

Telomerase Activity in Gastric Cancer¹

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Abstract

Although many genetic alterations have been reported in gastric cancer, it is not known whether all gastric tumors are capable of indefinite proliferative potential, *e.g.*, immortality. The expression of telomerase and stabilization of telomeres are concomitant with the attainment of immortality in tumor cells; thus, the measurement of telomerase activity in clinically obtained tumor samples may provide important information useful both as a diagnostic marker to detect immortal cancer cells in clinical materials and as a prognostic indicator of patient outcome. Telomerase activity was analyzed in 66 primary gastric cancers with the use of a PCR-based assay. The majority of tumors (85%) displayed telomerase activity, but telomerase was undetectable in 10 tumors (15%), 8 of which were early stage tumors. Most of the tumors with telomerase activity were large and of advanced stages, including metastases. Survival rate of patients of tumors with detectable telomerase activity was significantly shorter than that of those without telomerase activity. Alterations of telomere length (reduced/elongated terminal restriction fragments) were detected in 14 of 66 (21%) gastric cancers, and all 14 had telomerase activity. Cellular DNA contents revealed that all 22 aneuploid tumors had detectable telomerase activity. The present results indicate that telomerase activation may be required as a critical step in the multigenetic process of tumorigenesis, and that telomerase is frequently but not always activated as a late event in gastric cancer progression.

Introduction

Gastric cancer is one of the most common malignant tumors in the world, especially in Japan (1). The natural history of the disease is not fully established because gastric cancer is a heterogenous disease. Genetic analyses of gastric cancers suggest the involvement of mutations in several oncogenes and tumor suppressor genes (2). However, the specific types of mutations in these genes are poorly characterized, as is the frequency of these alterations in gastric cancer (2). There is a need to elucidate the molecular mechanisms of gastric carcinogenesis to understand its pathogenesis, to develop useful molecular markers for diagnosis, and ultimately for the development of therapeutic strategies.

Telomeres are specialized structures containing unique (TTAGGG)_n repeats at the ends of eukaryotic chromosomes that are thought to be important in the protection and replication of chromosomes (3). Lagging strand DNA synthesis at the end of linear chromosomes cannot be completed [referred to as the end replication problem (4, 5)], and this results in the progressive shortening of telomeric repeats with each cell division (5–8). Germline cells compensate for the end replication problem by expression of the enzyme telomerase (9, 10), which contains a RNA template complementary to (TTAGGG)_n repeats that permits the *de novo* synthesis of TTAGGG telomeric DNA onto chromosomal ends.

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Whereas germline cells expressing telomerase activity maintain approximately 15–20 kbp³ of these telomeric repeats, due to the repression of telomerase during development, telomeres in somatic cells *in vitro* and *in vivo* progressively shorten with each cell division (11, 12). This shortening of telomeres, in the absence of telomerase, has been proposed to be the mitotic clock by which cells count their divisions (11), and when telomeres are sufficiently short may contribute to replicative cellular senescence in somatic cells (6, 8). Although there is not a clear understanding of how shortened telomeres may contribute to cellular senescence, mechanisms proposed include a DNA damage signal from a rare telomere lacking repeats (5) or from altered expression of subtelomeric regulatory loci (12). The shortening of telomeres could, in part, lead to chromosome instability, which in turn could lead to additional genetic changes, leading to increased proliferation and reactivation of telomerase (7, 10, 12). Although the reactivation of telomerase may be insufficient for cells to proliferate indefinitely, the expression of telomerase and the stabilization of telomeres appear to be concomitant with the attainment of immortality in tumor cells (10, 11, 13).

We have previously studied the alterations of telomere length (shortened or elongated length compared with adjacent normal tissue) in a variety of malignant tumors (14–16). On the basis of the results of these studies, we proposed that telomerase activation occurs in the progression of various malignant tumors. Telomerase activity in human tumor tissues was first demonstrated in ovarian carcinoma (13). It has now been found in approximately 90% of more than 100 primary tumor biopsies from more than a dozen different tumor types (not including gastric cancer) but was absent in 50 normal somatic tissues as shown by a PCR-based telomerase assay (TRAP assay; Ref. 9). We have also studied telomerase activity in lung cancer (17) and neuroblastoma (18), which are common tumors in adult and children, and observed telomerase activation in 80 and 94% of the tumors, respectively. In addition, in cultured cells representing 18 different tissues, 98 of 100 immortal and none of 22 mortal populations expressed telomerase activity (9). Thus, telomerase activity appears to be repressed in somatic cells and tissues but is reactivated in most immortal cells and human cancers. Thus, in almost all instances immortal cells may ultimately be required to maintain tumor growth. In the present study, we measured telomere length and telomerase activity in gastric cancer.

Materials and Methods

Tissue Samples. Gastric mucosa and primary tumor samples were obtained from 66 patients with previously untreated gastric cancer undergoing operation at Hiroshima University Hospital. Tumor sizes were measured immediately after gastrectomy. All samples were obtained within 1 h after surgical removal, frozen, and stored at –80°C until use. All histological factors were evaluated by pathologists according to the criteria of the Japanese Research Society of

³ The abbreviations used are: kbp, kilobase pairs; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment.

Gastric Cancer. Because histology often varied within the same tumors, the diagnosis was based on the dominant pattern.

TRAP Assay. Frozen samples of 50–100 mg were homogenized in 200 μ l of 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate lysis buffer as described previously (9, 17, 18). After 25 min of incubation on ice, the lysates were centrifuged at $16,000 \times g$ for 20 min at 4°C, and the supernatant was rapidly frozen and stored -80°C . The concentration of protein was measured with the use of the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), and an aliquot of extract containing 6 μ g of protein was used for each TRAP assay. For RNase treatment, 5 μ l of extract was incubated with 1 μ g RNase Plus (5 Prime \rightarrow 3 Prime, Inc., Boulder, CO) for 20 min at 37°C. Assay tubes were prepared by sequestering 0.1 μ g of CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') under a wax barrier (Ampliwax; Perkin Elmer Cetus, Foster City, CA). Each extract was assayed in 50 μ l of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μ M dNTPs, 150 kBq [³²P]dCTP, 0.1 μ g of TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3'), 0.5 μ M T4 gene 32 protein (United States Biochemicals, Cleveland, OH) and 2 units of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). After a 30-min incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90°C for 90 s and then subjected to 31 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s. The PCR product was electrophoresed on a 10% polyacrylamide gel. For estimation of telomerase activity, positive extracts were reexamined by serial dilution. Extracts containing 6 μ g of protein were used for 10 \times dilution (0.6 μ g) and 100 \times dilution (0.06 μ g).

Southern Blot Analysis. Genomic DNA was isolated from normal and tumor tissues of untreated patients as described previously (14, 15). For TRF analysis, 2 μ g of DNA were digested to completion with 10 units of *Hinf*I, electrophoresed on 0.8% agarose gels, and then blotted onto nitrocellulose filters. The filters were hybridized to a ³²P-labeled (TTAGGG)₄ probe, washed as reported previously (14, 15), and then autoradiographed. We estimated the length of TRFs at the peak position of hybridization signal. To exclude the possible effect of DNA degradation, we electrophoresed undigested DNA. To exclude partial digestion, we hybridized the same filters with a β -globin or *K-ras* probe.

Flow Cytometric Analysis of Cellular DNA Content. Duplicate samples of the same frozen tumor tissues used in the TRAP assay were cut into small pieces with scissors. Suspensions of single nuclei were prepared with the use of a detergent-trypsin procedure (19) and stained with propidium iodide (Becton Dickinson, Mountain View, CA). Measurement of DNA cellular content was performed with the use of the FACScan flow cytometer (Becton Dickinson). In the analysis of 2×10^4 nuclei per each sample, a single parametric histogram was used to determine the DNA content of the tumor. The DNA index was determined by calculating the ratio of the modal channel number for tumor G₀-G₁ cells to that for normal diploid cells. A cytogram containing two or more G₀-G₁ peaks was considered evidence of aneuploidy. In some instances, when it is difficult to define the channel number of normal diploid cells, we added normal lymphocytes or normal gastric mucosa to increase the height of the normal G₀-G₁ peak. Tumors were considered tetraploid if a peak occurred with a DNA index of 1.9 to 2.1, consisting of more than 20% of the total number of analyzed cells, or if a peak corresponding to G₂-M cells of a tetraploid cell population was present.

Statistical Analysis. For statistical analysis, we divided gastric tumors into two groups: tumors with undetectable telomerase activity and tumors with telomerase activity. Between these two groups, clinical data, TRF alterations, and DNA ploidy were compared with the use of the Fisher's exact test or the χ^2 test when appropriate. Age at diagnosis and tumor sizes were compared with the use of the Mann-Whitney *U* test. The cumulative survival rates were calculated with the use of the Kaplan-Meier method, and survival curves were tested with the use of the Mantel-Cox method.

Results

Telomerase Activity and Clinical Data in Gastric Cancers. In the present study, 66 gastric cancers obtained from 66 patients without any preoperative treatments were analyzed with the use of the TRAP assay. Telomerase activity was detected in tumor tissues from 56 of 66 (85%) (Table 1). Analysis of the adjacent "normal" gastric mucosa in

Table 1 Telomerase activity in gastric cancer and adjacent gastric mucosa

	Telomerase activity ^a		
	Undetectable	Positive	Positive 10 \times diluted
Gastric cancer (<i>n</i> = 66)	10	56	34
Adjacent gastric mucosa (<i>n</i> = 66)	62	4 ^b	0

^a Positive/undetectable, positive or negative using extract containing 6 μ g of protein; positive 10 \times diluted, positive using extract containing 0.6 μ g of protein.

^b Of the 4 adjacent gastric mucosa with detectable telomerase activity, 2 had chronic gastritis.

the same patients revealed that almost all (94%) were negative for telomerase activity, and only 4 tissue samples of gastric mucosa adjacent to the cancer showed low telomerase activity levels. Because there was a variation in the intensity of the TRAP signals in the tumor specimens, we estimated the activity of telomerase by serial dilution of each extract as described previously (17, 18). The four noncancerous gastric mucosa extracts with weak telomerase activity did not have detectable activity when diluted 10-fold (with the use of extracts containing of 0.6 μ g protein). However, in the 56 tumors with telomerase activity, 34 tumors retained telomerase activity with the use of extracts of 0.6 μ g protein (Table 1) and 21 tumors retained the activity with the use of extracts of 0.06 μ g protein (Fig. 1a). This suggests that the enzyme may be activated in most gastric cancers and that a large proportion of tumors has high levels of telomerase activity.

The variations of telomerase activity may have resulted from Taq polymerase inhibitors present in the tissue extracts. To evaluate this possibility, each extract was mixed with an extract from a cell line with telomerase activity. Of 35 gastric cancer extracts with undetectable or low telomerase signals, 15 extracts inhibited the TRAP signals of the extracts to varying degrees (Fig. 1b). Normal gastric mucosa did not have evidence of inhibitors (data not shown). In this study, we divided gastric cancers into two groups: telomerase-positive tumors and those tumors in which we could not detect telomerase activity. Three samples of gastric cancers without telomerase activity completely inhibited the signals of telomerase in the mixing study, and we excluded these cases from this analysis.

We then compared clinical data in the two groups (Table 2). The age at diagnosis, sex, and histology were not different in the two groups. The degrees of tumor cell invasion in wall depth were not significantly different in the two groups, but the sizes of tumor extension with telomerase activity were significantly larger than those without telomerase activity ($P < 0.05$). The lymph node metastasis in tumors with telomerase was more frequent than those without telomerase activity ($P = 0.020$). Of the 4 cases with distant metastasis at operation, all had strong telomerase activity. In addition, 8 of 10 gastric cancers with undetectable telomerase activity were stage I. Extracts of 2 advanced stage cancers with undetectable telomerase inhibited telomerase activity incompletely in the mixing study. Thus, the possibility exists that these 2 tumors might have telomerase activity.

Overall survival for groups of patients with gastric cancer with detectable telomerase activity ($n = 56$) and without telomerase activity ($n = 10$) was computed by the Kaplan-Meier method (Fig. 2). A significantly shortened survival time ($P < 0.05$) was shown in the group with detectable telomerase activity.

TRF Analysis and Cellular DNA Content. TRFs, indicators of telomere length, were estimated at the peak position of hybridization signal with a ³²P-labeled (TTAGGG)₄ probe. To exclude possible DNA degradation, we electrophoresed undigested high molecular weight DNA and confirmed that no degradation was observed. The lengths of TRFs of adjacent gastric mucosa ranged between 7 and 15 kbp; those of gastric cancer ranged between 3 and 33 kbp. The

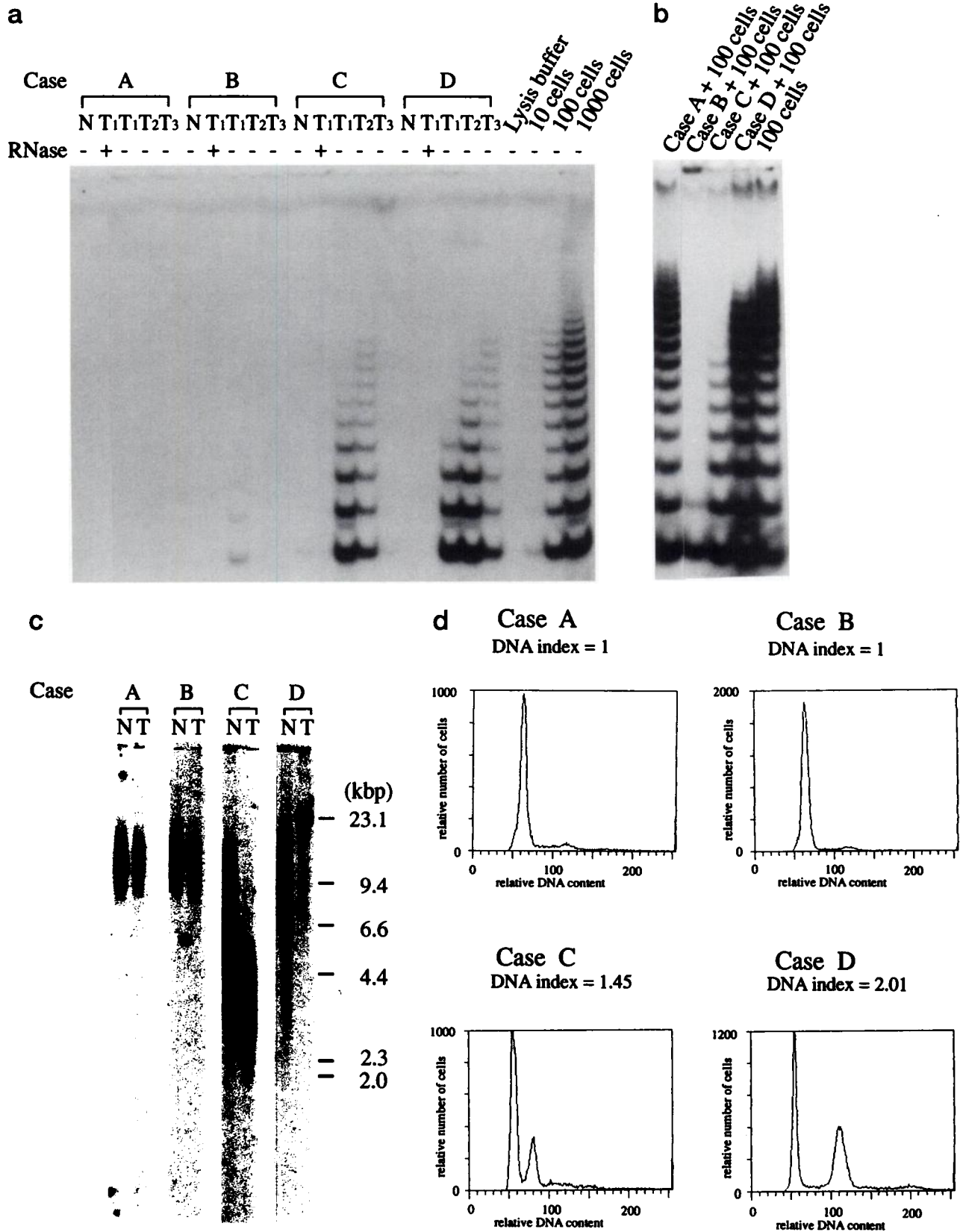


Fig. 1. Telomerase activity and terminal restriction fragments (TRFs) in gastric cancer (T) and matched adjacent gastric mucosa (N). a, telomerase activity signals with (+) or without (-) RNase pretreatment of the extract. Extracts of an human papillomavirus 16 E6/E7 immortalized human mammary epithelial cell line with telomerase activity were used as the standard. N, adjacent normal gastric mucosa; T₁, T₂, and T₃, tumor extracts that were assayed in aliquot containing 6, 0.6, and 0.06 μg of protein, respectively. Case A showed no detectable telomerase activity. Tumor samples (6 μg of protein) of case B showed weak 6-bp ladder signals, whereas cases C and D showed strong signals. Case C had weak signals using an extract containing 0.6 μg of protein, whereas case D showed strong signals using an extract containing 0.6 or 0.06 μg of protein. b, mixing of equal portions of extracts from gastric cancer tissues with extracts of a telomerase-positive cell line equivalent to 100 cells. The extract of case B had inhibitors against TRAP assay. c, analysis of TRF length in each case. Case C showed reduced TRFs, and case D showed elongated TRFs when compared to normal adjacent tissue. d, flow cytometric analysis of tumor cellular DNA content. Cases A and B showed a diploid pattern, whereas cases C and D showed an aneuploid pattern.

alterations of TRFs in gastric cancer were compared to that of paired normal adjacent gastric tissues in each patient (Fig. 1c). We have arbitrarily defined as reduced or elongated TRFs when TRF length of tumor tissues was shorter than 80% or longer than 120% of the normal adjacent tissues, respectively. In some samples, two different peaks of signals were obtained. One of the signals was the same length as normal tissues and was considered to be derived from admixture of normal cells. Thus, when there were two peaks of signals, we estimated the length of TRFs of the tumor with the peak that was different in length from that of adjacent normal tissue (15). TRF analysis revealed that 8 of 66 (12%) tumor samples had the reduced TRFs, and 6 of 66 (9%) had elongated TRFs (>15 kbp). These 14 tumors with alterations of TRF lengths (either increased or decreased in comparison with normal tissues) showed detectable telomerase activity (Table 3).

In addition, all tumor samples were examined for cellular DNA content of tumor cells by flow cytometry (Fig. 1d, Table 3). We defined samples as aneuploid when two distinct G₀-G₁ peaks were evident. In 66 tumors, 44 tumors (67%) were diploid and 22 tumors (33%) were aneuploid. All DNA indices of the aneuploid tumors

Table 3 TRF lengths and DNA ploidy pattern in untreated gastric cancer

	Telomerase activity		
	Undetectable (n = 10)	Positive (n = 56)	
Alteration of TRFs ^a (Reduced/Elongated TRFs)	0 (0/0)	14 (8/6)	
DNA ploidy ^b			P = 0.012
Diploid	10	34	
Aneuploid	0	22	

^a Alterations of TRFs were studied by Southern blot analysis (14, 15). Results of TRF lengths in 45 samples have been reported previously (15).

^b DNA ploidy was examined by flow cytometric analysis.

were larger than 1.20. All of the aneuploid tumors had telomerase activity. There was a significant correlation between DNA ploidy and telomerase activity (P = 0.012).

Discussion

In normal somatic cells without telomerase activity, telomeres progressively shorten and after many cell divisions undergo a process known as cellular senescence (11, 12). When cancer cells become immortal, telomeres are stabilized at a length that depends on a balance between the loss of telomeric repeats at each cycle of DNA replication and the telomere elongation due to telomerase activity. In the present studies, the majority (85%) of gastric cancers had telomerase activity. In 38 advanced staged tumors (stage II, III, and IV), 36 (95%) showed telomerase activity, whereas telomerase activity was undetectable in 8 (29%) of 28 early stage (stage I) gastric cancers (P = 0.012). Tumors with telomerase activity were generally of large size and with a high frequency of lymph node metastasis. The survival rate of the patients with telomerase activity was significantly shorter than those patients without telomerase activity (P < 0.05). These results suggest that telomerase-positive gastric cancers have more malignant potential than do those without telomerase activity.

Our results also suggest that telomerase is not always activated in gastric cancer, especially in early stage cancers. In gastric cancer, telomerase may occur as a late event of cancer progression as we demonstrated previously in non-small-cell lung cancer (17). However, because 20 of 28 (71%) stage I gastric tumors had telomerase activity, telomerase activation is not necessarily a late event in gastric cancer cell progression.

Once cancer cells acquire telomerase activity, their TRF lengths could be stabilized at any length, as observed in immortal cell lines with telomerase activity (9). In the present study, altered TRF lengths were found only in 14 tumors, and 12 of them showed high telomerase activity (positive using extracts containing 0.6 μg of protein). TRF length obtained by Southern blot analysis represents an average of the cells in the tumor, whereas TRAP analysis can detect even a small number of cells with telomerase activity in the tumor. Thus, if a tumor consisted of a large number of cells without telomerase (but with normal lengths of telomere) and a small number of tumor cells with telomerase (and altered telomere lengths), the overall TRF length would generally remain in the normal range, and overall telomerase level would be low. However, when the majority of tumor cells have telomerase activity, the tumor would exhibit a high level of telomerase activity, and altered TRF length would be detectable. This supposition is compatible with our data that most gastric tumors with altered TRF length had high telomerase activity, whereas most tumors with low or undetectable telomerase activity had normal telomere lengths.

The aneuploid pattern of gastric cancer cells correlates with a poor prognosis (20). We found that all aneuploid gastric cancers had telomerase activity, although many tumors that retained a diploid karyotype also expressed telomerase activity. A critical shortening of

Table 2 Clinical data in gastric cancer cases without preoperative treatments

	Telomerase activity		
	Undetectable (n = 10)	Positive (n = 56)	
Age at diagnosis (yr)	34-82	41-86	
Mean age at diagnosis	55	66	
Sex (male:female)	5:5	37:19	
Tumor invasion in depth ^a			
Early	3	14	
Advanced	7	42	
Tumor size ^b in mm (mean)	15-50 (24.5)	12-150 (53.2)	P < 0.05
Lymph node metastasis	2	34	P = 0.020
Histology ^c			
Pap	0	5	
Tub	3	21	
Por	3	22	
Sig	4	8	
Stage ^c			P = 0.012 ^d
I	8	20	
II	0	12	
III	2	15	
IV	0	9	

^a Early, proliferation within Tela submucosa; advanced, invade beyond Tunica muscularis propria.

^b Tumor size was defined as the largest size in extension on gastric mucosa.

^c According to the criteria of the Japanese Research Society for Gastric Cancer. Pap, papillary adenocarcinoma; Tub, tubular adenocarcinoma; Por, poorly adenocarcinoma; Sig, signet ring cell adenocarcinoma.

^d P value was calculated between stage I and others.

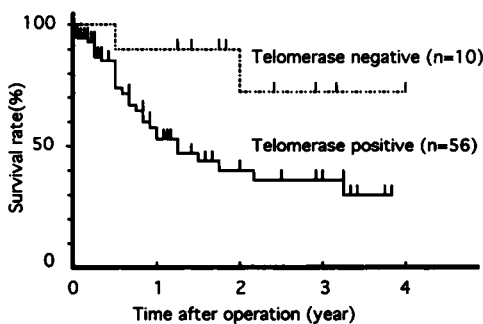


Fig. 2. The survival rates of patients with gastric cancers were compared between a telomerase-positive (n = 56) and a telomerase-negative (n = 10) group. Patients with telomerase-positive gastric cancers had significantly poorer prognosis than did the patients with telomerase-negative cancers (P < 0.05).

telomeres may cause chromosomal instability, leading to cellular senescence. The cells overcoming this critical phase acquire immortalization concomitant with telomerase activation and telomere stabilization, enabling the cells to continue to proliferate (10, 11).

A few extracts of adjacent normal gastric mucosa showed weak telomerase signals. There is a possibility that a microinvasion of cancer cells (occult metastasis) existed in the specimens that were resected as adjacent normal gastric mucosa. Alternatively, the basal cell layer, which is the regenerative origin in gastric mucosa, may have weak telomerase activity. Finally, inflammations such as gastritis may cause the infiltration of blood cells with weak telomerase activity. In 2 of 4 adjacent gastric mucosa samples with weak telomerase activity, there was evidence of chronic gastritis. At present we cannot distinguish between these possibilities.

In summary, telomerase activity was detected in 85% of gastric cancers. In the tumors without detectable telomerase activity, 80% were early stage gastric cancer. Moreover, the patients with telomerase-positive tumors showed poorer prognosis than did those with telomerase-negative tumors, indicating that telomerase-positive gastric cancers may have more malignant potential. Knowledge of telomerase activity in gastric cancer may be useful in cancer diagnosis, as well as a prognostic indicator of clinical outcome. Future development of drugs aimed at telomerase inhibition may potentially provide a therapy with relatively limited side effects.

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