

Altered mRNA Expression of Telomere-Associated Genes in Monoclonal Gammopathy of Undetermined Significance and Multiple Myeloma

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In this study, we explored changes in the expression of the telomere maintenance genes, *TRF1*, *TRF2* and *TANK1* in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). Results were correlated with human telomerase reverse transcriptase (*hTERT*) expression, telomere length (TL) and clinicopathological characteristics. Bone marrow (BM) samples from 132 patients, 64 with MGUS and 68 with MM, were studied. Real-time quantitative reverse transcription-polymerase chain reaction was used to quantify gene expression. TL was evaluated by terminal restriction fragment length analysis. MGUS patients showed increased *TRF1* levels ($P = 0.006$) and lower expression of *TRF2* ($P = 0.005$) and *TANK1* ($P = 0.003$) compared with MM patients. For *hTERT* analysis, patients were divided into three groups by use of receiver operating characteristics: low (group I (GI)), intermediate (group II (GII)) and high (group III (GIII)) expression. We observed increasing expression of *TRF2* and *TANK1* from GI to GIII in MGUS and MM, with differences for both genes in MM ($P < 0.01$) and for *TRF2* in MGUS ($P < 0.01$). GIII patients with the highest telomerase expression had the shortest TL. In both entities, a positive association between *TRF2-TANK1*, *TRF2-hTERT* and *TANK1-hTERT* ($P \leq 0.01$) was observed. In MM, the percentage of BM infiltration and Ki-67 index were positively associated with *TRF2*, *TANK1* and *hTERT* expression ($P \leq 0.03$) and negatively with TL ($P = 0.02$), whereas lactate dehydrogenase was significantly correlated with *TRF2* mRNA ($P = 0.008$). Our findings provide the first evidence of a modification in the expression of telomeric proteins in plasma cell disorders, and suggest that mechanisms other than telomerase activation are involved in TL maintenance in these pathologies.

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INTRODUCTION

Monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) are the two most common plasma cell disorders characterized by the presence of clonal bone marrow (BM) plasma cells and of a monoclonal protein in serum and/or urine. MM constitutes approximately 10 to 15% of all hematologic malignancies and about 1% of all forms of cancer. Clinical manifestations that include osteolytic lesions, ane-

mia, hypercalcemia, immunodeficiency and renal abnormalities can be attributed to the underlying plasma cell proliferation (1). The natural course of the disease may progress from MGUS, a presymptomatic phase, to MM. MGUS is characterized by serum M protein levels less than 3 mg/dL, BM plasma cell infiltration (BMPCI) less than 10% and no clinical manifestations related to monoclonal gammopathy (2). This entity is one of the most common premalignant disorders in

Western countries, with a prevalence of 3.2% in the population of white individuals age 50 years and older. The transformation rate of MGUS to MM is about 1% per year, with an actuarial probability of malignant evolution of 30% at 25 years. After a median of 10 years, about one-quarter of MGUS patients develop MM. Recent studies have identified markers that can be used to identify patients with high risk of progression: higher levels of monoclonal protein, non-IgG protein isotype and abnormal ratio of free light chains (3).

Human telomeres comprise tandem repeats of the noncoding DNA sequence TTAGGG and are involved in the maintenance of chromosomal stability and genome integrity by DNA-binding proteins, which associate with other

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proteins/complexes to achieve telomere-end protection and length control (4). Because of the end-replication problem, telomeres progressively shorten with repeated cell division, a process that leads to telomere dysfunction and, ultimately, contributes to tumorigenesis. In cancer cells, telomere length (TL) is maintained by the enzyme telomerase, a ribonucleoprotein complex that compensates for telomere reduction by adding new repeats to chromosome ends. Telomerase is composed of two subunits: human telomerase reverse transcriptase (hTERT), which has catalytic activity, and the RNA component (hTERC), which provides the template for telomeric synthesis. Activation of telomerase may therefore be a critical step in human cancer development because telomerase activity is absent in most normal somatic cells, but it is present in most malignant tissues and immortal human cell lines (5,6).

Telomerase activity is regulated in *cis* by the shelterin hexa-protein complex (TRF1, TRF2, POT1, RAP1, TIN2 and TPP1) and epigenetic factors (7,8). In particular, TRF1 and TRF2 bind to DNA as preformed homodimers, and despite the similarities in their sequence and architecture, TRF1 and TRF2 have different functions. TRF1 is involved in a negative feedback mechanism that allows telomere shortening by inhibiting the activity of telomerase (9). Although TRF2 is also involved in negative TL regulation, it participates in t-loop formation, capping and protecting the 3' single-strand overhang. Like TRF1, increased expression of TRF2 shortens telomeres, but loss of its activity leads to telomere-telomere fusion events, suggesting a protective role for TRF2 in the maintenance of telomere structure and function (10).

Nonshelterin proteins at chromosome ends may also play important roles at telomeres. Among them, the enzyme TANK1 (tankyrase-like protein 1) is a member of the growing family of poly (ADP-ribose) polymerases (PARPs) that interacts with and ADP-ribosylates the telomere-binding protein TRF1 (11). *In vitro*, ribosylation by TANK1 displaces

TRF1 from telomeric DNA, a process that suggests that TANK1 might be a positive regulator of TL in telomerase-expressing cells (9). Evidence has also confirmed this role *in vivo* (12). Tankyrase overexpression in human cells induces a progressive elongation of telomeres and is expected to be upregulated in all human tumors.

Widmann *et al.* (13) recently reported their finding of significantly short telomeres in both myeloid and lymphoid non-neoplastic cells of patients with aggressive non-Hodgkin lymphoma. Furthermore, telomere shortening in peripheral blood lymphocytes in patients suffering from various solid tumors has also been reported (14). The results of these studies suggest that chromosome instability associated with telomere dysfunction is an early event in tumorigenesis.

A number of reported studies have evaluated TL and telomerase activity in MM patients (15–19). However, information is very scarce regarding telomere dysfunction in patients with MGUS (15,17) (five and two patients, respectively), and no studies about changes in the expression of shelterin proteins in plasma cell disorders have been reported. Thus, the aim of this study was to investigate mRNA expression of a set of genes that encode the telomere-binding proteins TRF1, TRF2 and TANK1 in patients with MGUS and MM, to determine the role of telomere dysfunction as one of the steps involved in the progression of MGUS to MM. Results were correlated with *hTERT* expression, TL and clinicopathological characteristics of patients.

MATERIALS AND METHODS

Patients

The present study included 132 patients with plasma cell disorders: 64 patients with MGUS and 68 with MM. The diagnoses were based on standard criteria (1,2). MM staging was made according to the criteria proposed by Durie and Salmon (20) and the recently developed International Staging System (21). Pa-

tients were studied at diagnosis and all individuals provided their informed consent according to institutional guidelines. The study was approved by the ethics committee of our institution. Median follow-up was 17 months (range: 2–48 months). Detailed data about sex, age, disease stage and clinicopathological characteristics of all patients are summarized in Table 1.

RNA Extraction, Reverse Transcription and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted with the Trizol reagent (Invitrogen, Buenos Aires, Argentina) from mononuclear cells isolated from BM samples from patients and peripheral blood of controls and from the K-562 cell line. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 1 × RT Buffer (Promega, Madison, WI, USA), 200 U/μL of Moloney murine leukemia virus RT (Promega), 250 ng/μL random primer (Promega) and 10 mmol/L each deoxynucleoside-5'triphosphate (Invitrogen). The cDNA synthesis was performed in a total volume of 20 μL, containing 1 μg of the total RNA, for 10 min at 95°C, for 60 min at 37°C and 10 min at 95°C to inactivate the enzyme. cDNA was stored at –20°C until use.

The analyses of *TRF1*, *TRF2*, *TANK1* and *hTERT* mRNAs were performed using quantitative real-time PCR (QRT-PCR) in a LightCycler system (Roche Diagnostics, Mannheim, Germany), based on TaqMan methods. The mRNA expression levels were measured by using gene-specific fluorescent-labeled probes. 5-Carboxyfluorescein was added as the 5'-fluorescent reporter, and 5-carboxytetramethylrhodamine was added to the 3' end as a quencher. Primers and probe sequences have been previously described (22,23). The house-keeping gene *GAPDH* was used to normalize sample-to-sample differences in cDNA input, RNA quality and RT efficiency, and it was amplified by using the primers and probe described by Hu

Table 1. Clinical characteristics of patients with MM and MGUS.

Characteristics	MM	MGUS
No. cases	68	64
Sex, F/M, n	35/33	36/28
Age, mean (range), y	68.5 (30–87)	69.3 (39–88)
Durie-Salmon stage, %		
I	27.6	—
II	12.1	—
III	60.3	—
International Staging System stage, %		
1	31.1	—
2	33.4	—
3	35.5	—
BMPCI, %		
0–10	—	100
>10–30	42	—
>30–60	31.6	—
>60	26.4	—
Paraprotein isotype, %		
IgG	65.5	62.7
IgM	1.7	23.7
IgA	27.6	11.9
IgG + IgM	1.7	1.7
IgA + IgG	1.7	—
No secretor	1.7	—
Light chain, %		
κ	61	57.4
λ	39	42.6
Lytic bone lesions, %	47.6	0
β ₂ microglobulin, mean (range), μg/mL	0.7 (0.11–1.79)	0.3 (0.11–0.77)
LDH, mean (range), U/L	193.6 (84–1265)	167.3 (94–395)
Albumin, mean (range), g/dL	3.3 (1.7–4.6)	3.7 (3–4.44)
Calcium, mean (range), mg/dL	9.2 (7.3–14.6)	9.2 (7.8–10.6)
Creatinine, mean (range), mg/dL	1.7 (0.58–11.3)	0.9 (0.5–1.82)
Hemoglobin, mean (range), g/dL	10.9 (5.8–15.8)	12.7 (9–15.6)
M band, mean (range), g	3.1 (0.08–9.48)	0.7 (0.16–1.73)
Ki-67, mean (range), % positive cells	14.8 (0–40)	4.7 (0–25)

et al. (23). The PCR reaction was performed by using 4 μL of each RT reaction, 1 × TaqMan master mix (Roche Diagnostics), 200 nmol/L of the probe and 300 nmol/L of *TRF1* primers and 500 nmol/L of *TRF2*, *TANK1*, *hTERT* and *GAPDH* primers in a 20-μL final volume. For all targets, the thermal cycling conditions were 10 min at 95°C to activate the enzyme, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. All measurements included a determination of the standards and the use of a no-template as a negative control, in which water was substituted for the cDNA. Standard curves were constructed with five-fold serial dilutions of

the cDNA from the K562 cell line (0.15–5 μg total RNA). The ratio between copy numbers of *GAPDH* and *TRF1*, *TRF2*, *TANK1* or *hTERT* genes in samples and controls were calculated with the LightCycler software, by using these standard curves.

Telomere Length Evaluation

Telomere length was evaluated by use of terminal restriction fragments (TRF) assay as previously described (17). Briefly, for terminal restriction analysis, high molecular weight DNA was extracted from BM samples, followed by double digestion of 10 μg DNA with *HinfI* and *RsaI* restriction en-

zymes at 37°C, overnight. The digested products were separated by electrophoresis in 0.8% agarose gels during a 20-h period 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 for 10–15 d at –70°C before the film was developed. Hybridization signals were evaluated in the autoradiograph by densitometric scanning in each lane with respect to a λ/*HindIII* molecular weight standard and were analyzed using the Gel Pro software (Media Cybernetics, Bethesda, MD, USA). Telomere mean values were measured by estimating the band size corresponding to the point with the highest intensity. For comparative analysis, peripheral blood mononuclear cells from 18 normal controls (7 males and 11 females; median age 65 years, range: 33–86 years) defined as healthy individuals with no personal or family history of cancer and matched by sex and age, were also evaluated. All of the study participants who were controls provided their informed consent. In addition, as internal controls for telomere shortening we used the K-562 cell line (positive control of telomere shortening), and cord blood cells (negative control) as a very young tissue without telomere reduction.

Immunohistochemical Analysis of Ki67

BM biopsies were fixed and decalcified for 72 h in Bouin's solution and embedded in paraffin. Tissue sections of 4-μm thickness were stained with hematoxylin-eosin, reticulin and Giemsa. For immunohistochemical analysis, tissue sections were deparaffinized and brought to phosphate-buffered saline solution, pH 7.2. After blocking endogenous peroxidase with 3% H₂O₂ in methanol and antigen retrieval pretreatment with citrate buffer in a microwave

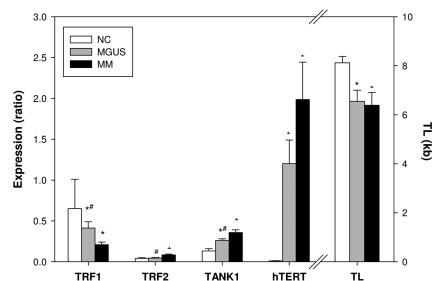


Figure 1. Histogram showing mean TL and mRNA expression profiles of *TRF1*, *TRF2*, *TANK1* and *hTERT* genes in patients and healthy individuals as normal controls (NC). Significant differences with respect to NC: * $P < 0.01$ and to MM: # $P < 0.006$.

oven, slides were incubated 1 h with monoclonal antibody against the Ki67 antigen (Dako, Carpinteria, CA, USA). Tissue sections were subsequently post-treated with a biotinylated antimouse immunoglobulin antiserum (Biogenex, San Ramon, CA, USA), followed by peroxidase-labeled avidin, and revealed with diaminobenzidine as chromogen. Counterstaining was then performed by using hematoxylin. Specificity tests, performed by omission of the specific antibody and incubation with nonimmune mouse serum, produced negative results.

Statistical Evaluation

The comparison of data from patients and controls and between subgroups was performed by using the Mann–Whitney test. Groupwise comparison of the distribution of clinical and laboratory variables was performed with the Student *t* test (for quantitative variables) and the χ^2 or Fisher exact test (for categorical variables). Correlations between gene expression and TL or clinical variables were assessed by using the Kendall coefficient. The cutoff point for *hTERT* expression was selected according to receiver operating characteristic (ROC) analysis. Overall survival was estimated by use of the Kaplan–Meier method and compared with the log-rank test. For all tests, $P < 0.05$ was regarded as statistically significant.

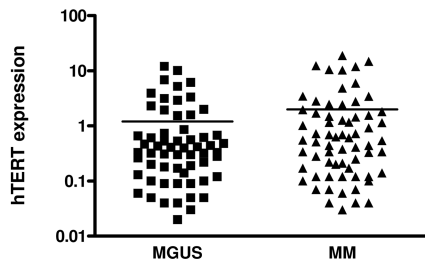


Figure 2. Scatter plot showing the wide distribution of *hTERT* mRNA levels in MM and MGUS.

RESULTS

Expression of Telomere-Binding Proteins by QRT-PCR

The mRNA expression of *TRF1*, *TRF2*, *TANK1* and *hTERT* was evaluated in 64 patients with MGUS and 68 with MM. A differential expression pattern with lower levels for *TRF1* and higher for *TRF2*, *TANK1* and *hTERT* in patients with respect to controls were observed (Figure 1). When both pathologies were compared, a significant increase of *TRF1* mRNA levels ($P = 0.006$) and a lower expression of *TRF2* ($P = 0.005$) and *TANK1* ($P = 0.003$) in MGUS with respect to MM patients were found. Although the mean *hTERT* mRNA level in MGUS patients was lower than that in MM, no significant difference between both entities was observed.

Analysis of data showed that *hTERT* expression varied considerably in both pathologies, with cases that overexpressed *hTERT* and many others with low mRNA transcripts (Figure 2). For a better analysis, patients were divided into three groups according to *hTERT* levels by using ROC curves: low (≤ 1.08) (group I [GI]), intermediate (< 1.08 to ≥ 5.0) (group II [GII]) and high (> 5.0) (group III [GIII]) mRNA expression (Table 2). MM and MGUS both demonstrated a similar distribution of patients per group, with most of the cases in GI (50 MGUS and 44 MM) and the least in GIII (5 MGUS and 7 MM). The evaluation of remaining genes according to *hTERT* groups showed an increased expression of *TRF2* and *TANK1* from GI to GIII in MGUS and MM. Significant differences for both genes in MM (GIII versus GI and GII: $P < 0.01$) and for *TRF2* in MGUS (GIII versus GI: $P < 0.01$) were observed. In addition, the comparison between GIII patients showed that both genes had lower mRNA expression in MGUS than MM, with significant differences for *TRF2* ($P = 0.01$). As for *TRF1*, a nonsignificant increase from GI to GIII in MM was observed. However, we observed heterogeneous behavior in MGUS, with higher values in GI and GII with respect to GIII, probably due to the presence of patients with elevated *TRF1*

Table 2. Analysis of telomeric gene expression and mean TL according to groups of *hTERT* expression in patients with MM and MGUS.

Groups	Telomeric genes (X ± ES)				TL, kb (X ± ES)
	<i>hTERT</i>	<i>TRF1</i>	<i>TRF2</i>	<i>TANK1</i>	
MGUS					
GI	0.27 ± 0.03	0.38 ± 0.08	0.04 ± 0.004	0.24 ± 0.01	7.17 ± 0.41
GII	2.53 ± 0.28	0.65 ± 0.28	0.05 ± 0.02	0.26 ± 0.08	6.92 ± 0.31
GIII	8.05 ± 1.28	0.23 ± 0.06	0.10 ± 0.02 ^{a,b}	0.39 ± 0.11	4.93 ± 1.01 ^c
MM					
GI	0.30 ± 0.04	0.18 ± 0.04	0.06 ± 0.01	0.28 ± 0.07	7.04 ± 0.51
GII	2.17 ± 0.24	0.21 ± 0.03	0.07 ± 0.01	0.39 ± 0.05	6.76 ± 0.79
GIII	12.10 ± 1.52	0.34 ± 0.08	0.22 ± 0.03 ^a	0.79 ± 0.16 ^a	4.14 ± 0.63 ^d

^a $P < 0.01$, significant differences of GIII with respect to GI and GII in MM and with respect to GI in MGUS;

^b $P = 0.01$, significant differences of GIII between MGUS and MM.

^c $P = 0.03$, significant differences of GIII with respect to GII.

^d $P = 0.04$, significant differences of GIII with respect to GI and GII.

mRNA expression in both groups (two cases in GII and five cases in GI).

In addition, we explored the relationship among mRNA expression of different genes. Both entities showed a positive association between *TRF2-TANK1* ($P < 0.0001$), *TRF2-hTERT* ($P < 0.0001$) and *TANK1-hTERT* genes ($P < 0.0001$ for MM and $P = 0.01$ for MGUS). The correlation between genes is shown in Figure 3.

TL Evaluation

TL measured by TRF assay was performed in 43 patients with MGUS and 47 with MM. As we have previously reported (17), all patients showed significant telomere shortening (MGUS: 6.55 ± 0.45 kb and MM: 6.39 ± 0.52 kb) compared with controls (8.12 ± 0.26 kb) ($P = 0.003$ and $P = 0.004$, respectively) (Figure 1). However, when the analysis of TL was performed according to *hTERT* groups, we found that patients from GIII, with the highest telomerase expression, had the shortest TL (Table 2). Significant differences in patients from GIII with respect to GII (MGUS: $P = 0.03$; MM: $P = 0.04$) and GI (MM: $P = 0.04$) were observed. Moreover, in MM the GIII mean TRF value was also significantly reduced compared with that for the K-562 cell line (5.02 ± 0.3 kb) ($P = 0.04$).

Correlation of Gene Expression and TL with Clinical Parameters and Ki67 Index

The correlations among gene expression and TL with known prognostic factors are shown in Figure 4. In patients with MM, the percentage of BMPCI was positively associated with *TRF2* ($P = 0.0002$), *TANK1* ($P = 0.005$) and *hTERT* ($P = 0.003$) expression and negatively with TL ($P = 0.02$), whereas lactate dehydrogenase (LDH) was significantly correlated with *TRF2* mRNA ($P = 0.008$). The Ki-67 index (Figure 5) was positively associated with *TRF2* ($P = 0.006$), *TANK1* ($P = 0.03$) and *hTERT* ($P = 0.03$) mRNA levels and negatively with TL ($P = 0.02$). When patients were distributed according to *hTERT* expression groups, significant differences in the percentage of

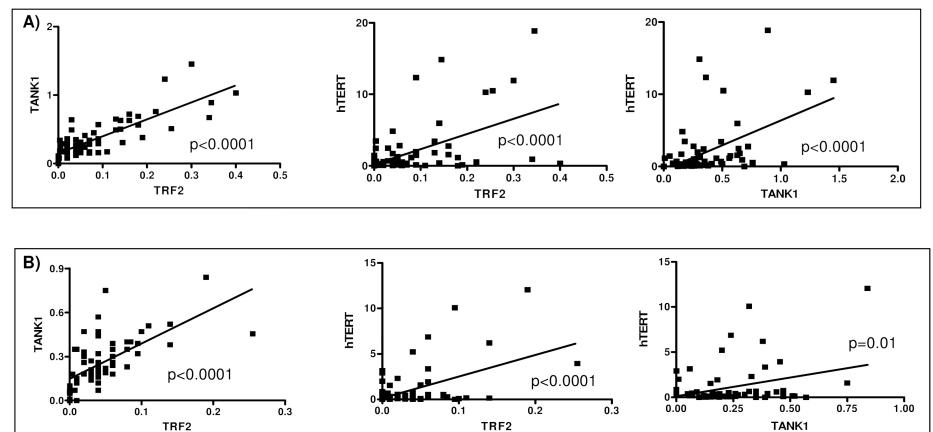


Figure 3. Significant correlation between *TRF2*, *TANK1* and *hTERT* mRNA expression in MM ($P < 0.0001$) (A) and MGUS ($P \leq 0.01$) (B).

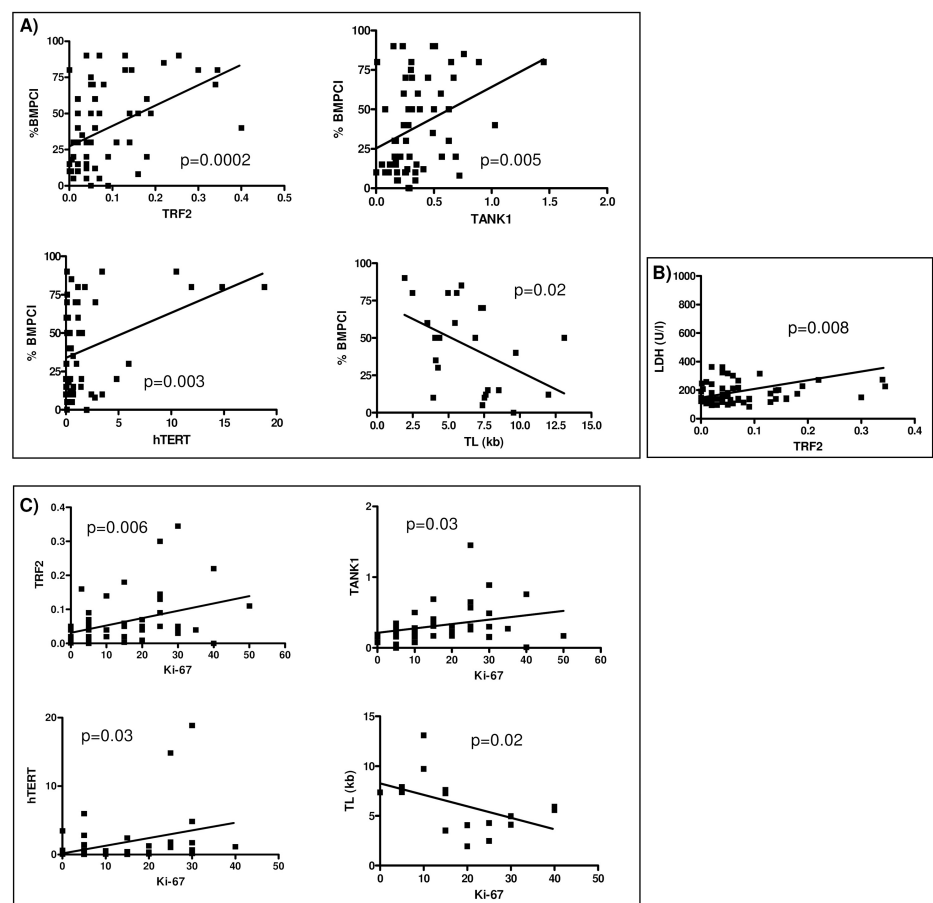


Figure 4. Evaluation of clinical parameters in MM. (A) Positive correlation between the percentage of BMPCI and *TRF2*, *TANK1* and *hTERT* mRNA levels ($P \leq 0.005$), and negative correlation with TL ($P = 0.02$); (B) positive correlation between *TRF2* expression and LDH ($P = 0.008$); (C) positive correlation between the percentage of Ki67-positive cells and *TRF2*, *TANK1* and *hTERT* expression ($P \leq 0.03$), and negative correlation with TL ($P = 0.02$).

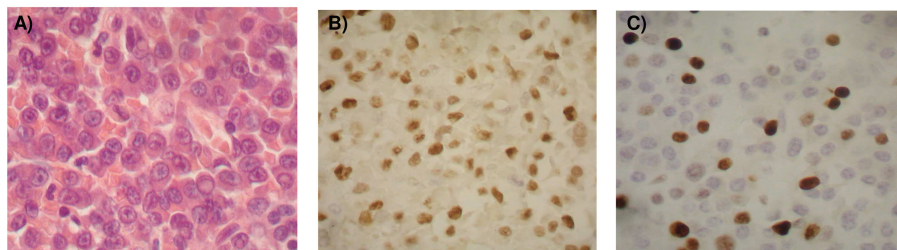


Figure 5. (A) Aggregates of atypical plasma cells in a BM sample from a patient with MM (hematoxylin and eosin, 400 \times). (B and C) Immunohistochemical determination of proliferative index with Ki-67 in two samples from patients with MM. (B) 75% of positive nuclei and (C) 15% of positive nuclei (3,3'-Diaminobenzidine, 400 \times).

BMPCI ($P = 0.04$) and albumin levels ($P = 0.03$) in GII/GIII with respect to GI were observed (Figure 6). The median overall survival was 17 months (range: 2–48 months). Although the number of patients was too small to allow valid statistical analysis, MM patients in GIII showed a shorter overall survival than GI patients (13.5 and 24.5 months, respectively), and similar results were observed when progression-free survival was evaluated (8 and 17 months, respectively). No association among genes and clinical parameters was found in MGUS.

DISCUSSION

The role of TL and telomerase activity in the development of human cancer has

been extensively investigated in recent years. In contrast, there are few reports of evaluations of the participation of telomere-binding proteins in tumorigenesis. In the present study, mRNA expression of *TRF1*, *TRF2* and *TANK1* genes was analyzed in a similar number of patients with MGUS and MM, and compared with *hTERT* expression and TL. As a whole, both entities showed shorter TL and higher *hTERT* expression in patients with MGUS and MM than in healthy controls, but no differences between MGUS and MM patients were observed. Analysis of data revealed that for both pathologies patients had a great heterogeneity in *hTERT* levels, with some cases in which this gene was overexpressed

and others in which *hTERT* mRNA expression was low. The use of ROC curves permitted us to divide patients into three different groups of *hTERT* expression. MGUS and MM exhibited a similar distribution of patients per group. In agreement with our data, heterogeneity in telomerase activity has been previously reported for MM patients (15,18). However, to our knowledge, this is the first study with results demonstrating a similar *hTERT* behavior in MGUS cases. As for TL, an inverse correlation with *hTERT* levels was observed in both MGUS and MM, with shortest telomeres for patients in GIII who had the highest *hTERT* expression. Our results showing a group of MGUS patients with very short telomeres and high *hTERT* mRNA levels suggest a role for these parameters in the progression from MGUS to MM. Supporting this idea, one MGUS patient of our series with high *hTERT* expression (6.87) and very short TL (4.28 kb) but without markers of high-risk disease, suffered progression to MM after 20 months of follow-up. More cases must be studied to confirm these data.

There have been a limited number of investigations evaluating the expression profile of telomere-binding factors in lymphoid malignancies. A number of studies have demonstrated increased mRNA levels of *TANK1*, *TRF2* and *hPif1* in high-grade non-Hodgkin lymphomas (22,24), and of *TRF1*, *TRF2* and *TIN2* in adult T-cell leukemia (25). In another reported study, investigators detected a low expression of many components involved in capping and telomere elongation in chronic lymphocytic leukemia (26). In our study, a nonsignificant decrease of *TRF1* and a significant increase of *TRF2* and *TANK1* levels in patients with respect to controls and in MM compared with MGUS patients were observed. *TRF1* was identified as a negative length regulator that limits telomere elongation and results in stable TL. According to a model proposed by Smogorzewska *et al.* (27), an unsuitably long telomere would recruit a large amount of *TRF1* protein, blocking the

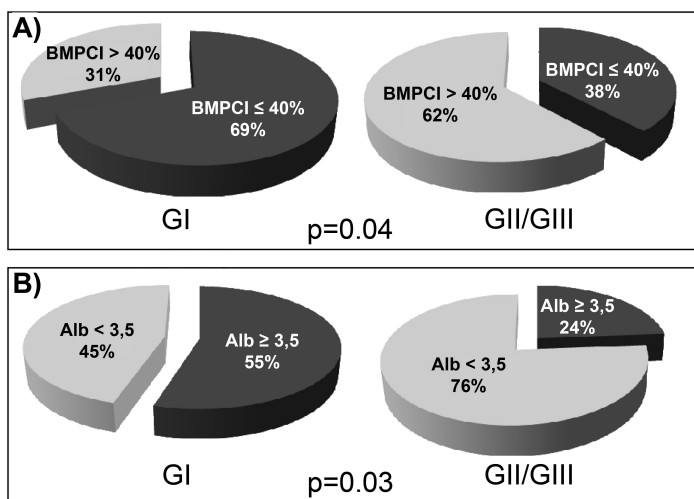


Figure 6. Distribution of MM patients according to *hTERT*-expression groups. Significant differences in patients from GII/GIII with respect to GI for (A) the percentage of BMPCI ($P = 0.04$) and (B) albumin (Alb) levels ($P = 0.03$).

telomerase-mediated elongation. In addition, van Steensel *et al.* (9) reported that long-term overexpression of *TRF1* in the telomerase-positive tumor-cell line HT1080 resulted in a gradual and progressive telomere shortening, supporting the role of TRF1 as a suppressor of telomere elongation. In MM patients, we have observed an increase of *TRF1* associated with a decrease of TL from GI to GIII, suggesting the possible participation of TRF1 in the maintenance of short telomeres. In MGUS, we have detected heterogeneous behavior, with higher values in GI and GII compared with GIII. One explanation for these results is that patients in GI and GII had elevated *TRF1* mRNA expression that raised mean values. More studies will be necessary to improve our understanding of *TRF1* function in MGUS.

As previously mentioned, our patients showed overexpression of *TRF2* and *TANK1* with significant differences between MGUS and MM. Both genes had a direct correlation with *hTERT* mRNA levels. The upregulation of *TANK1* and its association with telomerase expression was previously observed by Xu *et al.* (15) in a small number of MM and plasma cell leukemia patients, but no studies of *TANK1* expression in MGUS have been reported. Thus, our data confirm a previous observation in MM and, interestingly, showed similar behavior for MGUS patients. Simultaneously, our study results provide the first evidence of *TRF2* upregulation in MGUS and MM. In addition, we were able to demonstrate that *TRF2* and *TANK1* levels increased from GI to GIII in both MM and MGUS, showing a direct correlation between *TRF2* and *TANK1* expression. These findings indicate an interaction between positive and negative factors in the maintenance of TL in plasma cell disorders and support a role for *TRF2* and *TANK1* upregulation in disease progression. In a related study of gastric cancer, a significantly higher expression of *TRF2* in tumor tissue and in lymph-node metastasis compared with precancerous lesions and normal gastric mucosa was found (28). Changes in

telomeric gene expression and their negative correlations with telomere reduction, observed in our series, suggest that mechanisms other than telomerase activation are involved in TL maintenance in plasma cell disorders.

The present study is the first to correlate mRNA expression of telomere-associated genes and known prognostic factors in plasma cell disorders. In MM, we found that the percentage of BMPCI was positively correlated with the expression levels of *TRF2*, *TANK1* and *hTERT*, and negatively with TL, whereas LDH was associated with *TRF2*, suggesting these genes as probable prognostic markers in this entity. Furthermore, Ki67 was also positively associated with these genes and negatively with TL, supporting a high degree of telomere dysfunction in those myeloma cells with increased proliferative capacity. This finding supports the recent observation of Gastinne *et al.* (29), who found correlations between Ki67 and markers of intrinsic malignancy and tumor burden in MM patients, and supports previous data showing a strong correlation between telomerase activity and Ki67 (16). In our series, we also detected shorter survival in MM patients in GIII (with high levels of *hTERT* and very short TL) compared with GI, supporting the connection between dysfunctional telomeres and tumor progression. This observation is consistent with those reported by Wu *et al.* (18), who found that the combination of both short telomeres and high telomerase activity defined a subgroup of patients with poor prognosis.

In reference to MGUS, the molecular basis of its evolution to a malignant monoclonal gammopathy remains poorly understood (3). Different predictors of risk progression (size of M protein, type of immunoglobulin, BMPCI, serum free light chain ratio) have emerged, and there is growing evidence that the stromal component of the BM microenvironment may also play an important role in disease evolution (3,30). In our series of MGUS cases, no association with clinical characteristics was found, but the pres-

ence of a group of patients with both short TL and high expression of telomere-associated genes permits us to suggest that these parameters are probable prognostic factors that will likely be useful in allowing more accurate risk stratification for individual patients. The short time of transition from MGUS to MM in one of these patients supports this hypothesis. Nevertheless, more studies will be necessary to confirm these findings.

Telomere dysfunction is considered one of the molecular causes of genetic instability (31). Telomere shortening has been demonstrated to contribute directly to the presence of chromosomal abnormalities usually found in diverse types of cancer. This telomere reduction may trigger the formation of telomeric fusions between chromosome arms, which may result in novel karyotypic rearrangements (32,33). Different reports showed similar genetic alterations in MGUS and MM, which suggests a model for MGUS evolution based on genomic instability manifested by aneuploidy as the permissive event for the occurrence of chromosome alterations (34,35). Furthermore, gene expression profiling studies allowed the identification of a clear oncogenic pathway deregulation from normal plasma cells to relapsed MM characterized by increased chromosome instability and overexpression of *MYC* and *E2F* genes (36). The results of this study and a previously reported study by our group (17) in both MM and MGUS patients support the presence of genomic instability manifested by telomere dysfunction and the probable participation of these factors in the development and/or progression of the disease.

Results of a recently reported study (13) showed that short telomeres in nontransformed leukocytes are a risk factor for lymphomagenesis. In our study, we were able to find short telomeres and changes in the expression of telomere maintenance genes as well as a strong correlation among genes in mononuclear cells of patients with MGUS and MM, providing the first evidence of a modification in the expression of telomere-associated genes in

these pathologies. This finding is important, considering the new therapeutic approaches with diverse telomere-targeting inhibitors (37) and suggests that a number of MM patients may be particularly sensitive to telomere-damaging drugs.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Kyle RA, Rajkumar SV. (2004) Multiple myeloma. *N. Engl. J. Med.* 351:1860–73.
- Kyle RA, et al. (2006) Prevalence of monoclonal gammopathy of undetermined significance. *N. Engl. J. Med.* 354:1362–9.
- Bladé J, Rosiñol L, Cibeira MT, de Larrea CF. (2008) Pathogenesis and progression of monoclonal gammopathy of undetermined significance. *Leukemia.* 22:1651–7
- Xin H, Liu D, Songyang Z. (2008) The telosome/shelterin complex and its functions. *Genome Biol.* 9:232.
- Counter CM, et al. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* 11:1921–9.
- Shay JW, Zou Y, Hiyama E, Wright WE. (2001) Telomerase and cancer. *Hum. Mol. Genet.* 10:677–85.
- Blasco MA. (2007) The epigenetic regulation of mammalian telomeres. *Nat. Rev. Genet.* 8:299–309.
- Palm W, De Lange T. (2008) How shelterin protects mammalian telomeres. *Ann. Rev. Genet.* 42:301–34.
- van Steensel B, de Lange T. (1997) Control of telomere length by the human telomeric protein TRF1. *Nature.* 385:740–3.
- de Lange T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* 19:2100–10.
- Smith S, Giriati I, Schmitt A, de Lange T. (1998) Tankyrase, a poly (ADP-ribose) polymerase at human telomeres. *Science.* 282:1484–7.
- Smith S, de Lange T. (2000) Tankyrase promotes telomere elongation in human cells. *Curr. Biol.* 10:1299–302.
- Widmann TA, Herrmann M, Taha N, König J, Pfreundschuh M. (2007) Short telomeres in aggressive non-Hodgkin's lymphoma as a risk factor in lymphomagenesis. *Exp Hematol.* 35:939–46.
- Wu X, et al. (2003) Telomere dysfunction: a potential cancer predisposition factor. *J. Natl. Cancer Inst.* 95:1211–8.
- Xu D, et al. (2001) Telomerase activity in plasma cell dyscrasias. *Br. J. Cancer.* 84:621–5.
- Shiratsuchi M, et al. (2002) Clinical significance of telomerase activity in multiple myeloma. *Cancer.* 94:2232–8.
- Cottliar A, et al. (2003) Telomere shortening in patients with plasma cell disorders. *Eur. J. Haematol.* 71:334–40.
- Wu K-D, et al. (2003) Telomerase and telomere length in multiple myeloma: correlations with disease heterogeneity, cytogenetic status, and overall survival. *Blood.* 101:4982–9.
- Ladetto M, et al. (2004) Telomere length correlates with histopathogenesis according to the germinal center in mature B-cell lymphoproliferative disorders. *Blood.* 103:4644–9.
- Durie BG, Salmon E. (1975) A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. *Cancer.* 36:842–54.
- Greipp P, et al. (2005) International staging system for multiple myeloma. *J. Clin. Oncol.* 23:3412–20.
- Klapper W, Krams M, Qian W, Janssen D, Parwaresch R. (2003) Telomerase activity in B-cell non-Hodgkin lymphomas is regulated by hTERT transcription and correlated with telomere-binding protein expression but uncoupled from proliferation. *Br. J. Cancer.* 89:713–9.
- Hu LH, Chen FH, Li YR, Wang L. (2004) Real-time determination of human telomerase reverse transcriptase mRNA in gastric cancer. *World J. Gastroenterol.* 10:3514–7.
- MacNamara B, et al. (2001) Telomerase activity in relation to pro- and anti-apoptotic protein expression in high grade non-Hodgkin's lymphomas. *Haematologica.* 86:386–93.
- Bellon M, et al. (2006) Increased expression of telomere length regulating factors TRF1, TRF2 and TIN2 in patients with adult T-cell leukemia. *Int. J. Cancer.* 119:2090–7.
- Poncet D, et al. (2008) Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood.* 111:2388–91.
- Smogorzewska A, et al. (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.* 20:1659–68.
- Hu H, Zhang Y, Zou M, Yang S, Liang XQ. (2010) Expression of TRF1, TRF2, TIN2, TERT, KU70, and BRCA1 proteins is associated with telomere shortening and may contribute to multistage carcinogenesis of gastric cancer. *J. Cancer Res. Clin. Oncol.* 136:1407–14.
- Gastinne T, et al. (2007) Plasma cell growth fraction using Ki-67 antigen expression identifies a subgroup of multiple myeloma patients displaying short survival within the ISS stage I. *Eur. J. Haematol.* 79:297–304.
- Kyle RA, et al. (2010) Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia.* 24:1121–27.
- Gisselsson D, et al. (2001) Telomere dysfunction triggers extensive DNA fragmentation and evolution to complex chromosome abnormalities in human malignant tumors. *Proc. Natl. Acad. Sci. U. S. A.* 98:12683–8.
- Gisselsson D, et al. (2002) Centrosomal abnormalities, multipolar mitoses, and chromosomal instability in head and neck tumours with dysfunctional telomeres. *Br. J. Cancer.* 87:202–7.
- Höglund M, Gisselsson D, Hansen GB, Sähl T, Mitelman F. (2003) Ovarian carcinoma develops through multiple modes of chromosomal evolution. *Cancer Res.* 63:3378–85.
- Fonseca R, et al. (2002) Genomics abnormalities in monoclonal gammopathy of undetermined significance. *Blood.* 100:1417–24.
- Bochtler T, et al. (2008) Evaluation of the cytogenetic aberration pattern in amyloid light chain amyloidosis as compared with monoclonal gammopathy of undetermined significance reveals common pathways of karyotypic instability. *Blood.* 111:4700–5.
- Anguiano A, et al. (2009) Gene expression profiles of tumor biology provide a novel approach to prognosis and may guide the selection of therapeutic targets in multiple myeloma. *J. Clin. Onc.* 25:4197–203.
- Phatak P, Burger AM. (2007) Telomerase and its potential for therapeutic intervention. *Br. J. Pharmacol.* 152:1003–11.