

ORIGINAL PAPERS

Telomeric repeat factor 1 protein levels correlates with telomere length in colorectal cancer

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ABSTRACT

Background: colorectal cancer is the third cancer cause of death in Spain. It is important to investigate new tumoral markers for early diagnosis, disease monitoring and prevention strategies. Telomeres protect the chromosome from degradation by nucleases and end-to-end fusion. The progressive loss of the telomeric ends of chromosomes is an important mechanism in the timing of human cellular aging. Telomeric Repeat Factor 1 (TRF1) is a protein that binds at telomere ends.

Purpose: to measure the concentrations of TRF1 and the relationships among telomere length, telomerase activity, and TRF1 levels in tumor and normal colorectal mucosa.

Method: from normal and tumoral samples of 83 patients who underwent surgery for colorectal cancer we analyzed TRF1 protein concentration by Western Blot, telomerase activity, by the fluorescent-telomeric repeat amplification protocol assay and telomere length by Southern Blot.

Results: high levels of TRF1 were observed in 68.7% of tumor samples, while the majority of normal samples (59%) showed negative or weak TRF1 concentrations. Among the tumor samples, telomere length was significantly associated with TRF1 protein levels ($p = 0.023$).

Conclusions: a relationship was found between telomere length and TRF1 abundance protein in tumor samples, which means that TRF1 is an important factor in the tumor progression and maybe a diagnostic factor.

Key words: Telomeric repeat factor 1. Telomere length. Telomerase activity. Colorectal cancer.

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ABBREVIATIONS

ALT: alternative lengthening of telomeres.

Kb: kilobases.

TA: telomerase activity.

TI: telomerase index.

TL: telomere length.

TPG: total product generated.

TRF1: telomeric repeat factor 1.

TRF2: telomeric repeat binding factor 2.

INTRODUCTION

Telomeres, structures composed mostly of chromatin localized at the end of eukaryotic chromosomes (1), consist of 800-3000 tandem repeats of six nucleotides (5'TTAGGG3') along a 5-15 kilobases (Kb) sequence (2). Telomeres protect the chromosome from degradation by nucleases and end-to-end fusion. The progressive loss of the telomeric ends of chromosomes is an important mechanism in the timing of human cellular aging (3). When telomeres become sufficiently short, the majority of cells enter in senescence, but some escape because they are able to maintain their telomere length (TL).

Telomerase is a ribonucleoprotein that compensates for the shortening of telomeres by adding TTAGGG repeats onto the chromosome ends (4). In germ cells and renewing tissues, telomerase is activated and maintains the integrity and stability of the genome (5). Some immortalized mammalian cell lines and tumors maintain or increase the overall length of their telomeres in the absence of telomerase activity (TA) by one or more mechanisms referred to as alternative lengthening of telomeres (ALT) (6).

Previous studies have not uncovered a direct relationship between TA and TL (7), raising the possibility of telomere-binding proteins that regulate TL, TA, and maintain the protective structure of the telomere *in vivo* (8,9). Six proteins (TRF1, TRF2, POT1, TIN2, TPP1, and hRap1) bind at telom-

ere ends in a complex known as *shelterin*. TRF1, TRF2, and POT1 directly bind telomeric DNA. Telomeric Repeat Binding Factor 1 (TRF1) acts in *cis*, obstructing telomerase and preventing telomere elongation (10). The number of molecules of TRF1 bound to each telomere end is correlated with TL (11). Telomeric Repeat Binding Factor 2 (TRF2) induces T-loop structure, protecting telomeres from degradation by exonucleases. The telomere homeostasis hypothesis predicts that longer telomeres bind more TRF1; TRF1 obstructs telomerase and prevents telomere elongation (12). Short telomeres bind fewer TRF1 molecules, favoring an open conformation with more chances to be elongated by telomerase (13).

TL abnormalities appear to be one of the earliest prevalent genetic alterations acquired during the multistep process of malignant transformation (14). By demonstrating the participation of TRF1 in TL maintenance, we gain a useful new biomarker to employ in early diagnostics and disease monitoring and prevention strategies.

In this study, we investigate whether the telomere homeostasis hypothesis can be directly applied to understand various molecular characteristics of normal and cancerous tissues. We measured TA, TL, and TRF1 protein abundance in cancerous and noncancerous mucosal samples from 83 colorectal cancer patients. Finally, we applied statistical techniques to determine the relationships among these three variables and additional anatomopathological features.

PATIENTS AND METHODS

Patients

Fresh samples of cancer tissue and normal mucosa were obtained from 83 patients (46 male, 37 female) undergoing oncological tumor resection for colorectal cancer at Lleida Arnau de Vilanova University Hospital. We considered normal mucosa as having a macroscopically normal appearance and lying from 10 cm to the tumor. The patients ranged in age from 45 to 89 years, with an average age of 69.3 years. No patient's received neoadjuvant therapy. All tissue samples weighing 100 mg were immediately frozen in liquid nitrogen and stored at -80 °C.

Samples were analyzed by the hospital's Pathology unit. Tumors were staged according to the tumor-node-metastases classification of the International Union against Cancer (UICC-TNM classification). Clinical and pathological data were collected in a specific database. All research protocols used in this study were approved by the Hospital Clinical Research Ethics Committee, conforms Ethical Guidelines of Helsinki Declaration.

Evaluation of TRF1 protein concentration by Western Blot

Briefly, 40 mg of frozen tissue samples were homogenized with 400 µl protein lysis buffer (125 mM Tris,

pH 6.8), with RNase inhibitor (RNasin® Ribonuclease inhibitor, Promega) added to the tumor samples. The homogenates were centrifuged at 12,000 rpm at 4 °C for 2 minutes, and the supernatants were sonicated (Digital sonifier 450 Branson) to degrade DNA. Total protein concentration was determined using the Bio-Rad Micro Protein Assay (Bio-Rad Laboratories). 25 µg of total protein were separated by electrophoresis on 10% agarose, transferred to a PDVF membranes (polyvinylidene fluoride, Millipore) and incubated with 1:500 anti-TRF1 monoclonal mouse antibody into a 5% milk solution (clone TRF-78, Sigma-Aldrich) under the manufacturer's recommended conditions. The corresponding peroxidase-labeled secondary anti-mouse antibody, diluted 1:12,500 (Jackson ImmunoResearch Laboratories), was detected using an enhanced chemiluminescence system (EZ-ECL Chemiluminescence Detection Kit for HRP, Biological Industries Kibbutz Beit Haemek, Israel) and analyzed by Lumi-Imager (Boehringer Mannheim). Levels of monoclonal anti-α-tubulin (Sigma, Saint Louis, Missouri) used at 1/30,000 dilution were measured for protein normalization between samples. TRF1 levels were qualitatively compared with a TRF1 positive control (cell line 293, Human embryonic kidney cells) (15).

Levels of TRF1 protein in tumor and normal samples were determined relative to protein levels in cell line 293, our baseline positive control (designated "100%"). Samples were categorized by three separate observers as follows: "-" as low concentration relative to the positive control, "+" as weak concentration, "++" as medium concentration, and "+++" as high concentration. We further sub-classified TRF1 protein levels into two groups: samples with low and weak concentrations (-/+) and samples with medium and high concentrations (+/+++). To determine the TRF1 ratio we compared the protein levels in two samples from the same patient. The ratio was positive if the TRF1 level was higher in the tumor mucosa than the normal mucosa, and the ratio was negative if the TRF1 level was lower in the tumor mucosa than the normal mucosa. These TRF1 ratios were divided into two groups: negative and equal ratios (-/=), and positive ratios (+).

Fluorescent-telomeric repeat amplification protocol

TA was measured quantitatively by the fluorescent-telomeric repeat amplification protocol assay (TRAP-F), using a TRAPeze Telomerase Detection Kit (INTERGEN® Purchase) (16). We report TA in TPG (total product generated). Analysis was performed as described previously (17). Telomerase index (TI) was determined by the formula $TI = \log (TA \text{ of cancer tissue} - TA \text{ of normal mucosa})$.

Telomere length measurement by Southern Blot

Telomere restriction fragment (TRF) length was determined by Southern blot (Telo TAGGG Telomere length

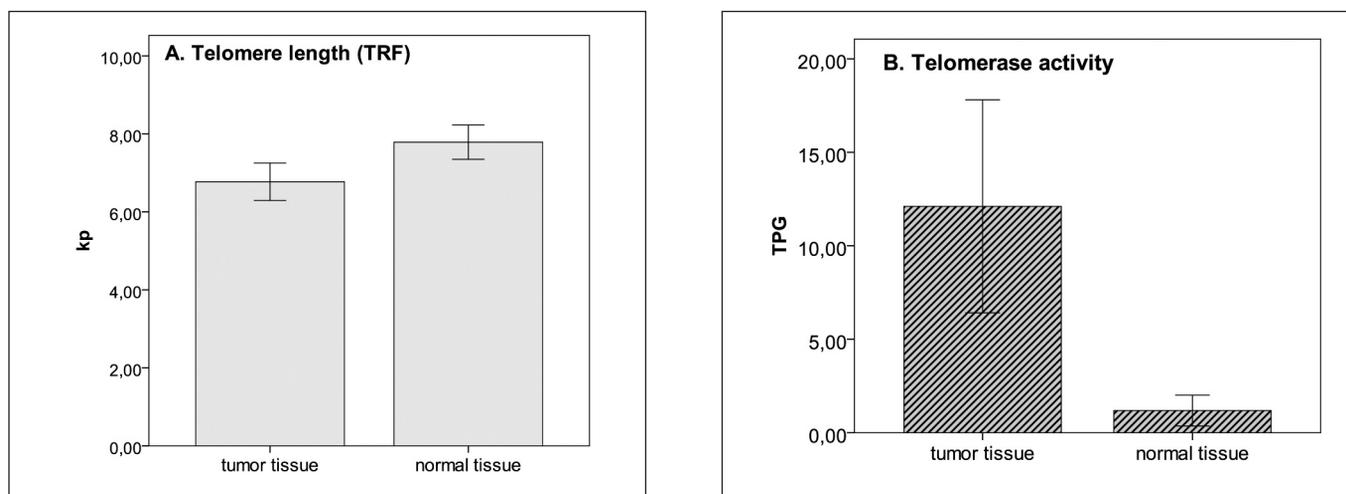


Fig. 1. Telomere length and telomerase activity in tumor and normal tissue in colorectal cancer patients. A. Telomere length levels in kb. B. Telomerase activity in TPG in tumor and normal tissue of colorectal cancer patients ($n = 83$). Data are shown as mean \pm standard error. In both $p < 0.001$.

assay, Roche Diagnostics) in tumor and normal tissues. Analysis was performed as described previously (18,19). The telomere length was expressed in Kb. The telomere length ratio (TLR) was determined as the quotient between the TL in the tumor tissue and the TL in normal mucosa from the same patient. Telomere shortening and elongation were defined as TLs of carcinoma less than 80% and greater than 120% of the corresponding normal mucosa, respectively.

Statistical analysis

We used the statistical program SPSS[®] 18.0 for Windows (SPSS Inc.) for analyses. Patient factors and TRF1 expression level were analyzed using the chi-square test. We used the non-parametric Mann-Whitney U-test and Fisher test. The cut-off TRF length ratio = 1 was chosen by means of the classification and regression tree technique (CART). A TI value of 0.85 was used to classify patients showing a high index (> 0.85) and patients with low index (< 0.85)

and this value was estimated using maximally selected chi-squared statistics (18). We considered results statistically significant when $p < 0.05$.

RESULTS

Molecular markers differ between cancerous and normal tissues

The anatomopathological characteristics studied from 83 colorectal cancer patients are shown in table I. Mean TL (Fig. 1A) in tumor samples was 6.77 kb (2.19-13.31 kb), while in normal mucosa the average TL was 7.78 kb (4.20-15.70 kb) ($p < 0.001$). The mean TA (Fig. 1B) in the tumor samples was 12.3 TPG (0-164.87 TPG) and in normal samples it was 1.18 TPG (0-24.67 TPG) ($p < 0.001$). The TI mean was 0.45. Additionally, we detected TRF1 protein in 95% and 89% of our cancerous and noncancerous samples, respectively. The majority of normal samples (59%) showed negative or weak TRF1 concentration, while 68.7% of tumor samples presented high levels of TRF1. To summarize, on average the cancerous samples had shorter telomeres, higher TA, and higher TRF1 protein levels than paired noncancerous samples.

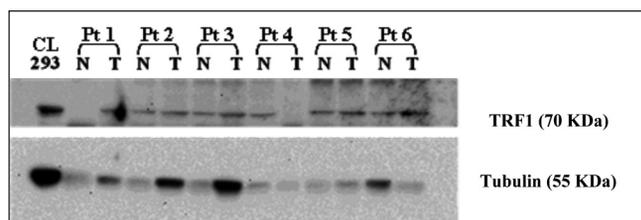


Fig. 2. TRF1 analysis by Western Blot. A representative Western Blot analysis is shown for telomeric repeat binding factor 1 (TRF1) expression in patients (Pt) with colorectal carcinoma. Results obtained from five tumor tissues (T) and non-tumor tissues (N) are included, and tubulin normalization also is indicated. First line is a TRF1 positive control (cell line 293, Human embryonic kidney cells).

TRF1 protein levels are significantly related to anatomopathological features (Fig. 2)

Two statistically significant relationships between the anatomopathological variables and molecular tissue markers emerged upon chi-square analysis (Tables I and II). First, normal mucosa from male patients exhibited lower relative TRF1 protein levels compared with the noncancerous samples from female patients (Table I, $p < 0.009$). Second, we

Table I. Relationships among TRF1 protein levels (low/weak or medium/high) and anatomopathological features analyzed by the Chi-square test

Variable (n)	TRF1 level, tumor (%)		TRF1 level, normal (%)		TRF1 Ratio (%)	
	-/+	++ /+++	-/+	++/+++	-/=	+
Patients (n = 83)	26 (31)	57 (69)	49 (59)	34 (41)	49 (59)	34 (41)
<i>Gender</i>						
Male (46)	18 (39)	28 (61)	33 (72)	13 (28)	20 (43.5)	26 (56.5)
Female (37)	8 (22)	29 (78)	16 (43)	21 (57)	17 (46)	20 (54)
<i>p</i>	0.087		0.009		0.822	
<i>Age (years)</i>						
≤ 70 (41)	9 (22)	32 (78)	25 (61)	16 (39)	15 (37)	26 (63)
> 70 (42)	17 (40.5)	25 (59.5)	24 (57)	18 (43)	22 (52)	20 (48)
<i>p</i>	0.069		0.723		0.148	
<i>Tumor site</i>						
Right colon (32)	10 (31)	22 (69)	17 (53)	15 (47)	17 (53)	15 (47)
Left colon (21)	4 (19)	17 (81)	15 (71)	6 (29)	5 (24)	16 (76)
Rectum (30)	12 (40)	18 (60)	17 (57)	13 (43)	15 (50)	15 (50)
<i>p</i>	0.284		0.393		0.083	
<i>Tumor histology</i>						
Adenocarcinoma (73)	23 (31.5)	50 (68.5)	45 (62)	28 (38)	31 (42.5)	42 (57.5)
Mucinous (10)	3 (30)	7 (70)	4 (40)	6 (60)	6 (60)	4 (40)
<i>p</i>	0.923		0.192		0.295	
<i>Macroscopic aspect</i>						
Ulcerous-infiltrative (42)	15 (36)	27 (64)	22 (52)	20 (48)	23 (55)	19 (45)
Vegetating (36)	10 (28)	26 (72)	25 (70)	11 (30)	12 (33)	24 (67)
Polypoid (5)	1 (20)	4 (80)	2 (40)	3 (60)	2 (40)	3 (60)
<i>p</i>	0.642		0.209		0.161	
<i>Dukes classification</i>						
A (1)	1 (100)	0 (0)	1 (100)	1 (0)	1 (100)	0 (0)
B (47)	16 (34)	31 (66)	26 (55)	21 (45)	24 (51)	23 (49)
C (24)	6 (25)	18 (75)	16 (67)	8 (33)	10 (42)	14 (58)
D (11)	3 (27)	8 (73)	6 (54.5)	5 (45.5)	2 (18)	9 (82)
<i>p</i>	0.410		0.652		0.156	
<i>T classification</i>						
Tis (2)	1 (50)	1 (50)	2 (100)	0 (0)	1 (50)	1 (50)
T2 (11)	3 (27)	8 (73)	5 (45.5)	6 (54.5)	6 (54.5)	5 (45.5)
T3 (63)	20 (32)	43 (68)	35 (56)	28 (44)	29 (46)	34 (54)
T4 (7)	2 (29)	5 (71)	7 (100)	0 (0)	1 (14)	6 (86)
<i>p</i>	0.932		0.060		0.373	
<i>N classification</i>						
N (-) (49)	17 (35)	32 (65)	27 (55)	22 (45)	25 (51)	24 (49)
N (+) (34)	9 (26.5)	25 (73.5)	22 (65)	12 (35)	12 (35)	22 (65)
<i>p</i>	0.427		0.382		0.156	
<i>M classification</i>						
M0 (72)	23 (32)	49 (68)	43 (60)	29 (40)	35 (49)	37 (51)
M1 (11)	3 (27)	8 (73)	6 (54.5)	5 (45.5)	2 (18)	9 (82)
<i>p</i>	0.756		0.745		0.059	
<i>TNM stage</i>						
0 (2)	1 (50)	1 (50)	2 (100)	0 (0)	1 (50)	1 (50)
I (11)	3 (27)	8 (73)	5 (45.5)	6 (54.5)	6 (54.5)	4 (45.5)
II (35)	13 (37)	22 (63)	20 (57)	15 (43)	18 (51)	17 (49)
III (24)	6 (25)	18 (75)	16 (67)	8 (33)	10 (42)	14 (58)
IV (11)	3 (27)	8 (73)	6 (54.5)	5 (45.5)	2 (18)	9 (82)
<i>p</i>	0.829		0.567		0.365	

Table II. Relationships among TRF1 protein levels (low/weak or medium/high) and telomere length, telomere length ratio and telomerase activity analyzed by the Chi-square test

Variable (n)	TRF1 level, tumor (%)		TRF1 level, normal (%)		TRF1 Ratio (%)	
	-/+	++ /+++	-/+	++/+++	-/=	+
Patients (n = 83)	26 (31)	57 (69)	49 (59)	34 (41)	49 (59)	34 (41)
<i>Telomere length</i>						
Shortened (32)	15 (47)	17 (53)	17 (53)	15 (47)	17 (53)	15 (47)
Elongated (7)	3 (43)	4 (57)	4 (57)	3 (47)	4 (57)	3 (43)
No change (44)	8 (18)	36 (82)	28 (64)	16 (36)	16 (36)	28 (64)
<i>p</i>	0.023		0.651		0.273	
<i>TLR</i>						
" 1 (63)	21 (33)	42 (67)	38 (60)	25 (40)	26 (41)	37 (59)
> 1 (20)	5 (25)	15 (75)	11 (55)	9 (45)	11 (55)	9 (45)
<i>p</i>	0.484		0.674		0.282	
<i>TI 0.85</i>						
" 0.85 (57)	17 (30)	40 (70)	31 (54)	26 (46)	27 (47)	30 (53)
> 0.85 (26)	9 (35)	17 (65)	18 (69)	8 (31)	10 (38.5)	16 (61.5)
<i>p</i>	0.662		0.202		0.449	

TLR: telomere length ratio; TI: telomerase index.

found among the tumor samples, TL had a significant association with TRF1 protein levels ($p < 0.023$); this association was not significant in the normal mucosal samples (Table II, $p < 0.651$) the tumors that did not change their TL had a high TRF1 expression than the others that elongated or shorten their TL.

TRF1 protein abundance, telomerase activity, and telomere length are related

We uncovered a relationship between TA (positive/negative) and TRF1 protein abundance in tumor samples: the 87.5% of tumors samples with negative TA showed medium/high TRF1 protein levels, as compared with 64% of positive telomere tumors. This result was not statically significant ($p < 0.071$). Tumors with high TRF1 protein abundance exhibited greater TA and longer telomeres than tumors with lower TRF1 protein levels. In noncancerous samples, however, the samples with low TRF1 protein levels displayed higher TA and shorter telomeres than the noncancerous samples with high TRF1 protein abundance, a difference that was not statistically significant ($p < 0.052$).

DISCUSSION

In this study we have shown on average that cancerous samples had shorter telomeres, higher TA, and higher TRF1 protein levels than paired noncancerous samples. While on average tumoral mucosa display elevated TA and shorter telomeres relative to normal mucosa, no statistically significant association between TA and TL has been identified (8,17,20). We identified TRF1 as a candidate protein for maintenance of telomere homeostasis. This protein, located

at the end of telomeres, maintains the T-loop structure necessary to protect chromosome ends from degradation (12,21).

TRF1 has been subjected to various studies in various tumor types (13,14,21,22). The main methodologies to study TRF1 have been western blotting (13) and immunohistochemistry (22,23). We detected TRF1 protein in 95% and 89% of our cancerous and noncancerous samples, respectively. The majority of normal samples showed low or weak TRF1 protein levels, while almost 70% of tumor samples presented high TRF1 protein abundance. This is an unexpected result; although we anticipated TRF1 protein in both tissues, we did not expect to observe elevated TRF1 levels in tumor samples relative to normal samples, since telomeres in tumor samples are shorter than normal mucosa and TRF1 protein was previously observed bound to the (longer) telomeres of normal mucosa (10,13). Given these previous studies, we expected a direct association between TL and levels of TRF1. This association was not identified in tumor samples due to altered telomere homeostasis mechanisms. Generally, in normal mucosa in people without a tumoral process, this mucosa show a relation between TL and TRF1 levels, since longer telomeres accept more TRF1 bound because this binding is sequence specific. In tumoral mucosa the telomere homeostasis was deregulated, lots of cellular mechanisms not function well (due to a transformation process from normal to tumor cell). In addition, shorter telomeres may be more difficult to stabilizer and need to bind higher levels of TRF1. Whereas, in normal mucosa which has longer telomeres they may be easier to stabilizer and other mechanisms which control the homeostasis of telomeres function well and needs less TRF1 to bind.

TRF1 protein abundance in normal mucosa showed a significant association with gender, with more samples from women displaying elevated protein levels. High quantity of TRF1 upon normal mucosa means more stability for telom-

eres and homeostasis of them. This situation determines healthier telomeric homeostasis in females than in males. Several studies reported that females have long telomeres than males due to faster attrition rate in males (in people without tumoral process) (24). Nawrot et al. (25) concluded that the difference in gender were due to estrogen-responsive hormone regulators in telomerase. Nawrot suggested that the process of ageing might be an X-linked trait. TL had a significant association with TRF1 protein levels in tumor mucosa: a high percentage of samples with average normal TL displayed high TRF1 protein levels. The binding of TRF1 on the telomeres may be a protection mechanism, as it can permit a better tolerance to shorter telomeres during tumor progression (26). TRF1 and TRF2 bind to the double-strand telomere DNA together with POT1, Rap1, TIN1, and PPT to form the shelterin complex, protecting chromosome ends and activating the telomere repair pathway (10). Under normal conditions, a direct relation exists between TRF1 levels and TL (12), but we have uncovered an indirect relation between TRF1 levels and TL in tumor samples. If telomeres in tumor samples have sufficient levels of bound TRF1, they may survive despite the short telomeres. TL and TA are altered in tumors (13); this study provides evidence that TRF1 levels are also altered in comparison to normal tissue. Previously, the percentage of TRF1-positive cells was correlated positively with tumor histology; differentiated tumors expressed more TRF1 –more differentiated level more TRF1 level (22). A significant negative relationship between TRF1 levels and histology degree was observed in brain tumors (27,28).

While we observed that, as a group, telomerase-negative tumors displayed higher TRF1 protein levels than normal tissue; this result did not pass our significance cutoff. Perhaps tumors with low TA require additional mechanisms to maintain TL; for example, many TRF1 molecules binding at the telomeres may increase survival, as suggested by the homeostasis telomere hypothesis (6). It is now known that TRF1 binds the telomeres lengthwise, hindering telomerase binding and lengthening the telomeres (10,11). Our results agree with a previous work demonstrating that longer telomeres recruited more TRF1 than shorter telomeres, and that TRF1 inhibited telomerase; in other words, telomerase tended to elongate shorter telomeres with less bound TRF1 (10). TRF1 seems to protect short telomeres short, maintaining their integrity and promoting tumor survival. Samples with medium to high TRF1 protein abundance showed longer telomeres than samples with low TRF1 expression in both normal and tumor tissues. These results agree with other studies affirming that the amount of TRF1 binding at the telomere end is proportional to the TL (12).

The statistical analyses did not reveal significant differences TA, TL, and TRF1 protein abundance. On the one hand, TL demonstrated a link with TRF1 protein abundance; on the other, TRF1 protein levels had no significant relation with TA. In general, we found that tumors with low TA had long telomeres and expressed high levels of TRF1, whereas tumors with higher TA had shorter telomeres and expressed lower TRF1 levels. A possible explanation for

this behavior is for samples with low or no telomerase activity, a high level of TRF1 could become essential for chromosome stability (11), thus affecting TA. Additionally, the telomeres in tumor cells may display different behavior from telomeres in normal cells, possibly as a result of protein regulation of TL and TA (8). It is known that three sequence-specific DNA binding proteins (TRF1, TRF2, and POT1) are recruited to chromosomal ends; they form a functional complex that caps telomere ends and prevents telomere dysfunction which initiates genomic changes and promotes the development of cancer (8,14).

We propose that some tumors elongate their telomeres using TA as opposed to ALT (6,26,29). ALT is a TL maintenance mechanism based on recombination, where telomeres use other telomeric DNA as a template for DNA synthesis. ALT cells are able to elongate their telomeres without any apparent intervention of other telomeres (30). *In vitro* experiments indicate that human cells may be capable of concomitantly utilizing both mechanisms of telomere maintenance without affecting growth and viability (31).

In summary, we observed that tumors that do not change their TLs have higher TRF1 protein levels than those that do change their TLs. We found a relationship between TL and TRF1 protein abundance, but not with TA. Tumor samples exhibited different molecular activities related to TRF1, TL, and TA when compared with normal samples. These results affirm that a direct relation exists between TL and TRF1 levels. Normal samples with elongated telomeres exhibited higher TRF1 protein levels than samples with short telomeres, a result coincident with the homeostasis telomere hypothesis, whereas tumor samples whose TLs were maintained presented higher TRF1 level. We believe that additional studies of TRF1 will bring to light additional key factors of cancer development. Our study concludes that TRF1 is an important factor in the regulation of the telomeres and TRF1 changes its behavior in cancer compared in normal samples. Then it maybe an important prognostic factor in the future together with telomere length and telomerase activity.

The alteration of the mechanism of TL maintenance (telomerase, TRF1, ALT) may represent a first step toward the proliferation of cells leading to carcinogenesis. TRF1 is an important factor in the homeostasis of telomeres; it is involved in both the telomerase and ALT mechanisms, since TRF1 and TRF2 are required to form ALT structures. TRF1, TL, and TA may therefore serve as new indicators of prognosis and as markers for new anti-cancer therapies.

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