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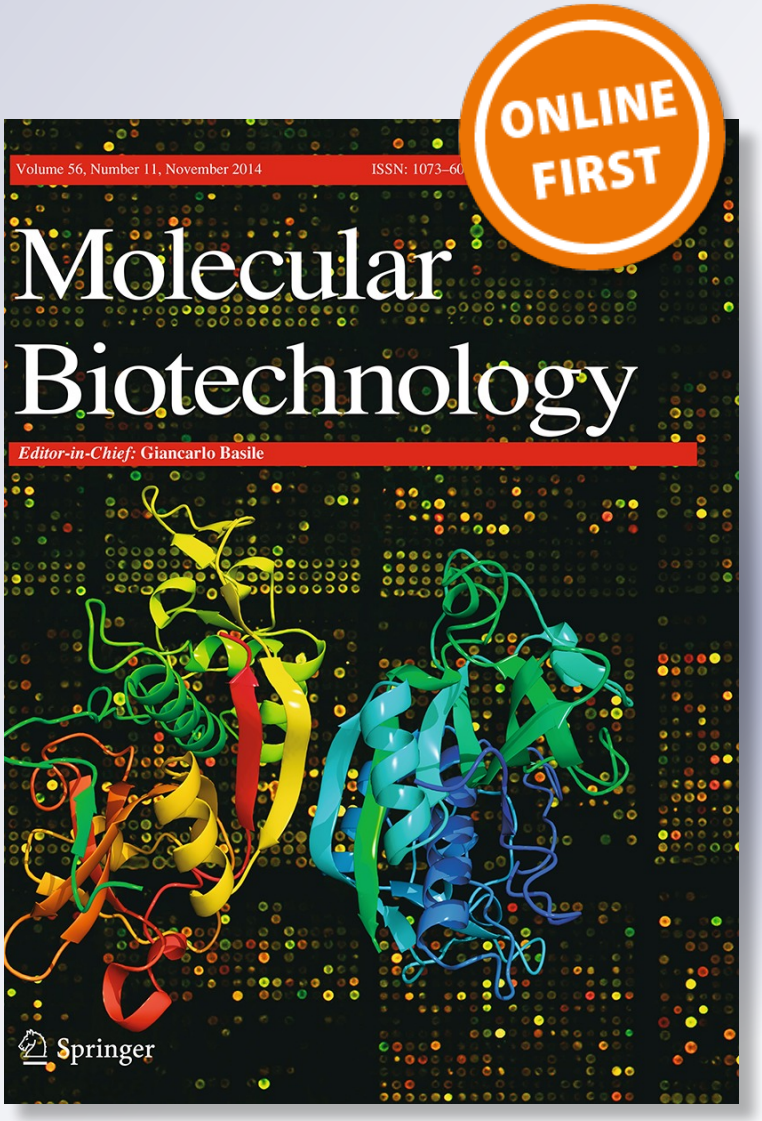

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RESEARCH

Absolute qPCR for Measuring Telomere Length in Bone Marrow Samples of Plasma Cell Disorders

Julieta Panero · Nathan J. O'Callaghan · Michael Fenech · Irma Slavutsky

Abstract Telomere length (TL) is currently used as an emerging biomarker in understanding the development/progression of hematological malignancies. The absolute quantitative PCR (qPCR) methodology has allowed the study of TL from a variety of mammalian tissues, but it has not been tested for bone marrow (BM) samples. In this study, we have examined the relationship between TL data generated by absolute qPCR versus those obtained by terminal restriction fragments (TRF) in 102 BM samples from patients with plasma cell disorders. A significant linear correlation between both methodologies was observed ($p < 0.0001$; $r^2 = 0.70$). Results were also analyzed in relation to clinical characteristics and significant associations between telomere shortening and parameters of adverse prognosis were observed. Furthermore, another set of 47 BM samples from patients with low quantity of DNA for TRF assay were suitably analyzed by qPCR, indicating the usefulness of the absolute qPCR methodology for the inclusion of patients with scarce material to the study. Taken together, these findings are of interest considering the importance of telomere dysfunction in the pathogenesis of cancer and give a new alternative to measure TL in hematologic disorders with substantial time and cost savings.

Keywords Telomere length · Absolute quantification · Bone marrow samples · Multiple myeloma · MGUS

Introduction

Telomeres are specialized structures, located at the end of eukaryotic chromosomes, constituted by $(TTAGGG)_n$ DNA tandem repeats and associated proteins. They are involved in the protection of chromosome ends from degradation, fusion, and recombination. Due to the end replication problem, telomeres shorten with each cell division, leading to senescence in human cells [1]. This telomere shortening can result in an increased level of chromosomal instability, associated with cancer development [2], and age-related diseases [3, 4]. Different methodologies have been described for the study of telomere length (TL), including terminal restriction fragments (TRF), fluorescence in situ hybridization, flow cytometry, and quantitative-polymerase chain reaction (qPCR). Among them, the TRF analysis [5] is considered the gold standard technique and the one used as reference for developing new strategies. However, this methodology has several disadvantages as larger amount of DNA are required and is time-consuming, and the result includes the unknown length of the subtelomeric region.

The measurement of relative average telomere lengths by qPCR was developed by Cawthon [6], using a carefully designed pair of primers. In this assay the telomere signal is normalized to the signal from a single copy gene (SCG) to generate a Telomere/SCG ratio. More recently a qPCR assay to measure absolute TL was developed [7], in which

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J. Panero (✉) · I. Slavutsky
Laboratorio de Genética de Neoplasias Linfoides, Instituto de Medicina Experimental (IMEX), CONICET-Academia Nacional de Medicina, J.A. Pacheco de Melo 3081, C1425AUM Buenos Aires, Argentina
e-mail: jpanero@unq.edu.ar

N. J. O'Callaghan · M. Fenech
Commonwealth Scientific and Industrial Research Organization (CSIRO)-Food and Nutritional Sciences, Adelaide, Australia

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Table 1 Telomere length measurement of patients with plasma cell disorders

Patients (no cases)	Sex		Mean age (years) (range)	TRF (kb)	Log [qPCR] (kb/genome)	Converted TRF (kb)
	M	F				
MGUS (48)	18	30	69.5 (41–84)	7.20 ± 0.46	4.27 ± 0.24	6.69 ± 0.48
MM (54)	15	39	67.4 (30–87)	6.15 ± 0.41	3.65 ± 0.26	5.76 ± 0.37

Results are shown as mean ± SE

MGUS monoclonal gammopathy of undetermined significance, *MM* multiple myeloma, *M* male, *F* female, *TRF* terminal restriction fragments, *qPCR* quantitative-polymerase chain reaction

the incorporation of a synthesized standard oligomer generates an absolute value for TL, instead of a relative measure. This methodology also normalizes the quantity of telomere product to a SCG to provide a mean TL for the cell population. The advantage of this qPCR is that, small amounts of DNA are required and it can easily and rapidly be performed with a high throughput of samples. In addition, the capability to generate absolute TL values will allow a more direct comparison of results between experiments within and between laboratories [8]. Initially, this technique was described in peripheral blood, cell lines, and a variety of human tissues [7], but to date, it has not been evaluated in bone marrow (BM) samples.

Multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) are the two most common plasma cell disorders, characterized by the presence of clonal BM plasma cells, and of a monoclonal protein in serum and/or urine. Measures of TL in these pathologies became important due to the emergence of new therapeutic strategies based on telomere-targeting inhibitors [9]. In this study, we have (a) examined the relationship between TL data obtained from TRF versus that derived from the absolute qPCR method in a sample from BM cells of MM and MGUS patients and (b) evaluated the usefulness of this new qPCR approach for BM samples with scarce DNA material. Results were also analyzed in relation to clinical parameters and overall survival.

Materials and Methods

Patients

Comparison of TL measurements by TRF and absolute qPCR was performed in a cohort of 102 patients with plasma cell disorders: 54 with MM and 48 with MGUS (Table 1). In addition, another cohort of 47 patients: 28 with MM and 19 MGUS (22 males; mean age 69.2 years, range 43–88 years) without TRF analysis, were also studied. The diagnosis was based on the International Myeloma Working Group Criteria [10]. All individuals provided their written informed consent according to institutional guidelines. The study was approved by the local Ethics Committee.

Measurements of Telomere Length

Absolute TL measurement was carried out by qPCR in a LightCycler system (Roche Diagnostics, Mannheim, Germany). Briefly, genomic DNA was isolated from bone marrow cells. For each DNA sample, two consecutive reactions were performed: the first to amplify a single copy gene, *RPLP0* (ribosomal protein, large, P0) and the second for telomeric sequence. The primers sequences (written 5′→3′) were as follows: TELf, CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT; TELr, GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT; RPLP0f, CAGCAAGTGGGAAGGTGTAATCC; RPLP0r, CCCATTCTATCATCAACGGGTACAA [7]. Both PCRs were performed in a final volume of 20 μL containing 20 ng DNA, 1× SYBRGreen Master Mix (Roche Diagnostics, Mannheim, Germany) and 250 nM *RPLP0* primers or 100 nM telomere primers. The PCR conditions were as follows: 95 °C 10 min followed by 45 cycles of 95 °C 15 s, 60 °C 1 min. The melting curve was performed with 1 cycle of 95 °C 20 s, 50 °C 15 s, and 98 °C with a temperature ramp of 0.1 °C/s. Each sample was analyzed in duplicate and all measurements included the determination of the standards and no-template as a negative control, in which water was substituted by the DNA. Standard curves were constructed using five-fold serial dilutions of synthesized oligonucleotides containing only TTAGGG repeats for telomere PCR and an oligomer containing the *RPLP0* product for SCG PCR (Figure S1).

Mean TRF lengths were determined by Southern Blot (SB) as previously described [11]. Briefly, for terminal restriction analysis, high-molecular weight DNA was extracted from BM samples followed by double digestion of 10 μg DNA with *Hin*fI and *Rsa*I restriction enzymes at 37 °C, overnight. The digested products were separated by electrophoresis in 0.8 % agarose gels during 20 h at 35 V. Gels were first depurinated, then denatured and neutralized, and finally transferred to nylon membranes by Southern blotting. The telomeric fragments were detected through

hybridization with a 5′ end-labeled telomeric probe $(TTAGGG)_7$. The hybridized membranes were exposed to films during 10–15 days at −70 °C before developing. Hybridization signals were evaluated in the autoradiographs by densitometric scanning with respect to a λ/*Hin*dIII molecular weight standard. The mean TL of a sample was calculated by integrating the signal intensity over the entire TRF distribution as a function of TRF length using the formula: $\sum(OD_i)/\sum(OD_i/L_i)$, where OD_i and L_i are the signal intensity and TRF length, respectively, at position "i" on the autoradiogram. In addition, peripheral blood mononuclear cells from healthy controls, K-562 cell line as internal positive control of telomere shortening, and cord blood cells as very young tissue without telomere reduction, were used.

The absolute TL values of 102 plasma cell disorders samples which were also analyzed by TRF assay were used to perform a regression analysis in order to convert absolute TL (kb per diploid genome) to SB TL values (kb).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 5.0 (2008). Linear regression was used to obtain the correlation between the method of absolute TL and the TRF length. Overall survival measured from diagnosis to last follow-up or death was estimated by Kaplan–Meier and compared with the log-rank test. For all tests, $p < 0.05$ was considered as statistically significant.

Results and Discussion

Validation of Absolute qPCR Method in BM Samples

Absolute telomere length was measured in BM samples of MM patients that had been previously analyzed for TRF lengths by SB, in order to test the performance of the qPCR method. A significant linear correlation between TRF and the absolute qPCR approach was observed ($p < 0.0001$; $r^2 = 0.70$) (Fig. 1). The regression formula ($y = 0.1316\ x + 0.2370$, where $x =$ Log [TL (qPCR)] and $y =$ Log [TL (TRF)]) was used to convert the qPCR TL values (kb per diploid genome) into the equivalent SB TL (kb) values. To verify the reliability of the absolute qPCR method, a set of 15 BM samples of MGUS patients were analyzed by both methodologies. The qPCR TL values (kb per diploid genome) were converted into the equivalent SB TL (kb) values using the regression curve in Fig. 1, showing consistent results with TRF data (Table S1). These findings validate the prediction regression model and indicate the usefulness of the new absolute qPCR methodology for telomere measurements in BM samples.

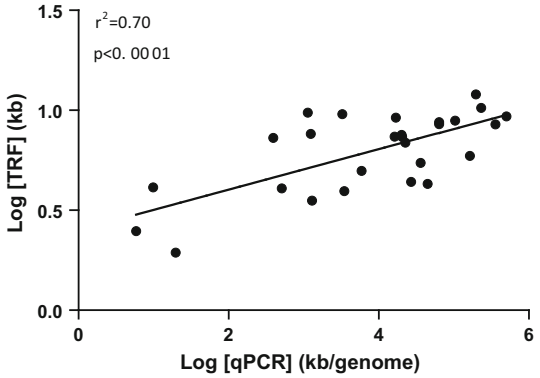

Fig. 1 Comparison of telomere length measurement methods in bone marrow samples of multiple myeloma patients. Graph represents correlation between absolute qPCR and TRF assay ($p < 0.0001$; $r^2 = 0.70$)

Telomere Length in Plasma Cell Disorders and Correlation with Clinical Parameters

Analysis of TL was performed in a total of 54 patients with MM and 48 with MGUS. A general decrease in TL in MM compared to MGUS was observed, but no significant differences were reached. Results are summarized in Table 1. Although the converted values for qPCR TL were slightly shorter for all samples, the average TL was comparable to SB data in both MM and MGUS patients. These findings are in agreement with previous reported data [6, 7, 12]; the difference is mainly attributed to the presence of subtelomeric regions or non-telomeric components that remain attached to the TTAGGG sequence after restriction enzyme digestion, and that are still detected by SB but not amplified by qPCR.

Furthermore, clinical characteristics were available for 45 MM cases (Table 2). The analysis of correlation with absolute TL and TRF values showed significant differences for β_2-microglobulin (qPCR: $p = 0.04$; TRF: $p = 0.02$) and hemoglobin levels ($p = 0.01$ for both methods) (Fig. 2), both parameters related to adverse prognosis in MM patients [10]. The median overall survival for MM patients was 28 months (range 2–50 months), calculated from the date of diagnosis. For a better analysis, patients were divided according to TL less than versus more than or equal to the median converted TRF value (5.76 kb). Although the analysis did not reveal any significant difference in survival rate, probably related to the small number of cases, shorter survival for patients with less than the median TL value (31 months) compared to cases with higher telomere lengths (46 months) was observed. These results are in concordance to those previously observed by Wu et al. [13] in MM patients, and support the participation of telomere dysfunction in disease progression. No association between TL and clinical parameters in MGUS patients was found.

Table 2 Clinical characteristics in MM patients

Characteristics	Median (range)	Low TL	High TL	*p* value
Age (years)	68.5 (30–85)	66.1 (30–85)	71.15 (52–82)	0.78
ISS stage (%)				
I	41.6	12.5	29.1	
II	25.0	16.6	8.4	0.34
III	33.4	16.7	16.7	
BMPCI (%)	51 (10–90)	52 (10–90)	46 (15–85)	0.75
β_2 microglobulin (mg/dL)	4.59 (0.5–18.2)	5.52 (0.5–18.2)	3.81 (0.8–17.9)	0.19
LDH (U/L)	187.8 (107–361)	180.63 (116–324)	180 (107–272)	0.83
Serum albumin (g/dL)	3.26 (2.0–4.6)	3.14 (2.09–4.12)	3.37 (2–4.6)	0.64
Serum calcium (mg/dL)	9.58 (7.7–14.6)	9.57 (7.7–14.6)	9.59 (8.8–14)	0.90
Creatinine (mg/dL)	2.30 (0.58–11.3)	2.31 (0.6–6.2)	2.29 (0.58–11.3)	0.17
Hemoglobin (g/dL)	11.27 (6.9–15.1)	10.57 (6.9–14.1)	11.96 (9.4–15.1)	0.28
Paraprotein M (g)	2.72 (0.08–9.45)	3.93 (0.08–9.45)	1.34 (0.11–2.52)	0.20

Total sample: 45 patients. Female patients constituted 62 % of sample

ISS international staging system, *BMPCI* bone marrow plasma cell infiltration, *LDH* lactate dehydrogenase

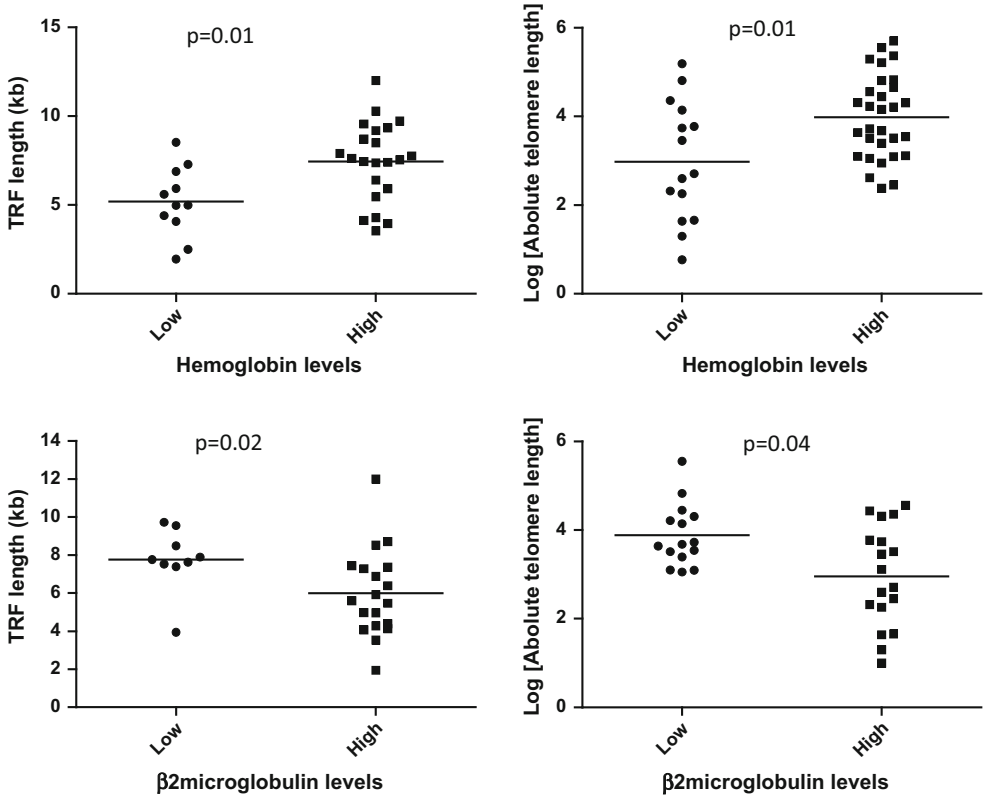

Fig. 2 Analysis of telomere length and clinical parameters in multiple myeloma patients. High β_2-microglobulin levels (*above* 3.5 mg/dL) and low hemoglobin concentration (*below* 10 g/dL) was found associated with telomere reduction performed by both TRF and qPCR methods

Telomere Length in BM Samples Not Suitable for TRF Assay

As previously referred, one of the disadvantages of TRF analysis is the requirement of high quantity of unfragmented DNA which is problematic when working with BM samples. Thus, we further analyzed whether absolute qPCR would permit the measurement of those types of samples. In this regard, we have evaluated another set of 47 BM samples that could not be previously analyzed by SB due to low quantity of DNA. All of them amplified suitably the *RPLP0* gene and showed a mean converted TRF value of 5.45 ± 0.52 kb for MM cases and 6.88 ± 0.80 kb for MGUS patients. These results are in concordance with mean TRF values observed in Table 1 and also with those found by our group in other cohort of patients with plasma cell disorders [14]. Although

our series is not so large, we have obtained promising results with this methodology, allowing the inclusion of patients with low quantity of DNA to the study.

In conclusion, we have used for the first time the methodology of absolute qPCR to quantify TL successfully in BM samples. This opens a new alternative to measure TL in hematologic malignancies with substantial time and cost savings. Interestingly, this new absolute qPCR method showed a good correlation with results analyzed by TRF, which would permit a direct comparison of previous data by interpolating the absolute value in the correlation curve. Furthermore, the low quantity of DNA needed for qPCR compared to TRF will allow the analysis of an increased number of BM samples, including those with small amount of DNA. As known, dysfunctional telomeres may be a key event driving genomic instability and disease progression in tumors and correlate with the emergence of increased genomic complexity [15]. Thus, the assessment of telomere length becomes important not only in understanding biological characteristics of the disease but also provides useful prognostic information. Particularly in MM, the association of telomere length with clinical parameters was demonstrated using the recently described absolute qPCR method as well as the gold standard TRF assay. In this respect, different studies, including those of our group, showed that short telomeres were associated with known prognostic factors and in combination with high telomerase activity define a subgroup of patients with poor prognosis [11, 13, 14]. Taken together, these observations highlight the evaluation of TL as well as telomere-related proteins, providing the opportunity to choose the best patient population for new therapeutic approaches with telomere inhibitors.

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Conflict of interest No competing financial interests exist.

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