High Telomerase Activity Is an Independent Prognostic Indicator of Poor Outcome in Colorectal Cancer

Naokuni Tatsumoto, Eiso Hiyama, Yoshiaki Murakami, Yuji Imamura, Jerry W. Shay, Yuichiro Matsuura, and Takashi Yokoyama

ABSTRACT
Telomerase activity and altered telomere length have been extensively studied in many kinds of malignant tumors for clinical diagnostic and/or prognostic utilities. In the present study, we investigated telomerase activity and telomere length in colorectal cancers and noncancerous colonic mucosa specimens in 100 patients between 1991 and 1996. To determine whether the level of telomerase activity or telomere length is a prognostic indicator of patient outcome, we followed these patients more than 3 years after surgery. Among 100 primary colorectal cancer specimens, 96 specimens had telomerase activity. Because noncancerous mucosa has some detectable telomerase activity, we divided the levels of telomerase activity into three categories: high (>50-fold more than that in noncancerous mucosa); moderate (10- to 50-fold); and low (<10-fold) levels. Among 100 cancer tissues, 28 showed moderate telomerase activity and 44 showed high telomerase activity. The frequency of tumors with moderate or high telomerase activity showed no significant relationship with any clinicopathological factors. The prognosis of the patients with high telomerase activity was significantly worse than that for patients with moderate and low telomerase activity (P < 0.01). Among the 87 patients with curative surgery, disease-free survival rate of those with high telomerase activity was also significantly poorer (P < 0.01). These results indicate that a high level of telomerase activity may be an independent prognosis-predicting factor in the patients with colorectal cancer.

INTRODUCTION
Telomeres are specialized structures containing unique guanine-rich hexameric repeat sequences at the ends of human and other eukaryotic chromosomes and are important in the protection and replication of chromosomes (1, 2). DNA synthesis at the end of linear chromosomes cannot be completed (referred to as the end-replication problem) with each cell division (3), and it is proposed that the loss of telomeres eventually induces antiproliferative signals that result in cellular senescence (4). In human somatic cells with low or without detectable telomerase activity, the ends of chromosomes consisting of the telomeric repeats TTAGGG progressively shorten with each cell division. In germline and immortal cells, telomerase activity maintains telomere length and thus compensates for the end-replication problem. The expression of telomerase and the stabilization of telomeres appear to be concomitant with the attainment of immortality in cancer cells (4–9). Whereas germline cells and immortal cells express telomerase activity to maintain telomeric repeats, all somatic cells including stem cells of renewal tissues exhibit progressive erosion of telomeres with each cell division, which may be due to the repression or down-regulation of telomerase activity during development (10, 11). The highly sensitive PCR-based telomerase assay, called the TRAP assay, 3 was developed for the detection of telomerase activity (8, 12). With the use of this method, telomerase activity has been found in ~90% of cancer tissues examined, covering a large variety of cancer types including colorectal cancer (13–17). Thus, detection of telomerase activity may have utility in the early diagnosis of cancer, and telomerase may be a new target for therapeutic intervention. Recently, telomerase activity has been detected in human self-renewal tissues such as hematopoietic progenitor cells (18), proliferative basal cells in the epidermis (19), and intestinal crypt cells (20). It is believed that the proliferation of stem cells balances the loss of differentiated cells in renewal tissues such as in the blood, skin, and gastrointestinal tract (21). In the intestinal cell renewal system, the putative stem cells are located in the lower regions of the intestinal crypts and their descendant cells migrate up the crypt-villus axis, losing proliferative ability, acquiring differentiation, and finally resulting in cell loss at the villus tip (22). We reported that telomerase activity was found only in the lower proliferative zone of the intestinal crypt where putative stem cells and/or their immediate descendants were located (20). In the present study, we quantitatively measured telomerase activity levels and telomere lengths in colorectal cancer tissues to determine whether there was a correlation between telomerase activity levels or telomere length and other clinicopathological features.

3 TRAP, telomeric repeat amplification protocol; LIC, internal control; TRF, terminal restriction fragment; RTA, relative telomerase activity.
MATERIALS AND METHODS

Samples. Among the patients who underwent surgery between 1991 and 1996 in Hiroshima University Medical Hospital or other two affiliated institutions, a total of 100 paired colon cancer and adjacent noncancerous colonic tissues were obtained at the time of surgery. Each adjacent noncancerous tissue was obtained from a distant portion of each cancer. These patients were between 21 and 91 years old; 54 were male and 46 were female. The disease staging and histological findings were classified using the Dukes classification (23) and the standard clinical and pathological criteria in Japan (Japanese Society for Cancer of the Colon and Rectum 1994) (24). Follow-up periods after surgical resection ranged between 1 and 96 months (mean, 41 months).

Telomerase Assay. Telomerase extracts and assays of its activity were done as described earlier (8, 12). Briefly, tissue samples of 50–100 mg were homogenized in 100–200 μl of 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid lysis buffer. After 25 min of incubation on ice, the lysates were centrifuged at 16,000 × g for 20 min at 4°C, and the supernatant was rapidly frozen in liquid nitrogen and stored at −80°C. The concentration of protein was measured using the BCA (bicinchoninic acid) protein assay kit (Pierce Chemical Co., Rockford, IL), and an aliquot of extract containing 6 μg of protein was used for each TRAP assay. For RNase treatment, 5 μl of extract were incubated with 1 μg of RNase (Boehringer Mannheim, Indianapolis, IN) for 20 min at 37°C. Telomerase activity was measured using a commercial kit, TRAPeze kit (Intergen, Purchase, NY) that enables a semiquantitative estimation of telomerase activity with the use of a PCR internal control. In our previous studies, the standard TRAP assay for gastrointestinal samples frequently showed false negative results because of the presence of PCR or Taq polymerase inhibitors in the extracts (12, 16). To remove the inhibitors in those samples, we used a modified TRAP assay. Each extract was incubated at 30°C for 30 min in 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 68 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μM dNTPs, and 0.1 μg of TS primer (5'-AATCCGTGCAGAGATT-3'). After this telomerase-mediated extension of the TS primer, the mixture was treated with phenol-chloroform followed by ethanol precipitation. The precipitated products were resuspended and then subjected to PCR assay as previously described (12, 20). Each assay contained 0.1 μg of TS primer, 0.1 μg of ACX primer (5'-GGGCGG[CTTACC]CCAACC-3'), 0.1 μg of NT primer (5'-ATCGCTCTCAGCAGTT-3'), 0.01 attomol of LIC, 2 units of Taq polymerase (Takara, Tokyo, Japan), and 150 mBq of [α-32P]dCTP. The internal control (LIC) is amplified by the primer of TS and NT that gives a 173-bp product, which is coamplified with telomerase activity products and is sufficiently long so that it does not interfere with the visualization of the telomerase ladder. This LIC was recommended by the manufacturer as the most sensitive indicator of PCR inhibitor and is believed to be the best choice for tumor and clinical research samples. The mixture was subjected to 28 PCR cycles of 94°C for 40 s and 60°C for 50 s. The PCR product was electrophoresed on a 10% polyacrylamide gel and then autoradiographed. For semiquantitative estimation of telomerase activity in tissue
samples, negative control using lysis buffer and 0.1 and 0.2 attomol of the quantitative standard R8 (5'-AATCCGTCGAG-CAGAGTTTGGTGTTTTT3') products were run in each gel. The polyacrylamide gels were exposed to a Phospholmage screen, and the intensities of the amplified products were measured with a Bioimage Analyzer (BAS 2000; Fuji, Tokyo, Japan) and MacBass software (Fuji). For semiquantification of the levels telomerase activity, the intensity of the TRAP ladder was estimated by comparing the ratio of the entire TRAP ladder with the signal of amplified LIC and by then determining the radioactivity of each TRAP ladder corrected for the background and the quantitative standard R8 with the formula:

$$RTA = \frac{(T - B)CT}{(R8 - B)CR8} \times 100$$

The term used are: $T$, total intensity of telomerase-mediated bands from the tested extract; $B$, intensity from the negative control (background); $R8$, intensity from R8; $CT$, intensity from of the tested extract; $CR8$, intensity from ILC of R8. Final quantified telomerase activity levels were expressed as RTA.

**Telomere Length Analysis.** Genomic DNA was isolated from colonic cancer and paired noncancerous colonic mucosa tissues. For the analysis of TRF length, 2 μg of DNA were digested to completion with 10 units of HindIII, electrophoresed on 0.8% agarose gels, and then blotted onto nitrocellulose filters. The filters were hybridized to a 32P-labeled (TTAGGG)4 probe, washed, and then autoradiographed, as previously reported (25). We estimated the mean length of TRFs at the peak position of hybridization signal using the BAS 2000 Bioimage Analyzer and MacBass software. To confirm complete digestion, the same filters were rehybridized with a globin or HinfI probe. To exclude the possible effect of DNA degradation, the integrity of undigested DNA was analyzed by gel electrophoresis.

**Statistical Analysis.** Correlations between telomerase activity levels and each of the other factors were analyzed by Wilcoxon’s t test, $x^2$, or Fisher’s exact test, where appropriate. The overall survival curve for each group of patients was estimated by the Kaplan-Meier method, and the resulting curves were compared using the Cox-Mantel test. Differences were considered significant at $P < 0.05$.

**RESULTS**

**Clinicopathological Findings.** Among the 100 patients studied, 87 underwent curative surgery. Surgical resection was considered curative when no distant metastases were evident, and the clearance of cancer was determined as complete by standard histological analysis. The remaining 13 cases underwent noncurative surgery due to distant metastasis or peritoneal dissemination. In these 13 patients and some other patients with advanced stage tumors, postoperative chemoadjuvant therapy was administrated.

Histological classification was assessed by light microscopy according to the pathological criteria in Japan (24). The most common histological types were the well-differentiated adenocarcinoma (51%) and moderately differentiated adenocarcinoma (43%). The remaining cases were mucinous adenocarcinomas (3%), poorly differentiated adenocarcinomas (2%), and goblet cell carcinoma (1%).

**Telomerase Activity Levels of Colorectal Cancer Specimens.** Among 100 primary colorectal cancer specimens obtained, 96 specimens displayed prominent telomerase-mediated 6-bp ladders using modified TRAP assay (Fig. 1A). As previously reported (20), normal colonic mucosa has some detectable telomerase activity derived from the intestinal crypt basal cells. The mean ± SD of the RTA value in noncancerous colonic mucosa was 1.1 ± 0.9 ($n = 100$), whereas that of cancer specimens was 55.2 ± 59.6 ($P < 0.001$). Because the TRAP assay is PCR based, we defined distinguishable telomerase activity when the RTA was $>10$, indicating $>10$ times of that in normal colon mucosa. We also defined 10–50 RTA as moderate telomerase activity and $>50$ RTA classified as high telomerase activity. Among 100 cancer tissues, 28 showed moderate telomerase activity and 44 showed high telomerase activity. Table 1 shows the correlation between telomerase activity levels and clinicopathological features of the patients. The frequency of tumors with moderate or high telomerase activity did not significantly differ in clinicopathological features. In stage classification, the level of telomerase activity gradually in-
creased in advanced stages and Dukes’ B–D. The frequencies of the tumors with the moderate or high activity were higher in advanced stages than those in early stages, but not significantly. From the 100 primary tumors, 33 (83%) of 40 tumors with lymph node metastasis and 39 (65%) of 60 tumors without lymph nodes metastasis had moderate or high telomerase activity (statistically not significant).

**Telomere Length of Colorectal Cancer Specimens.**
Telomere lengths were examined for all primary cancer tissue and the adjacent noncancerous mucosa samples. The TRF lengths of the all adjacent tissues ranged between 8 and 15 kb, whereas those of colorectal cancer tissues varied between 3.0 and 37 kb. We arbitrarily defined TRFs as shortened or elongated when TRF length of tumor tissues was shorter than 80% or longer than 120% of the normal adjacent tissues, respectively (26, 27) (Fig. 1B). In some samples, two peaks of signals were obtained. One of the two peaks showed the same length with normal tissues and was considered to be derived from admixtures of noncancerous cells. Thus, when there were two peaks of signals, we estimated the length of TRFs of the tumor with the peak which was different in length from that of adjacent normal tissue (27). Among these primary cancers, the shortened TRF lengths were detected in 38 tumors (38%) and elongated TRF lengths in 7 tumors (7%). There was no significant relationship between altered TRF length and sex, age, tumor site, tumor size, lymph node metastasis, stage, Dukes’ classification, or histology (Table 2).

In addition, Fig. 2 shows the relationship between telomere length and telomerase activity. There are no significant correlations between these two parameters. However, all 7 tumors with elongated TRFs showed high telomerase activity. The remaining 93 tumors showed various levels of telomerase activity.

**Telomerase Activity and Patient Prognosis.** The median follow-up in the series of patients examined was 41 months (range, 1–96 months). Kaplan-Meier overall survival curves of all patients (Fig. 3A) showed that the 5-year survival rate in the patients with high telomerase activity (RTA > 50) was 43%, whereas that in all remaining patients was 81%. The prognosis of the patients with high telomerase activity was significantly worse than those for other patients \( (P < 0.01) \). Although there were significant differences in survival rates of the patients according to the disease stages and Dukes’ classification, high telomerase activity showed the most significant correlation with prognosis of the patients. For example, among 33 cases with stage 2 tumors, 7 (64%) of 11 cases that resulted in death had tumors with high telomerase activity, whereas only 4 (18.8%) of 22 tumor-free survived cases had \( (P = 0.017) \). Thus, telomerase activity was an independent prognosis-associated factor in the patients with stage 2 tumors.

Among 87 patients with “curative” surgery, the recurrence rate was 25%. Disease-free survival curves of these 87 patients (Fig. 3B) showed significant difference between the tumor-free survival rates with and without high telomerase activity \( (P < 0.01) \). Among these patients who underwent “curative” surgery, 13 (38%) of 34 tumors with high telomerase activity recurred, whereas only 7 (13%) of 52 other tumors did \( (P = 0.016) \). Thus, the curative tumors with high telomerase activity had significantly higher recurrence rates than other tumors. Consequently, among these patients, 12 (35%) who had tumors with high telomerase activity died of colorectal cancer and 6 (12%) others died.

**DISCUSSION**

The prognosis of patients with colorectal cancer correlates with the depth of primary tumor invasion (Dukes’ classification), lymph node metastasis, and pathological findings (23, 28). However, these factors are not sufficient to predict the overall survival of the patients with colorectal cancer. To identify high risk patients for postoperative adjuvant therapies, many investigators have searched for additional useful prognostic markers (29).

Telomerase activity has been reported for many kinds of tumors, including gastric cancer (16), hepatocellular carcinoma (30, 31), pancreatic cancer (32–34), and colorectal cancer (15, 35, 36). Approximately 80–90% of all primary tumors show telomerase activity (37). In some types of tumors, high telomerase activity has been reported as a marker of tumor aggressiveness and poor prognosis (13, 16, 38, 39). In colorectal cancer, several investigators have reported high detection rate of telomerase activity and its correlation with clinicopathological

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Telomere length and clinicopathological data a</th>
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<tr>
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<tr>
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<td>Female</td>
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<td>≥80 yr</td>
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<tr>
<td><strong>Tumor size</strong></td>
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<td>Moderate</td>
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<tr>
<td>Others</td>
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</table>

a The disease staging and histological findings were classified using the standard clinical and pathological criteria of the Japanese Society for Cancer of the Colon and Rectum (24).

b Numbers in parentheses, range.

c Numbers in parentheses, percentage.

d LN meta, lymph node metastasis.
features (15, 35, 36). In agreement with these reports, in the present study, telomerase activity was detected in 96% of colorectal cancer specimens. However, no previous reports have shown a correlation between high telomerase activity and patient outcomes. Thus, this is the first report to show an association between telomerase activity levels and patient prognosis. Telomerase activity levels did not significantly correlate with stage of disease or Dukes’ classification in the present study. Thus, up-regulation of telomerase activity is an independent prognosis-associated factor in patients with colorectal cancer. In the present study, 12 (23%) of 51 early stage colorectal cancer patients died due to the recurrence of tumors, and 9 (75%) of these 12 cases showed high telomerase activity (RTA > 50). In contrast, 3 (20%) of 15 stage 4 patients remain disease free. Because all stage 4 cases underwent postoperative chemoadjuvant therapy, one explanation for this result is that the adjuvant therapy might have been effective to prevent recurrence in these three cases. Thus, we predict in early stage tumors that selection of patients for chemoadjuvant therapy based on high telomerase activity (RTA > 50) might be an effective method to improve the prognosis of this category of patient. Moreover, the exclusion of low risk patients from postoperative chemoadjuvant therapy could spare serious side effects.

Telomere lengths showed various sizes in colorectal cancers. In 1990, telomere shortening of colorectal cancer was reported by Hastie et al. (40). In the present study, there was no significant correlation between telomere length and clinicopathological features. Interestingly, all tumors with elongated telomeres showed high telomerase activity. This suggests that elongation of telomeres in colorectal cancer may require up-regulation of telomerase activity. Moreover, mean telomere length and mean telomerase activity in advanced stages tumors were slightly longer and higher than those of early stage tumors, indicating that telomerase might successfully stabilize telomere in late stage tumors.

In summary, we show that increased level of telomerase activity is a prognostic indicator of poor outcome in the patients with colon cancer, independent of disease stages and Dukes’ classification. Thus, high telomerase activity may risk-stratify patients who are likely to have cancer recurrence and may give an indication of postoperative standard chemoadjuvant therapy or in the future telomerase-targeting therapy.

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Fig. 2 Correlation between the levels of RTA and telomere length (TRF length). We defined as shortened (○) or elongated TRFs (■) when TRF length of tumor tissues was shorter than 80% or longer than 120% of the normal adjacent tissues, respectively (26, 27). There is no correlation between these two parameters. However, all seven tumors with elongated telomere length showed high telomerase activity.

Fig. 3 A, overall survival rates of patients with colorectal cancers compared between a high telomerase group (n = 44) and others (n = 56). Patients with high telomerase colorectal cancers showed significantly poorer prognosis (P < 0.01). B, disease-free survival rates of patients who underwent curative surgery for colorectal cancers compared between a high telomerase group (n = 35) and others (n = 52). Patients with high telomerase colorectal cancers showed significantly poorer prognosis (P < 0.01).
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