcinomas. Considering that telomerase activity correlates to cellular immortality, a patient with pancreatic cancer presenting with high telomerase activity will have a poor prognosis. It has been reported that the survival rate of gastric cancer tumors with telomerase activity is significantly shorter compared to gastric cancer tumors without telomerase activity (12). Similarly, neuroblastoma patients presenting with high telomerase activity have an unfavorable prognosis compared to cases of neuroblastoma with low telomerase activity (34). To our knowledge, there have been no reports of a clear relationship between histological differentiation and outcome of pancreatic cancer. Furthermore, no previous study has demonstrated an unfavorable prognosis for intraductal papillary and well-differentiated adenocarcinomas. The group of intraductal papillary and well-differentiated adenocarcinomas consisted of a relatively dense cellular component of carcinoma. On the other hand, the group of moderately to poorly differentiated adenocarcinomas consisted of relatively abundant stromas. The difference in telomerase activity among cancer cell populations may indeed be affected by such proportional differences between malignant cells and normal cells.
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