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Increased Telomerase Activities in Human Pancreatic Duct Adenocarcinomas

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Telomerase is a key enzyme with regard to immortalization of cancer cells and increased activity has been demonstrated in various human malignant neoplasms. Since little is known of its role in pancreatic cancers, we investigated changes in telomerase activity in human pancreatic duct adenocarcinomas and compared the frequency of increased telomerase activity with the presence of K-*ras* gene mutations. The samples were obtained from 38 pancreatic duct adenocarcinomas and 7 tumor surrounding tissues at surgical resection. Telomerase activity was examined by telomeric repeat amplification protocol assay and terminal restriction fragment (TRF) length was examined by Southern analysis. K-*ras* mutation was examined by means of polymerase chain reaction-single strand conformation polymorphism analysis. Among 38 pancreatic carcinomas, 32 (84%) exhibited increased telomerase activities with no apparent relation to the histological type of tumor, tumor size, regional lymphnode involvement and distant metastasis or clinical stage. In tissue surrounding the tumor, telomerase activity was not detected. TRF length tended to be reduced in pancreatic carcinomas. Mutations of K-*ras* gene were found in 24 out of the 38 (63%) cases. Among the 38 cases, 14 showed increased telomerase activity without K-*ras* mutation and 4 cases showed K-*ras* mutation without telomerase activity. These results suggest that increased telomerase activity might be a sensitive genetic diagnostic marker and could be a target for future therapy of pancreatic duct carcinomas.

Key words: Telomerase — K-*ras* — Pancreatic carcinoma — Genetic diagnosis

Adenocarcinoma of the pancreatic duct is the fifth leading cause of cancer death in the USA and Japan.[1,2] Development of the disease is clinically silent so that at the time of diagnosis, the vast majority of cases are incurable with a very poor prognosis. Therefore it is important to establish new methods for early detection and therapy of pancreatic carcinomas.

Telomeres are tandem arrays of guanine-rich repetitive motifs at the ends of chromosomes. They have been highly conserved throughout evolution and are functionally necessary for chromosome stability.[3,4] The ribonucleoprotein enzyme, telomerase, which synthesizes TTAGGG nucleotide repeats in vertebrates, has been suggested to be required for chromosome stabilization and acquisition of immortality.[5,6] Recently, Kim *et al.* demonstrated an intriguing link between neoplasia and increased telomerase using a new, highly sensitive assay for activity of the enzyme.[5]

In various human neoplasias, telomere reduction and increased telomerase activity have been observed. Telomere reduction has been described to occur with aging of human fibroblasts[7,8] and in colorectal,[9] ovarian,[10] renal cell,[11] and hepatocellular[12] carcinomas as well as leukemias.[13,14] High frequencies of elevated telomerase have also been found for carcinomas of the lung,[15] prostate,[16] stomach,[17] colon,[18] liver,[19] brain,[20] and ovary,[10] in addition to lymphomas,[21] the Wilms tumor,[5] rhabdomyosarcomas,[5] and leiomyosarcomas.[5] A correlation between shortened telomere length and augmented telomerase activity has been established for hepatocellular and ovarian carcinomas.[10,19] The results suggest that telomerase may play a critical role in progression or maintenance of the malignant state.

Mutations of the K-*ras* gene are more frequently detected in pancreatic carcinomas than in other cancers, constituting the most common genetic alteration in pancreatic carcinomas. Therefore, in the present study, we investigated telomerase activity and K-*ras* gene mutations to obtain basic genetic information which might be useful for early diagnosis and therapy of pancreatic duct adenocarcinomas.

MATERIALS AND METHODS

Tissues The samples were obtained by surgical resection of 38 pancreatic ductal adenocarcinomas and 7 samples of adjacent tissues. Details of TNM classification, tumor size (TS), regional lymphnode metastasis (N), and distant metastasis (M) according to criteria of the Japanese Pancreatic Society[22] and UICC clinical stages[23] are

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shown in Table I. The histological types of pancreatic cancers diagnosed according to the criteria of the Japan Pancreatic Society,[22] are given in Table II. Small portions of tumor and non-tumor tissues weighing approximately 20 mg, were taken, with care to avoid contamination with necrotic areas, and frozen immediately in liquid nitrogen. All samples were stored at −80°C until used. The frozen tissue was powdered in liquid nitrogen and divided into 2 tubes for the assay of telomerase activity and for the detection of K-*ras* mutations.

Telomerase assay Powdered tissue was homogenized by hand with a Teflon pestle in cold lysis buffer as described by Kim *et al.*[5] After 30 min on ice, the lysates were centrifuged at 12,000 rpm for 20 min at 4°C and the supernatants stored at −80°C. The protein concentration was determined with a DC protein assay kit (Bio Rad Laboratory, Richmond, CA). Extracts containing 6 μg of protein were used for telomerase assay, activity being assayed by the telomeric repeat amplification protocol (TRAP) method[5,18] with minor modifications. In brief, the tissue extract was incubated with 20 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 0.005% Tween-20, 1 m*M* EGTA, 50 m*M* deoxynucleoside triphosphate, 0.1 μg of TS primer sequence (5′-AATCCGTCGAG-CAGATTT-3′), 1 μg of T4g32 protein (Boehringer Mannheim, Mannheim, Germany), 0.1 mg/ml of BSA, 3 units of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden) and 0.4 μl of [α-^{32}P]dCTP at 20°C for 30 min. Then the mixture was heated to 90°C for 3 min to inactivate the telomerase activity and 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) was added. The polymerase chain reaction (PCR) procedure was performed with 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 45 s, followed by 72°C for 8 min. To determine the sensitivity to RNase, some samples were incubated with 1 μg of RNase A for 30 min at 37°C, and used for the TRAP assay. As negative controls, mixtures without TS or CX primers were also included. The PCR products (15 μl) were electrophoresed on 10% non-denaturing polyacrylamide gels in 0.5× Tris-borate EDTA buffer. The gels were dried and processed for autoradiography with overnight exposure at −80°C. Relative telomerase activity was estimated according to the criteria described previously[19]: strong, detectable in ×100 diluted samples (0.06 μg protein/assay); moderate, detectable in ×10 diluted samples (0.6 μg protein/assay); weak, detectable in ×1 diluted samples (6 μg protein/assay); negative, not detectable in ×1 diluted samples (6 μg protein/assay) after exposure for 2 days. Relative telomerase activity was confirmed by semi-quantitation of telomerase activity using a Fujix BAS 1000 phosphoimager (Fuji Photo Film Co., Ltd., Kanagawa).[24]

Southern blot analysis Terminal restriction fragment (TRF) length was examined by Southern blot analysis, as described previously.[12] Briefly, DNA was prepared from

Table I. Incidences of Increased Telomerase Activity and K-*ras* Mutation in Pancreatic Adenocarcinomas Classified by UICC Classification and TNM Classification[a]

		No. of cases (total: 38 cases)	No. with increased telomerase activity	No. with K-*ras* mutation
UICC stage	I	4	3	3
	II	5	5	3
	III	15	12	10
	IV	14	12	8
TS	1	5	5	3
	2	18	14	13
	3	7	6	5
	4	8	7	3
N	0	10	9	6
	1	18	14	12
	2	10	9	6
M	0	23	19	15
	1	15	13	9

a) TNM classification was according to the criteria of the Japanese Pancreatic Society. TS: tumor size (TS1≤2 cm, 2 cm<TS2≤4 cm, 4 cm<TS3≤6 cm, TS4>6 cm). N: regional lymphnode metastasis. M: distant metastasis.

Table II. Level of Telomerase Activity in Pancreatic Adenocarcinomas

Histological type	No. of cases	Level of telomerase activity			
		strong	moderate	weak	negative
Invasive ductal carcinoma	38	6	11	15	6
Papillary adenocarcinoma	3	0	2	1	0
Tubular adenocarcinoma					
well differentiated type	7	2	1	3	1
moderately differentiated type	22	3	5	9	5
poorly differentiated type	6	1	3	2	0
Tumor-surrounding tissue	7	0	0	0	7

Histological type was classified according to the criteria of the Japanese Pancreatic Society.

frozen tissues of 14 pancreatic carcinomas, 2 samples of adjacent pancreatic tissues, and 8 non-tumor tissues (stomach or colon) in age-matched patients. *Hinf* I-digested DNA was electrophoresed in 0.5% agarose gels, then transferred to nylon membranes. Southern hybridization was performed using ^{32}P-labeled $(TTAGGG)_4$ oligonucleotide probe. The profile of TRF length was analyzed with a Fujix BAS 1000 phosphoimager.

K-*ras* assay DNA extraction and PCR-single strand conformation polymorphism (SSCP) analysis to detect K-*ras* gene mutations were performed using the procedures described previously.[25,26] The primers applied for amplification of K-*ras* exon 1 were 5′-GGAATTCGACTGAATATAAACTTGTGG-3′ and 5′-GGAATTCCTGCACCAGTAATATGC-3′ which yield a 159-base-pair amplified DNA fragment including codons 12 and 13. PCR products were heated at 80°C for 3 min in formamide-containing loading buffer and electrophoresed on 5% polyacrylamide gels containing 0.5× Tris-borate EDTA buffer and 10% glycerol at 30 W for about 5 h at a constant temperature of 30°C. The results of SSCP analysis were confirmed by direct DNA sequencing using established methods.[24,25]

Statistics Comparison of frequencies and homogeneity among groups was done by using Fischer's exact test and the criterion of statistical significance was set conventionally at $P<0.05$.

RESULTS

The incidences of increased telomerase activity and K-*ras* mutation in pancreatic duct carcinomas are shown in Table I. Increased telomerase activities were detected in 32 of the 38 cases (84%). There was no correlation with clinical stage, or TS, N, and M factors. Representative patterns of telomerase activity under the standard conditions of the TRAP assay are shown in Fig. 1. Data for histology and the level of telomerase activity are shown in Table II. Relative telomerase activity according to the criteria described by Tahara *et al.*[19] was well correlated with the densitometric quantitation of telomerase activity. The results of densitometric quantitation of telomerase activity were as follows: in the weak group, 8.2–22.1 phosphostimulating luminescence–background (P–B)/mm^2 (mean±SD was 13.2±4.3); in the moderate group, 22.3–50.1 (P–B)/mm^2 (28.7±8.3); in the strong group, 65.8–120.4 (P–B)/mm^2 (89.2±20.5). No link between the telomerase activity and histological type of adenocarcinoma, clinical stage or TS, N, and M factors was found (data not shown).

PCR-SSCP analysis demonstrated K-*ras* gene mutations in 63% (24 out of 38) of the pancreatic adenocarcinomas examined. Direct sequencing revealed G-to-A transitions at the second position of codon 12 in 16 cases and G-to-T transversions at the second position of codon 12 in 8 cases. A comparison of the frequencies of increased telomerase activity and K-*ras* mutations is shown in Table III. The enzyme activity was elevated in 12 cases without K-*ras* mutation and K-*ras* mutations were found in 4 cases without increased telomerase. In these 4 cases, specific findings were not observed. The histological type of these 4 cases was moderate differentiated tubular adenocarcinoma. In 3 cases, regional lymphnode metastasis and in one case, liver metastasis were observed. There was no positive correlation between increased telomerase activity and K-*ras* mutation.

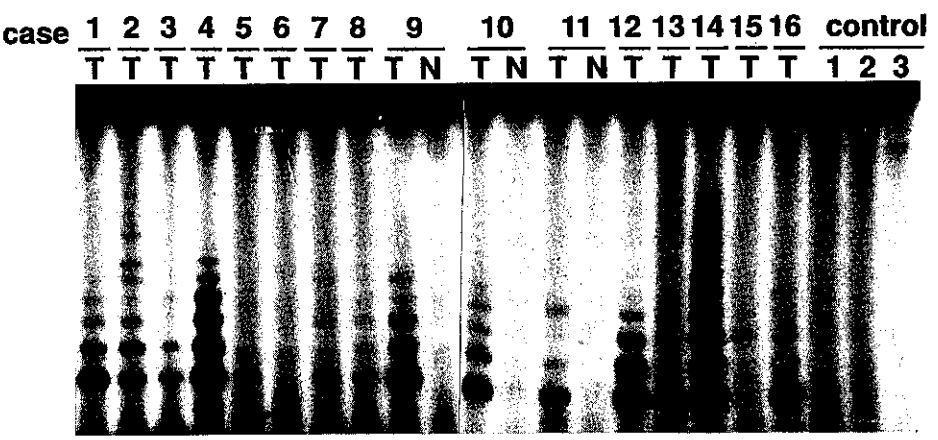

Fig. 1. Representative results of examination of telomerase activity in pancreatic adenocarcinomas using the standard TRAP assay. Case numbers refer to the patients' code; T indicates cancer and N noncancerous tissue from the same patient. Control: lane 1, TRAP assay with RNase A treatment; lane 2, TRAP assay without TS primer; lane 3, TRAP assay without CX primer (see "Materials and Methods").

Table III. Comparison of Increased Telomerase Activity and K-*ras* Mutation in Pancreatic Adenocarcinomas

Telomerase activity (%)		K-*ras* mutation	
		Negative (%) 14/38 (37)	Positive (%) 24/38 (63)
Not increased	6/38 (16)	2/38 (5)	4/38 (10)
Increased	32/38 (84)	12/38 (32)	20/38 (53)

Table IV. Peak of TRF Length in Pancreatic Carcinomas

	No. of cases examined	Peak of TRF length (kb, mean±S.D.)
Pancreatic carcinoma	14	9.0±1.1
telomerase activity (−)	3	8.8±1.1
telomerase activity (+)	11	9.1±1.2
Non-tumor tissue	10	9.6±0.7

Results of TRF length analysis are shown in Table IV. TRF length tended to be reduced in pancreatic carcinomas, compared to non-tumor tissues. There was no correlation between TRF length and telomerase activity. Moreover, there was no correlation between TRF length and clinical stage or histological type of carcinoma (data not shown).

DISCUSSION

In the present study, we investigated changes in telomerase activity and the presence of K-*ras* mutations in pancreatic carcinomas of the most common type. Telomerase may be a key player in cancer cell immortalization.[27] It has been reported that some 90–95% of late-stage malignant tumors are telomerase-positive but so far no data have been available for pancreatic duct adenocarcinomas. Our series of 38 cases included 4 at stage 1 and 5 at TS 1, which are thought to be early pancreatic cancers, but no correlation was found between positivity and tumor progression. With pancreatic cancers, the 5-year survival has been found to be only 36%, even for so-called early TS 1 cancers, less than 2 cm in diameter.[28] The present results imply that an increase in telomerase activity can occur not only in an early stage, but also in a late progression stage of human pancreatic carcinogenesis. This is in line with the detection of telomerase activation in precancerous lesions for gastric cancers, such as intestinal metaplasia and adenomas.[29] To clarify the role of telomerase in pancreatic carcinogenesis, further studies are needed to determine the critical step for telomerase activation and the underlying mechanisms. In pancreatic carcinomas, marked reduction of telomere length was not observed, compared with other carcinomas.[9, 12, 13] One possibility is that there are abundant normal cells, such as fibroblasts and lymphocytes, in pancreatic carcinomas. This idea is supported by findings in experimental pancreatic cancers and cell lines.[24] In pancreatic carcinomas without telomerase activation, other mechanisms might exist for the maintenance of telomere length. However, in this study, no specific biological behavior of pancreatic carcinomas without telomerase activity was observed.

Mutations of the K-*ras* gene are more frequently detected in pancreatic carcinomas (60–90%) than in other cancers, and they constitute the most common genetic alteration in pancreatic carcinomas.[30, 31] In this study, the frequency of K-*ras* mutations is relative low, compared with previous reports. The reason for this is not clear, though it was recently reported that the frequency of K-*ras* mutations is not so high (approximately 60%) in pancreatic carcinomas.[32] K-*ras* gene mutation can be detected by examination of the pancreatic juice, peripheral blood, and stool.[33–35] However, such mutation may also be found in duct epithelial hyperplasia, which is not cancer, but rather a possible pre-neoplastic lesion.[36, 37] Therefore, the use of K-*ras* mutation assay for diagnosis of pancreatic cancer is controversial. Animal model studies using nitrosamine-induced pancreatic duct adenocarcinoma in hamster clearly show that K-*ras* mutation is an early stage and *p53* mutation is a late stage of pancreatic duct carcinogenesis.[38–40]

The lack of a positive correlation between increased telomerase activity and K-*ras* mutation in the present study suggests that telomerase activation and K-*ras* mutation occurred independently through different pathways. Telomerase activation probably plays some role in the immortalization and eternal proliferative capacity of cancer cells. The occurrence of a K-*ras* mutation may be more directly related to the transformation of duct epithelial cells during pancreatic carcinogenesis.

Recently, alterations of *p53*, *APC*, *p16*, and *DPC4* genes have been detected in pancreatic cancers.[41–45] It is evident that changes of telomerase activity are important alterations in pancreatic duct carcinogenesis and the significance of this enzyme for the detection and therapy of pancreatic duct adenocarcinomas needs to be fully established.

For measuring telomerase activity, the TRAP assay is very sensitive and can be applied to a small volume of sample, such as pancreatic juice, aspirate, or ascitic fluid. The present study suggests that combination assays of telomerase activity with the TRAP method and K-*ras* mutations with PCR-SSCP might be a sensitive and reasonably specific approach for the diagnosis of pancreatic cancer, suitable for use with various and minimal materials. Moreover, telomerase also has potential as a target for anticancer therapy[46] in pancreatic cancer.

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