Correlations of Telomere Length, Telomerase Activity, and Telomeric-Repeat Binding Factor 1 Expression in Colorectal Carcinoma

Prognostic Indications

Cristina Garcia-Aranda[1]
Carmen de Juan, Ph.D.[1]
Antonio Diaz-Lopez[1]
Andres Sanchez-Pernaute, M.D., Ph.D.[2]
Antonio-Jose Torres, M.D., Ph.D.[2]
Eduardo Diaz-Rubio, M.D., Ph.D.[3]
Jose-Luis Balibrea, M.D., Ph.D.[2]
Manuel Benito, Ph.D.[1]
Pilar Iniesta, Ph.D.[1]

[1] Departamento de Bioquimica y Biologia Molecular, Facultad de Farmacia, Universidad Complutense, Madrid, Spain.

[2] Servicio de Cirugia, Hospital Clinico San Carlos, Madrid, Spain.

[3] Servicio de Oncologia, Hospital Clinico San Carlos, Madrid, Spain.

Supported by grants from FIS PI020193, Fundacion de Investigacion Medica Mutua Madrilena, Red Tematica de Investigacion Cooperativa de Centros de Cancer C03/010, and Sanofy Synthelabo.

Address for reprints: Pilar Iniesta, Ph.D., Departamento de Bioquimica y Biologia Molecular, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain; Fax: (011) 34 913941779; E-mail: insepi@farm.ucm.es

Received May 11, 2005; revision received July 11, 2005; accepted August 17, 2005.

BACKGROUND. Telomere maintenance has been proposed as an essential step for tumor cell immortalization. The objectives of the current study were to investigate the mechanisms implicated in telomere length in colorectal carcinoma (CRC) and to evaluate the prognostic impact of telomere status.

METHODS. Ninety-one colorectal carcinoma samples that were obtained from patients who underwent surgery were analyzed to investigate the factors related to telomere function. The authors studied telomerase activity, terminal restriction fragment (TRF) length, and telomeric-repeat binding factor (TRF1) expression and analyzed the prognostic implications of those factors.

RESULTS. Most tumors (81.3%) displayed telomerase activity. Overall, telomeres in CRC specimens were significantly shorter compared with telomeres in normal, adjacent specimens ($P = 0.02$). Moreover, tumors that demonstrated shortened telomeres displayed higher TRF1 levels than tumors without telomere shortening. In relation to patient prognosis, a significantly poor clinical course was observed in the group of patients who had tumors with longer telomeres ($P = 0.02$), and this finding emerged as an independent prognostic factor in a Cox proportional hazards model ($P = 0.04$; relative risk, 6.48). Among patients with tumors classified as telomerase-positive, telomere length ratios (the ratio of tumor tissues to normal tissues) ≤ 0.66 or TRF1 over-expression conferred a favorable outcome ($P = 0.03$ and $P = 0.05$, respectively).

CONCLUSIONS. The majority of CRC specimens in the current study displayed telomerase reactivation. However, only those tumors that displayed telomere elongation conferred a poor prognosis. Conversely, colorectal tumors that over-expressed TRF1 demonstrated telomere shortening, and patients with those tumors had a better clinical course. ***Cancer* 2006;106:541–51.**

KEYWORDS: telomerase activity, telomere status, telomeric-repeat binding factor 1 expression, colorectal carcinoma, prognosis.

Colorectal carcinoma (CRC) represents the second to fourth most common malignancy in industrialized countries.[1] Genetics plays a key role in predisposition to CRC and in its initiation and progression. Considerable advances in the molecular understanding of colorectal tumors have been made with the identification of mutations in multiple protooncogenes and tumor suppressor genes.[2] In addition to these mutations, malignant cells must escape from the control of cell senescence to reach immortality.

It was shown recently that telomerase and telomere length are

DOI 10.1002/cncr.21625
Published online 30 December 2005 in Wiley InterScience (www.interscience.wiley.com).

involved in the control of cell proliferation, the regulation of cell senescence in most somatic cells, and the unlimited proliferation capacity of malignant cells.[3] According to telomere hypothesis, normal somatic cells permanently exit from the cell cycle and become senescent after a limited number of cell divisions. Cells may escape temporarily from the senescence barrier if they lose the function of key tumor suppressor genes, especially *TP53* and/or *RB*, but most eventually will die, at which stage the cell population is described as in "crisis." Cells may bypass crisis and become immortalized if a telomere maintenance mechanism, telomerase or alternative lengthening of telomeres (ALT), is activated.[4]

Recent observations provide strong evidence that telomere maintenance plays an important role in governing the life span of human cells.[5] Although these observations support the hypothesis that maintenance of telomere length is the critical parameter that permits cell immortalization, recent evidence indicates that this simple model fails to describe completely the role of telomeres in regulating cell life span.[6]

Tumor cells and stem cells remain immortal by maintaining their telomeres, either by expressing an active telomerase enzyme or by other mechanisms. Therefore, the telomere maintenance pathway appears to contribute directly to human oncogenesis. Active areas of translational research in telomere biology include the development of diagnostic and prognostic markers for carcinoma and the clinical utilities of telomerase inhibition as antitumor therapy.[7]

In addition, telomere length abnormalities appear to be one of the earliest and most prevalent genetic alterations acquired in the multistep process of malignant transformation. These findings support a model in which telomere dysfunction induces chromosomal instability as an initiating event in many (perhaps most) human epithelial malignancies. Together with previous findings, the percentage of intraepithelial neoplasia lesions that shows telomere length abnormalities is $>$ 90%. The implications of these findings include the potential that telomere length assessment may be a widely useful biomarker for monitoring disease prevention strategies and for improved early diagnosis.[8]

To assess the role of telomeric function in CRC, we studied molecular factors that have been described as important in the regulation of telomere function. Therefore, we analyzed the correlation between telomerase activity and telomere length in 91 samples of sporadic CRC. Moreover, taking into account the negative role of telomeric-repeat binding factor 1 (TRF1) in telomere lengthening,[9] we evaluated the impact of TRF1 expression on telomere status. Finally, we analyzed the prognostic implications for all of the markers that were considered in the current study.

MATERIALS AND METHODS

Patients and Tissue Samples

Ninety-one primary colorectal carcinomas and their corresponding control tissue samples were obtained from patients who underwent surgery at San Carlos Hospital in Madrid. This study was approved by the hospital's Ethical Committee, and informed consent was obtained prior to investigation. Of 91 patients, 46 were female and 45 were male, and their average age was 68.60 years $\pm$ 10.29 years. The median follow-up for patients was 43.86 months $\pm$ 22.82 months (range, 1–77 months). None of the patients had received neoadjuvant therapy prior to undergoing surgical resection. After surgical resection, samples were frozen immediately and were kept at $-$ 80 °C until they were used. Cryostat sectioned, hematoxylin and eosin-stained samples from each tumor block were examined microscopically by 2 independent pathologists to confirm the presence of $>$ 80% tumor cells. Paired normal tissues from the same patient that were used as controls were obtained at least 10 cm away from the distal margin of the tumor and also were confirmed microscopically. Colorectal tumors were staged pathologically as Dukes Stage A–D, according to the modification of the original Dukes staging scheme by Turnbull et al.,[10] and included 10 patients with Dukes Stage A tumors, 32 patients with Dukes Stage B tumors, 29 patients with Dukes Stage C tumors, and 20 patients with Dukes Stage D tumors. Therefore, 71 patients with Dukes Stage A–C tumors underwent curative surgery, whereas patients who had more extensive disease underwent biopsy only. Twenty-three tumors were located in the right colon, 13 tumors were located in the left colon, and 55 tumors were located in the rectum. The histologic classification of tumors was established according to previous criteria.[11] Therefore, 75 tumors were well differentiated, 8 tumors were moderately differentiated, and 8 tumors were poorly differentiated.

Evaluation of Telomerase Activity

Telomerase activity in tumor tissues and in paired normal tissues was evaluated as described previously[12] using a telomerase polymerase chain reaction/enzyme-linked immunoadsorbent assay (ELISA) kit (Roche Molecular Biochemicals, Mannheim, Germany) that included a telomeric repeat amplification protocol (TRAP) assay and detection by ELISA in two steps. This method is an extension of the original TRAP procedure.[13] Telomerase-positive levels were es-

tablished according to data obtained previously from our laboratory.[12] For positive controls, we used extracts of the telomerase-positive embryonic kidney cell line 293, and negative controls were prepared for each specimen by treating cell extracts with DNase-free RNase. To avoid the effect of Taq polymerase inhibitors present in the tissue extracts, we estimated the activity of telomerase by serial dilutions of each extract, as described previously.[13]

Terminal Restriction Fragment Length Measurement

Terminal restriction fragment (TRF) length measurements in tumor specimens and in normal samples were obtained by using the *Telo* TTAGGG telomere length assay kit (Roche Molecular Biochemicals). Genomic DNA was isolated from tumor and nontumor tissues, as described previously.[14] Three micrograms of genomic DNA were digested with 20 U each of *Hinf*I/*Rsa*I at 37 °C for 2 hours and were run on 0.8% agarose gels. DNA samples were depurinated in 0.25 M HCl, denatured in 0.5 M NaOH/1.5 M NaCl, and neutralized in 0.5 M Tris-HCl/3 M NaCl. Fractioned DNA fragments were transferred to a positively charged nylon membrane by capillary blotting over 12 hours. Then, the DNA fragments were fixed by ultraviolet cross-linking (120 megajoules). Membranes were prehybridized at 42 °C for 30 minutes and hybridized with digoxygenin-labeled, telomere-specific probe $(TTAGGG)_4$ at 42 °C for 3 hours. After washing in 2 × standard saline citrate (SSC) and 0.2 × SSC containing 0.1% sodium dodecyl sulfate (SDS), membranes were incubated with a specific-specific antibody covalently coupled to alkaline phosphatase. Finally, the immobilized telomere probe was visualized by a highly sensitive chemiluminescent substrate for alkaline phosphatase (CDP-star). Membranes were then exposed to X-ray film (Hyperfilm; Amersham Biosciences Buckinghamshire, England), and TRF lengths were determined by comparing the signals relative to a standard molecular weight using Image Gauge software (version 3.46; Fujifilm, Tokyo, Japan). Figure 1 shows a representative X-ray film for telomere length analysis. All lanes were divided into intervals, and the mean TRF length was defined as $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, in which OD_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position *i*. The TRF length ratio was determined as the ratio between the length of tumor tissue TRF and their paired normal tissue TRF from the same patient (T/N ratio). Shortening or lengthening of the TRF was defined if the TRF length of tumor tissues was > 20% shorter or > 20% longer than the corresponding nontumor tissues, respectively.

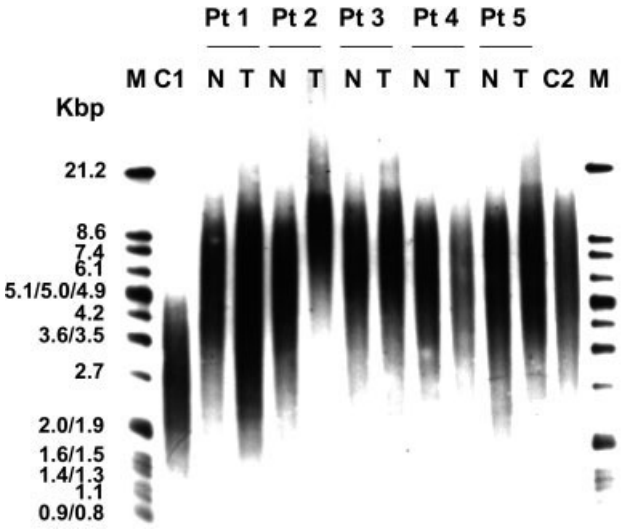


FIGURE 1. These representative X-ray films for telomere length analysis from five patients (Pt) (Pt1–Pt5) with colorectal carcinoma compare tumor tissues (T) with their paired normal samples (N). C1: low-molecular-weight positive control; C2: high-molecular-weight positive control; M: DNA molecular weight marker; Kbp: kilobase pairs.

Evaluation of TRF1 Expression by Western Blot Analysis

Briefly, 50 mg of frozen tissue samples were homogenized with 400 μL lysis buffer. Samples were then incubated on ice for 30 minutes, and the homogenates were centrifuged for 30 minutes (× 13,000 *g*) at 4 °C. The supernatants were transferred to a fresh tube, and the protein concentration was determined by using the Bradford assay. Tumor and nontumor tissue homogenates that contained 100 μg of total proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Einbeck, Germany) and were incubated with 4 μg/mL anti-TRF1 monoclonal antibody (Sigma, St. Louis, MO) under the manufacturer's recommended conditions. The corresponding peroxidase-labeled secondary antibody was detected by using an enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's recommendations. β-Actin expression data were considered for protein normalization. Levels of TRF1 were semiquantified by using Quantity One software (Bio-Rad Laboratories, CA), and differences in TRF1 expression between normal tissues and tumor tissues were evaluated. A representative experiment for TRF1 expression analysis is shown in Figure 2.

Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 11.5). The molecular mark-

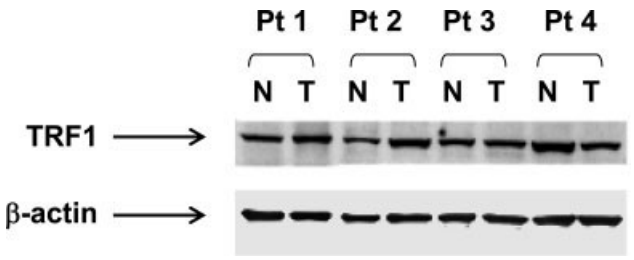


FIGURE 2. A representative Western blot analysis is shown for telomeric-repeat binding factor 1 (TRF1) expression in patients (Pt) with colorectal carcinoma. Results obtained from four tumor tissues (T) and nontumor tissues (N) are included, and β-actin normalization also is indicated.

ers investigated in this work were assessed for potential associations with a number of clinicopathologic parameters, including patient gender, age, Dukes stage, tumor location, and differentiation grade of tumors. Moreover, possible associations between telomerase activity, telomere length, and TRF1 expression were evaluated. Therefore, correlations between telomerase activity and clinicopathologic variables were assessed by using the chi-square test. Differences in telomere length and TRF1 expression among various groups of tumors discriminated for clinicopathologic parameters and telomerase activity were analyzed by analyses of variance and Student *t* tests. Differences in telomere length and TRF1 expression between matched tissue samples were determined with the Wilcoxon test for matched pairs. *P* values < 0.05 were considered significant. Distributions of disease-free survival (DFS) were estimated with the Kaplan–Meier method, and comparisons were made with log-rank statistics. Results were considered significant for *P* values < 0.05. DFS was calculated from the date of surgery until the time of tumor recurrence. For survival analysis, as established in the literature, only patients who had undergone potentially curative surgery (patients with Dukes Stage A–C tumors) and who did not die during the postoperative period were considered. Thus, in total, 68 patients were included in the survival analysis. A multivariate analysis was performed using a Cox proportional hazards model to identify which independent factors jointly had a significant influence on survival.

RESULTS

Seventy-four colon carcinoma samples (81.3%) displayed telomerase activity with low, moderate, and high telomerase levels in 18 patients, 22 patients, and 34 patients, respectively. Very low to borderline telomerase activity was observed in 14 of 91 normal adjacent tissue samples (15%). A positive correlation

TABLE 1
Telomerase Activity and Clinical Variables in Colorectal Carcinoma

Variable	No. of patients (%)			*P* value
	Total no.	Positive for telomerase activity	Negative for telomerase activity	
Gender	91	74 (81.3)	17 (18.7)	0.401
Female	46	38 (82.6)	8 (17.4)	
Male	45	36 (80.0)	9 (20.0)	
Age	91	74 (81.3)	17 (18.7)	0.144
≤ 69 yrs	46	40 (87.0)	6 (13.0)	
> 69 yrs	45	34 (75.6)	11 (24.4)	
Dukes stage	91	74 (81.3)	17 (18.7)	0.568
Stage A	10	7 (70.0)	3 (30.0)	
Stage B	32	27 (84.4)	5 (15.6)	
Stage C	29	25 (86.2)	4 (13.8)	
Stage D	20	15 (75.0)	5 (25.0)	
Tumor location	91	74 (81.3)	17 (18.7)	0.426
Right colon	23	17 (73.9)	6 (26.1)	
Left colon	13	12 (92.3)	1 (7.7)	
Rectum	55	45 (81.8)	10 (18.2)	
Tumor differentiation	91	74 (81.3)	17 (18.7)	0.166
Well	75	62 (82.7)	13 (17.3)	
Moderate	8	8 (100.0)	0 (0.0)	
Poor	8	4 (50.0)	4 (50.0)	
Recurrence	91	74 (81.3)	17 (18.7)	0.068
Negative	75	58 (77.3)	17 (22.7)	
Positive	16	16 (100.0)	0 (0.0)	

was not observed between enzyme activity and clinical variables. Thus, we did not observe significant differences when we evaluated patient gender or age or when we considered Dukes stage, tumor site, or tumor differentiation. Only tumor recurrence emerged as a borderline-significant parameter, because all patients who were positive for recurrence during follow-up demonstrated telomerase activity ($P = 0.068$) (Table 1). Analysis of telomerase activity in the 74 positive patients showed significant differences in relation to patient age. In fact, the higher levels of telomerase were detected in the group of patients older than age 69 years ($P = 0.003$). Moreover, elevated levels of telomerase were more frequent in females compared with males ($P = 0.057$) (Table 2).

Telomeres in colorectal tumors were significantly shorter compared with telomeres in normal adjacent tissue specimens ($P = 0.02$). Therefore, average values for mean TRF lengths (mean ± standard error) were 8.33 kilobase pairs (kbp) ± 0.36 Kbp for nontumor samples and 7.10 kbp ± 0.38 kbp in colorectal tumor samples, and the mean telomere length (T/N ratio) was 0.87 ± 0.03. A significant positive correlation was observed between nontumor telomere length (NTL)

TABLE 2
Clinicopathologic Variables and Levels of Telomerase Activity in Patients with Telomerase Activity-Positive Colorectal Tumors

	No. of patients (%)				
		Level of telomerase activity			
Variable	Total no.	Low	Intermediate	High	*P* value
Gender	74	18 (24.3)	22 (29.7)	34 (46.0)	0.057
Female	38	10 (26.3)	7 (18.4)	21 (55.3)	
Male	36	8 (22.2)	15 (41.7)	13 (36.1)	
Age	74	18 (24.3)	22 (29.7)	34 (46.0)	0.003
≤ 69 yrs	40	16 (40.0)	12 (30.0)	12 (30.0)	
> 69 yrs	34	2 (5.9)	10 (29.4)	22 (64.7)	
Dukes stage	74	18 (24.3)	22 (29.7)	34 (46.0)	0.575
Stage A	7	0 (0.0)	4 (57.1)	3 (42.9)	
Stage B	27	7 (25.9)	7 (25.9)	13 (48.2)	
Stage C	25	8 (32.0)	6 (24.0)	11 (44.0)	
Stage D	15	3 (20.0)	5 (33.3)	7 (46.7)	
Tumor location	74	18 (24.3)	22 (29.7)	34 (46.0)	0.110
Right colon	17	3 (17.7)	5 (29.4)	9 (52.9)	
Left colon	12	1 (8.3.0)	2 (16.7)	9 (75.0)	
Rectum	45	14 (31.1)	15 (33.3)	16 (35.6)	
Tumor differentiation	74	18 (24.3)	22 (29.7)	34 (46.0)	0.779
Well	62	14 (22.6)	19 (30.6)	29 (46.8)	
Moderate	8	2 (25.0)	3 (37.5)	3 (37.5)	
Poor	4	2 (50.0)	0 (0.0)	2 (50.0)	
Recurrence	74	18 (24.3)	22 (29.7)	34 (46.0)	0.739
Negative	58	13 (22.4)	19 (32.8)	26 (44.8)	
Positive	16	5 (31.3)	3 (18.7)	8 (50.0)	

and tumor telomere length (TTL) (TTL = 1.96 + 0.61 * NTL; correlation coefficient [*r*] = 0.35; $P = 0.01$; Pearson correlation). Overall, there was a statistically significant correlation noted between TTL and tumor location ($P = 0.005$) and between TTL and tumor differentiation ($P = 0.046$) (Table 3).

Next, the tumor samples were classified into three groups according to their telomere status. Based on established criteria, 36 of 91 tumors (39.6%) had telomere shortening, and 11 tumors (12.1%) had telomere elongation. In 44 tumors (48.3%), we did not detect significant differences in telomere length with respect to control tissues, and those tumors were classified into the group with telomere maintenance. Table 4 shows the correlation between telomere status and clinicopathologic variables in patients with CRC. Thus, borderline-significant differences were observed for tumor differentiation ($P = 0.098$). It is interesting to note that most well differentiated and moderately differentiated tumors demonstrated telomere maintenance. However, telomere dysfunction was detected in the majority of poorly differentiated tumors.

We then performed a correlation analysis of telomere length and telomerase activity. In this respect, our data indicated higher TRF lengths in telomerase-positive tumors versus telomerase-negative tumors (Fig. 3). However, differences between both groups were not significant ($P = 0.302$).

We also evaluated TRF1 expression in tumor tissues and control samples. Overall, tumor tissues showed greater TRF1 expression than their paired control tissues. Mean values for TRF1 in tumors (fold increase) were 2.2 ± 0.24. In relation to telomerase activity, although the differences did not reach significance, we detected greater TRF1 over-expression values in telomerase-positive samples (2.77 ± 0.35 vs. 1.81 ± 0.85 in telomerase-negative tissues; $P = 0.20$). Moreover, within the group of telomerase-positive samples, tumors that demonstrated shortened telomeres displayed greater TRF1 over-expression levels (2.96 ± 0.43) than tumors without telomere shortening (1.54 ± 0.11; $P = 0.04$).

A prognosis study then was conducted to assess the impact on survival of the molecular factors considered in the current study. To analyze the impact of telomere length on patient prognosis, we considered two groups after comparing mean levels across quartiles. Therefore, Group 1 included patients in the first and second quartiles (TTL ≤ 6.12 kbp), and Group 2 included patients in the third and fourth quartiles (TTL > 6.12 kbp). Therefore, we observed a significantly poor clinical course in the group of patients who had tumors with longer telomeres ($P = 0.02$) (Fig. 4A). Using the multivariate Cox proportional hazards model, this parameter was found to be a significant prognostic factor that was independent of tumor stage ($P = 0.04$). The relative risk (RR) was 6.48 in patients who had tumors with TTL > 6.12 kbp. Moreover, telomerase-positive activity was identified as a marker of a trend toward a poor prognosis. During follow-up, no recurrences were detected in patients who had telomerase-negative tumors ($P = 0.110$) (Fig. 4B). In addition, patients who had tumors with reactivated telomerase with telomere length ratios > 0.66 had a significantly shorter DFS compared with patients who had smaller telomere length ratios ($P = 0.03$) (Fig. 5).

With regard to prognostic information in patients with Dukes Stage C tumors, our results on telomere-related markers were particularly relevant. Thus, because all telomerase-negative tumors conferred a good outcome independent of tumor stage, when considering patients with telomerase-positive tumors only, both a TTL > 6.12 kbp (Fig. 6A) and a T/N ratio > 0.66 (Fig. 6B) were associated with a poor clinical course ($P = 0.03$ and $P = 0.04$, respectively).

In the group of patients with tumors that had been classified as telomerase-positive, TRF1 over-ex-

TABLE 3
Telomere Length in Colorectal Tumors and Their Paired Normal Tissues

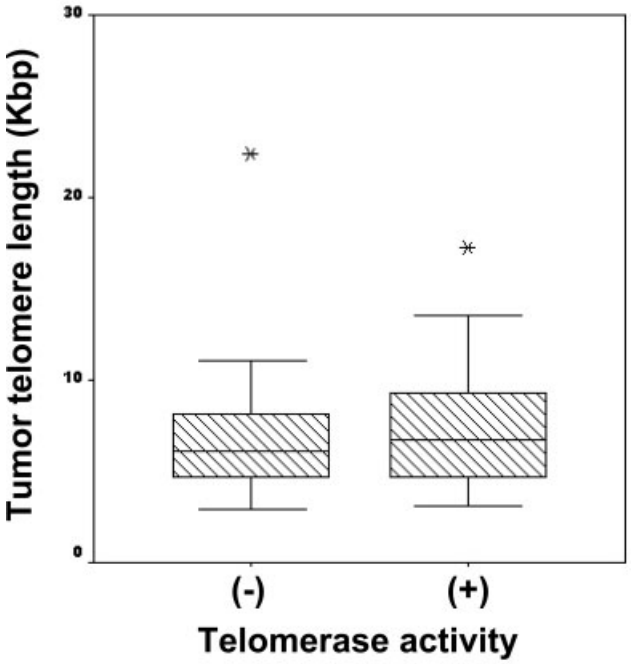
	Telomere length (kilobase pairs)[a]					
Variable	**Control tissues (N)**	***P* value**	**Tumor samples (T)**	***P* value**	**T/N ratio[b]**	***P* value**
Gender		0.184		0.943		0.180
Female	8.84 ± 0.60		7.07 ± 0.60		0.82 ± 0.04	
Male	7.89 ± 0.40		7.12 ± 0.47		0.92 ± 0.05	
Age		0.452		0.319		0.541
≤ 69 yrs	9.11 ± 0.51		7.87 ± 0.56		0.89 ± 0.05	
> 69 yrs	8.48 ± 0.69		6.92 ± 0.78		0.84 ± 0.06	
Dukes stage		0.609		0.523		0.620
Stage A	8.38 ± 1.18		5.99 ± 0.67		0.75 ± 0.05	
Stage B	7.77 ± 0.66		6.68 ± 0.66		0.89 ± 0.06	
Stage C	8.98 ± 0.60		7.85 ± 0.83		0.90 ± 0.07	
Stage D	8.49 ± 0.84		6.98 ± 0.72		0.83 ± 0.04	
Tumor location		0.125		0.005		0.115
Right colon	7.40 ± 0.76		5.34 ± 0.54		0.79 ± 0.05	
Left colon and rectum	8.65 ± 0.41		7.77 ± 0.48		0.91 ± 0.04	
Tumor differentiation		0.016		0.046		0.671
Well	8.63 ± 0.50		7.55 ± 0.52		0.89 ± 0.04	
Moderate and poor	5.85 ± 0.45		5.27 ± 0.35		0.93 ± 0.08	
Recurrence		0.446		0.410		0.785
Negative	8.31 ± 0.43		6.93 ± 0.48		0.86 ± 0.04	
Positive	9.19 ± 1.07		7.94 ± 0.73		0.89 ± 0.05	

[a] Data shown are the mean ± standard error.

[b] The ratio between the length of tumor tissue terminal restriction fragment (TRF) and the paired normal tissue TRF from the same patient.

TABLE 4
Clinicopathologic Variables in Colorectal Carcinoma in Relation to Telomere Status

	No. of patients (%)				
Variable	**Total no.**	**Telomere shortening**	**Telomere elongation**	**Telomere maintenance**	***P* value**
Gender	91	36 (39.6)	11 (12.1)	44 (48.3)	0.489
Female	46	22 (47.8)	4 (8.7)	20 (43.5)	
Male	45	14 (31.1)	7 (15.6)	24 (53.3)	
Age	91	36 (39.6)	11(12.1)	44 (48.3)	0.466
≤ 69 yrs	46	16 (34.8)	6 (13.0)	24 (52.2)	
> 69 yrs	45	20 (44.4)	5 (11.2)	20 (44.4)	
Dukes stage	91	36 (39.6)	11 (12.1)	44 (48.3)	0.562
Stage A	10	4 (40.0)	0 (0.0)	6 (60.0)	
Stage B	32	12 (37.5)	5 (15.6)	15 (46.9)	
Stage C	29	13 (44.8)	5 (17.3)	11 (37.9)	
Stage D	20	7 (35.0)	1 (5.0)	12 (60.0)	
Tumor location	91	36 (39.6)	11 (12.1)	44 (48.3)	0.495
Right colon	23	12 (52.2)	0 (0.0)	11 (47.8)	
Left colon	13	4 (30.8)	1 (7.7)	8 (61.5)	
Rectum	55	20 (36.4)	10 (18.2)	25 (45.4)	
Tumor differentiation	91	36 (39.6)	11 (12.1)	44 (48.3)	0.098
Well	75	32 (42.7)	8 (10.6)	35 (46.7)	
Moderate	8	0 (0.0)	0 (0.0)	8 (100.0)	
Poor	8	4 (50.0)	3 (37.5)	1 (12.5)	
Recurrence	91	36 (39.6)	11 (12.1)	44 (48.3)	0.175
Negative	75	31 (41.3)	11 (14.7)	33 (44.0)	
Positive	16	5 (31.2)	0 (0.0)	11 (68.8)	



Telomerase activity	Telomere length (Kbp)*
Negative	6.52 ± 0.62
Positive	7.38 ± 0.37
P	0.302

* Data are indicated as Mean ± Standart Error

FIGURE 3. This chart illustrates the correlation between telomerase activity and telomere length in colorectal tumors. Kbp: kilobase pairs; −: negative; +: positive.

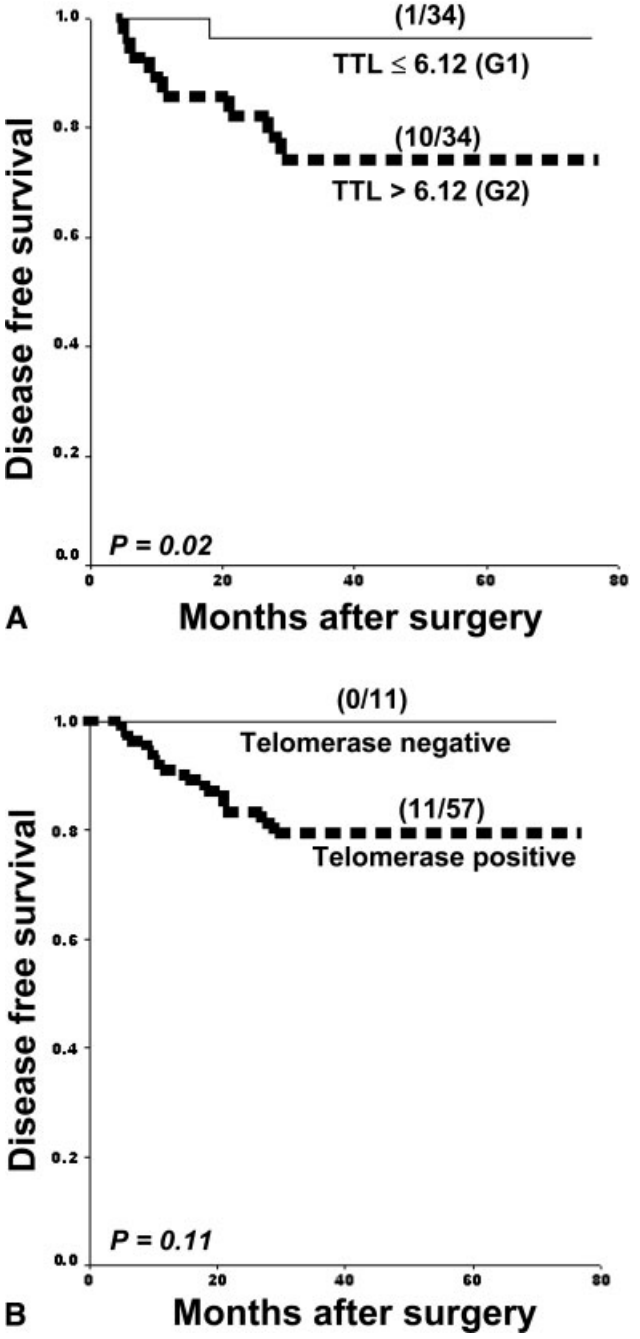


FIGURE 4. Kaplan–Meier survival curves for patients with colorectal carcinoma were determined according to (A) the function of tumor telomere length (TTL) expressed in kilobase pairs and (B) telomerase activity. G1: Group 1 (tumors in the first and second TTL quartiles); G2: Group 2 (tumors the third and fourth TTL quartiles).

pression in tumor samples was associated with a better prognosis, and tumors that had lower TRF1 expression (T/N levels $\leq$ 1.1) were associated with a poorer prognosis ($P = 0.05$) (Fig. 7).

Finally, it is important to note that the differences in DFS observed in the current study translated into differences in overall survival. Therefore, the current study data revealed that no deaths were detected in the group of patients with telomerase-negative tumors, and the differences in the overall survival of patients with telomerase-positive tumors was of borderline significance ($P = 0.11$). In relation to TTL, our overall survival analysis indicated that patients who displayed telomere elongation had the worst prognosis. Thus, considering the overall survival data, the group of patients who had tumors with TTL $>$ 6.12 kbp demonstrated a poorer survival probability compared with patients who had tumors with TTL $\leq$ 6.12 kbp ($P = 0.06$). The differences found in the survival analyses that considered DFS and overall survival derive from the finding that three patients who had recurrent tumors did not die during follow-up. Moreover, one patient without tumor recurrence died of further complications.

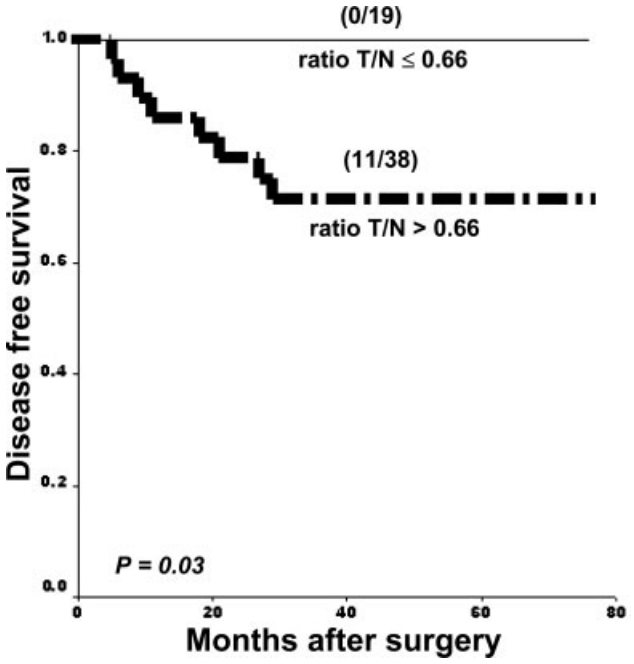


FIGURE 5. These survival curves show good outcomes in patients who had colorectal tumors with telomerase activity and at least 34% telomere shortening in relation to control samples. Ratio T/N: telomere length ratios for tumor tissues (T) to normal tissues (N).

DISCUSSION

Recent studies have assigned a putative usefulness to telomerase in identifying lesions with a malignant phenotype. Therefore, it has been suggested that telomerase status may be included as a prognostic marker to identify subgroups of patients who have carcinoma with the worst course of the disease and are possible candidates for new therapeutic protocols.[15,16] However, additional studies are necessary to assess the role of telomeric function in establishing the clinical outcome of patients with CRC. To clarify the potential clinical application of telomeric function as a prognostic indicator, we evaluated three important molecular parameters—telomerase activity, TTL, and TRF1 expression—in a large group of patients with CRC: To our knowledge, the current study is the first series of survival studies to include those three factors.

The telomerase activity analysis in the current study demonstrated that 81.3% of CRC samples displayed positive activity, which had a borderline association with tumor recurrence. These results support the observation made by Shay and Bacchetti,[17] indicating that approximately 80–90% of all primary tumors show telomerase activity. In fact, all recurrences were detected in the group of patients who had telomerase-positive tumors.

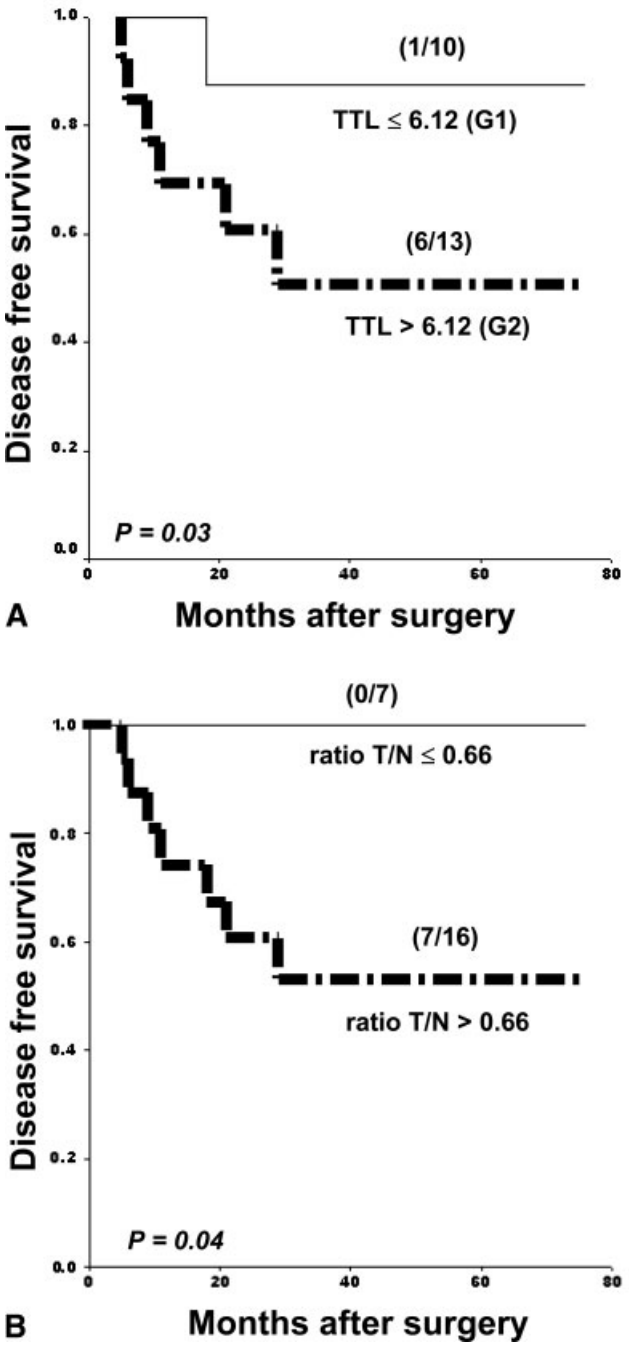


FIGURE 6. These Kaplan–Meier survival curves for patients with Dukes Stage C colorectal carcinoma show telomerase reactivation in relation to (A) tumor telomere length (TTL) expressed in kilobase pairs and (B) telomere length ratios for tumor tissues to control tissues (Ratio T/N). G1: Group 1 (tumors in the first and second TTL quartiles); G2: Group 2 (tumors in the third and fourth TTL quartiles).

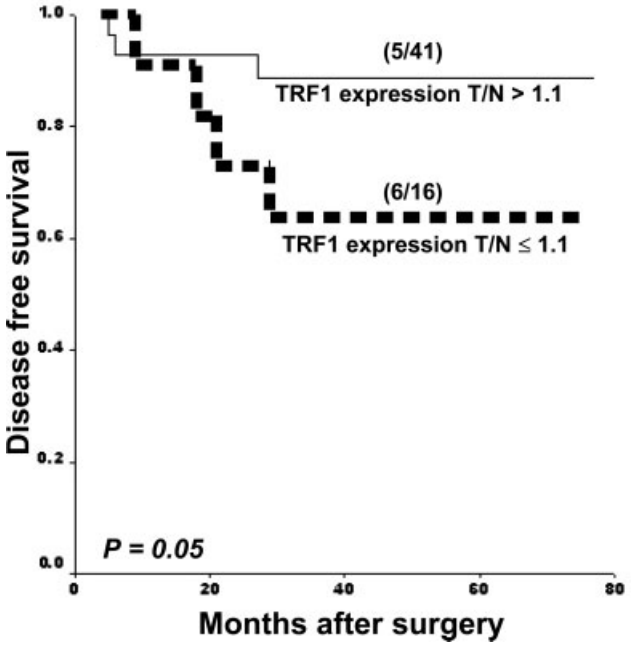


FIGURE 7. The prognosis for patients with colorectal carcinoma who had telomerase-positive tumors is illustrated in relation to telomeric-repeat binding factor 1 (TRF1) expression. T: tumor tissue; N: nontumor tissue.

In the current telomere length analyses, our results reflected significant telomere shortening in tumor tissues compared with corresponding normal tissues. Only 12% of tumors had longer telomeres compared with the adjacent normal mucosa. Other investigators[18,19] also reported shorter telomeres in the majority colon tumors that were submitted for analysis. Our data indicate that the median difference between normal tissues and tumor tissues was 1.23 kbp; and, in agreement with other reports,[19] telomere reduction appeared to occur in parallel in noncancerous mucosa and colon carcinoma, with a correlation between both.

In relation to clinicopathologic variables, tumors of the right colon displayed significantly shorter telomeres compared with tumors located in other sites. This correlation was reported previously by Takagi et al.[20] In contrast, other investigators did not observe this association.[3,18] It is interesting to note that, in the latter studies, no comparisons were made between different tumor sites in the colon, and only differences between sites in the colon and the rectum were analyzed.

With regard to the correlation between telomere lengthening or shortening and telomerase activity in human malignancies, contradictory data can be found in the literature. Some authors reported a significant positive correlation between both parameters,[3,18,21,22] whereas others described the lack of a correlation[23,24] or a negative association between telomere length and telomerase activity.[25] Several factors may explain the disagreements in the literature regarding telomeric results other than the inclusion of different tumor types in those studies. With regard to methodological criteria, in some articles, the telomere length changes reported were derived from a calculation procedure in which computer-based simulations were used to assess the effects of tumor heterogeneity on TRF values.[3,21] Moreover, some authors analyzed human telomerase reverse transcriptase (hTERT) expression to derive telomerase activity data.[18] Finally, reports of an inverse correlation between telomere length and telomerase activity in tumors implied the existence of ALT-like mechanisms.[22]

To provide a better explanation of our results on telomere length in telomerase-positive and telomerase-negative tumors, we performed Western blot analysis to evaluate TRF1 expression. Higher TRF1 levels were observed in the colorectal tumors that had telomere shortening. It has been established that telomeres are shortened gradually during cell division. Telomere shortening down to a critical length leads to chromosomal instability and, eventually, cell senescence and death. Most carcinomas and carcinoma cell lines maintain their telomeres through telomerase. Nevertheless, in the control of telomere length, other proteins apart from telomerase have to be considered. Therefore, TRF1 was identified as a suppressor of telomere elongation and is involved in the negative feedback mechanism that stabilizes telomere length.[26] To our knowledge, no reports regarding TRF1 expression in patients with CRC have been published to date. In patients with gastric carcinomas, it has been suggested that tumors with short telomeres need high levels of telomerase activity and large quantities of TRFs and TIN2; whereas tumors with long telomeres do not require high levels of telomerase activity or telomere-associated proteins.[27] In addition, Ohyashiki et al.[28] reported that TRF1 may be used to monitor telomere length under the condition of up-regulated telomerase activity in some neoplastic cells. The results of the current study in patients with CRC suggest that TRF1 over-expression correlates remarkably with telomere shortening, most likely by inhibiting telomerase action. Previous functional studies have implicated human TRF1 in telomere length homeostasis by acting as a negative regulator of telomere maintenance.[26,29] According to those reports, human TRF1 controls telomere length by affecting the ability of telomerase to extend individual chromosome ends.

Finally, we evaluated whether the molecular factors related to telomeric function that were investi-

gated in the current study had any prognostic usefulness for patients with CRC. Therefore, patients who had telomerase-positive CRC had a poorer prognosis compared with patients who had telomerase-negative CRC. Differences between both groups were of borderline significance. To date, telomerase activity has been analyzed to assess the prognostic relevance of telomere regulation in patients with CRC. Thus, Tatsumoto et al. identified high telomerase activity as an independent prognostic indicator of poor outcome in patients with CRC.[30] Moreover, Gertler et al. established the prognostic potential of hTERT expression in patients with CRC.[31] The same group also evaluated the prognostic impact of telomere length on patients with CRC.[18] Those authors determined that patients who had tumors with telomere length T/N ratios ≤ 0.9 had a significantly poorer course compared with patients who had tumors with telomere length T/N ratios > 0.9.

The current study data indicated a significantly poorer outcome for patients who had tumors with telomeres > 6.12 kbp, a finding that emerged as an independent prognostic marker in multivariate analysis. Moreover, considering telomere length T/N ratios, we found the worst prognosis for patients who had telomerase-positive tumors with T/N ratios > 0.66. In fact, no recurrences were detected in the group of patients who had tumors with T/N ratios ≤ 0.66. Therefore, the current results may be useful for identifying a subgroup of patients with CRC who have a good clinical course among patients with telomerase-positive tumors. Moreover, the prognostic information that emerged from the telomere function study regarding Dukes Stage C tumors is noteworthy. Currently, the study data support the molecular evidence that may lead us to discriminate between two subgroups of patients who have different clinical courses within the group of patients who have tumors with pathologic evidence in the lymph nodes. In addition, TRF1 over-expression was associated with a good prognosis in the group of patients with telomerase-positive tumors. These data may indicate that CRC tumors with shortened telomeres that overexpress TRF1 override the telomerase function and confer a better clinical course.

The results of the current study are in agreement with findings from previous functional studies on telomere status in different cellular systems. Therefore, the telomere hypothesis states that critical telomere shortening prevents somatic cells from dividing, whereas maintenance of telomeres allows tumor cells and stem cells to continue dividing. Inhibition of telomerase activity results in progressive telomere shortening and eventual senescence, whereas ectopic expression of telomerase in cells that normally lack telomerase maintains telomere length and extends the capacity of cells to divide.[7] Therefore, a failure in telomerase activity, among other factors, by over-expression of TRF1, leads to telomere shortening and cell senescence. Data from the current study indicate the usefulness of analyzing different parameters related to telomere function as prognostic indicators in patients with CRC. Based on the finding that most CRC tumors display telomerase reactivation, the capacity to identify patients who have tumors with elongating telomeres, which indicate a poorer prognosis, may have clinical relevance.

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