

Altered mRNA expression of telomere-associated genes in monoclonal gammopathy of undetermined significance and multiple myeloma

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Running head: Telomere associated genes in MGUS and MM

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Abstract

In this study, we explored changes in the expression of telomere maintenance genes, TRF1, TRF2 and TANK1 in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). Results were correlated with hTERT expression, telomere length (TL) and clinico-pathological characteristics. Bone marrow (BM) samples of 132 patients: 64 with MGUS and 68 with MM were studied. Real-Time Quantitative RT-PCR was used to quantify gene expression. TL was evaluated by Terminal Restriction Fragments Length Analysis. MGUS patients showed increased TRF1 levels ($p=0.006$) and lower expression of TRF2 ($p=0.005$) and TANK1 ($p=0.003$) with respect to MM cases. For hTERT analysis, patients were divided into three groups using ROC curves: low (Group I; GI), intermediate (Group II; GII) and high (Group III; GIII) expression. Increased 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 ($p<0.01$) were found. 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 telomere length 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 including osteolytic lesions, anemia, 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 pre-symptomatic phase, to MM. MGUS is characterized by serum M protein level less than 3 mg/dL, BM plasma cells infiltration (BMPCI) less than 10%, and no clinical manifestations related to their monoclonal gammopathy (2). This entity is one of the most common premalignant disorders in Western countries with a prevalence of 3.2% in the Caucasian population 50 years of age or 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 select a group of patients with high risk of progression: higher levels of monoclonal protein, non IgG protein isotype, and abnormal ratio of free light chain (3).

Human telomeres comprise tandem repeats of the non-codificant DNA sequence TTAGGG. They are involved in the maintenance of chromosomal stability and genome integrity by DNA-binding proteins, which associate with other proteins/complexes to achieve telomere-end protection and length control (4). Because of the end-replication problem telomeres progressively shorten with repeated cell division, leading to telomere dysfunction and, ultimately, contributing to tumorigenesis. In cancer cells, telomere length (TL) is maintained by the enzyme telomerase, a ribonucleoprotein complex that compensates the telomere reduction by adding new repeats to chromosomes ends. Telomerase is composed of two subunits: the human telomerase reverse transcriptase (hTERT), which has the catalytic activity and the RNA component (hTERC) that provides the template for the telomeric synthesis. Activation of telomerase may be therefore, a critical step in human cancer development, since telomerase activity is absent in most normal somatic cells, but it is present in most of 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, both TRFs 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 the t-loop formation, capping and protecting the 3' single strand overhang. Like TRF1, an 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).

Non-shelterin proteins at chromosome ends can play also 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, suggesting that TANK1 might be a positive regulator of TL in telomerase-expressing cells (9). There are also evidences that confirm this role *in vivo* (12). Tankyrase over-expression in human cells induces a progressive elongation of telomeres and is expected to be upregulated in all human tumors.

In a recent article, Widmann et al (13) have found significantly short telomeres in both myeloid and lymphoid non-neoplastic cells of patients with aggressive non-Hodgkin's lymphoma. Furthermore, telomere shortening in peripheral blood lymphocytes in patients suffering from various solid tumors, was also reported (14). These studies suggest chromosome instability associated with telomere dysfunction as an early event in tumorigenesis.

A number of reports have evaluated TL and telomerase activity in MM patients (15-19). However, there is very scarce information about 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 were reported. Thus, the aim of this study was to investigate the mRNA expression of a set of genes encoding telomere-binding proteins TRF1, TRF2 and TANK1 in patients with MGUS and MM, in order 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, telomere length (TL) and clinico-pathological characteristics of patients.

Materials and Methods

Patients

The present study included 132 patients with plasma cell disorders: 64 cases with MGUS and 68 with MM. The diagnosis was 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). Patients 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 clinico-pathological characteristics of all patients are summarized in Table 1.

RNA extraction, reverse transcription and Real-Time Quantitative RT-PCR

Total RNA was extracted with the Trizol reagent (Invitrogen), from mononuclear cells isolated from BM samples of patients and peripheral blood of normal controls, and from K-562 cell line. RT-PCR was carried out using 1X RT Buffer (Promega), 200 U/ μ l of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega), 250ng/ μ l random primer (Promega) and 10 mM each dNTP (Invitrogen). The cDNA synthesis was performed in a total volume of 20 μ l, containing 1 μ g of the total RNA, for 10 minutes at 95°C, for 60 minutes at 37°C and 10 minutes at 95°C to inactivate the enzyme. cDNA was stored at -20°C until use.

The analysis of TRF1, TRF2, TANK1 and hTERT mRNAs were performed using real-time quantitative PCR (QRT-PCR) in a LightCycler system (Roche Diagnostics), based on TaqMan methodology. The mRNA expression levels were measured using gene-specific fluorescent-labeled probes. FAM was added as the 5'-fluorescent reporter while TAMRA was added to the 3' end as a quencher. Primers and probes sequences were previously described (22, 23). The housekeeping gene GAPDH was used to normalize sample-to-sample differences in cDNA input, RNA quality and RT efficiency, and it was amplified using the primers and probe described by Hu et al (23). The PCR reaction was done using 4 μ l of each RT reaction, 1X 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 μ l final volume. For all targets, the thermal cycling conditions were 10 minutes at 95°C to activate the enzyme, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. All measurements included a determination of the standards and no-template as a negative control, in which water was substituted for the cDNA. Standard curves were constructed with fivefold serial dilutions of the cDNA from the K562 cell line (5 μ g to 0.15 μ g 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, using these standard curves.

Telomere length evaluation

Telomere length was evaluated by 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 μ g DNA with *HinfI* and *RsaI* restriction enzymes at 37°C, overnight. The digested products were separated by electrophoresis in 0.8% agarose gels during 20h at 35V. 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 autoradiograph by densitometric scanning in each lane respect to a λ /*HindIII* molecular weight standard and were analyzed using the Gel Pro software (Media Cybernetics). 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 matched by sex and age, were also evaluated. All of them provided their informed consent. In addition, as internal controls for telomere shortening we used 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. Four micrometer thickness tissue sections 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 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 anti-mouse immunoglobulin antiserum (Biogenex), followed by peroxidase-labeled avidin and revealed with diaminobenzidine as chromogen. Counterstained was then performed using hematoxylin. Specificity tests, performed by omission of the specific antibody and incubation with non-immune mouse serum, produced negative results.

Statistical evaluation

The comparison of data from patients and controls and between subgroups was performed using 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's exact test (for categorical variables). Correlations between gene expression and TL or clinical variables were assessed by using the Kendall's coefficient. The cut-off point for hTERT expression was selected according to receiver operating characteristic (ROC) analysis. Overall survival (OS) was estimated by the Kaplan-Meier method and compared by the log-rank test. For all tests, $p < 0.05$ was regarded as statistically significant.

Results

Expression of telomere-binding proteins by QRT-PCR

The mRNA expression of TRF1, TRF2, TANK1 and hTERT was evaluated in 64 cases 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 was found. No difference in hTERT values between MGUS and MM was observed.

Analysis of data showed that hTERT expression varied considerably in both pathologies, with cases that over-expressed hTERT and many others with low mRNA

transcripts (Figure 2). For a better analysis, patients were divided into three groups according to hTERT levels using ROC curves: low (≤ 1.08) (Group I; GI), intermediate ($1.08 < y \leq 5.0$) (Group II; GII) and high (> 5.0) (Group III; GIII) mRNA expression (Table 2). Both entities demonstrated a similar distribution of patients per group, with most of cases in GI (50 MGUS and 44 MM) and the less proportion 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 vs. GI and GII: $p < 0.01$) and for TRF2 in MGUS (GIII vs. 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 non-significant increase from GI to GIII in MM was observed. However, a heterogeneous behavior in MGUS, with higher values in GI and GII respect to GIII were found, probably due to the presence of patients with elevated TRF1 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.

Telomere length evaluation

TL measured by TRF assay was performed in 43 cases with MGUS and 47 with MM. As we have previously reported (17), whole patients showed a significant telomere shortening (MGUS: 6.55 ± 0.45 kb and MM: 6.39 ± 0.52 kb) compared to 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 to 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 correlation among gene expression and TL with known prognostic factors is 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 LDH was significantly correlated with TRF2 mRNA ($p=0.008$). In reference to Ki-67 index (Figure 5), it 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 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 of GIII showed a shorter OS than GI cases (13.5 and 24.5 months, respectively) and similar results were observed when progression free survival was evaluated (8 months and 17 months, respectively). No association among genes and clinical parameters was found in MGUS.

Discussion

The role of TL and telomerase activity in human cancer development has been extensively investigated in recent years. In contrast, there are few reports evaluating the participation of telomere-binding proteins in tumorigenesis. In the present study, mRNA expression of TRF1, TRF2 and TANK1 genes were 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 than normal controls, but no differences between MGUS and MM patients were observed. Analysis of data revealed that both pathologies had a great heterogeneity in hTERT levels, with cases that over-expressed this gene and others with low mRNA expression. The use of ROC curves permitted us to divide patients in three different groups of hTERT expression. Both entities exhibited a similar distribution of cases per group. In agreement with our data, heterogeneity in telomerase activity has been previously referred for MM patients (15, 18). However, to our knowledge, this is the first study 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 that had the highest hTERT expression. Our results showing a group of MGUS 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, progressed to MM after 20 months of follow-up. More cases must be studied to confirm this 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 (ATL) (25). Another report has detected a low expression of many components involved in capping and telomere elongation in chronic lymphocytic leukemia (26). In our study, a non-significant decrease of TRF1 and a significant increase of TRF2 and TANK1 levels in patients with respect to controls and in MM compared to MGUS 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 inappropriately long telomere would recruit a large amount of TRF1 protein, blocking the telomerase-mediated elongation. In addition, van Steensel et al (9) reported that long-term over-expression of TRF1 in the telomerase-positive tumor-cell line HT1080 resulted in a gradual and progressive telomere shortening, supporting that TRF1 acts as a suppressor of telomere elongation. In MM patients, we have observed an increase of TRF1 associated to a decrease of TL from GI to GIII, suggesting its possible participation in the maintenance of short telomeres. In MGUS, we have detected a heterogeneous behavior, with higher values in GI and GII compared to GIII. One explanation for these results could be the presence of patients in GI and GII with elevated TRF1 mRNA expression that raised mean values. More studies will be necessary in order to a better understanding of TRF1 function in MGUS.

As previously referred, our patients showed over-expression of TRF2 and TANK1 with significant differences between MGUS and MM. Both genes had a direct correlation with hTERT mRNA levels. The up-regulation 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 about TANK1 expression in MGUS were reported. Thus, our data confirm a previous observation in MM and, interestingly, showed a similar behavior for MGUS patients. Simultaneously, our studies provide the first evidence of TRF2 up-regulation in MGUS and MM. In addition, we were able to demonstrate that TRF2 and TANK1 levels increased from GI to GIII in both entities, 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 up-regulation in disease progression. In this aspect, in gastric cancer, a significant higher expression of TRF2 in tumor tissue and in lymph node metastasis compared to precancerous lesions and normal gastric mucosa was found (28). Changes in telomeric gene expressions and their negative correlations with telomere reduction observed in our series, may suggest that mechanisms other than telomerase activation are involved in telomere length maintenance in plasma cell disorders.

The present study is the first one to correlate mRNA expression of telomere-associated genes and known prognostic factors in plasma cell disorders. In MM, we found that the percentage of BMPC1 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 results of interest taking into account the recent observation of Gastinne et al (29) that found correlation between Ki67 with markers of intrinsic malignancy and tumor burden in MM patients, and support previous data showing strong correlation between telomerase activity and Ki67 (16). In our series, we also detected a shorter survival in those myeloma patients in GIII (with high levels of hTERT and very short TL) compared to GI, supporting the connection between dysfunctional telomeres and tumor progression. This is consistent with those observed 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, bone marrow plasma cell infiltration, serum free light chain ratio) have emerged and, there is growing evidence that the stromal component of the bone marrow 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 presence of a group of patients with both short TL and high expression of telomere-associated genes permit us to suggest these parameters as probable prognostic

factors that will likely result in a more accurate risk stratification for individual patients. The short time of transition from MGUS to MM of one of these cases support this hypothesis. Nevertheless, more studies will be necessary to confirm it.

Telomere dysfunction is considered one of the molecular causes of genetic instability (31). It was demonstrated that telomere shortening 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 that may result in novel karyotypic rearrangements (32, 33). Different reports showed similar genetic alterations in MGUS and MM suggesting 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 identifying 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). This study and a previous report of our group (17) support the presence of genomic instability manifested by telomere dysfunction in both MM and MGUS patients and their probable participation in the development and/or progression of the disease.

A recent article (13) showed short telomeres in non-transformed leukocytes as 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 good 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 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 reported no potential conflicts of interest

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Legends

Figure 1: Histogram showing mean telomere length (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 (multiple myeloma): # $p < 0.006$. MGUS: monoclonal gammopathy of undetermined significance.

Figure 2: Scatter plot showing the wide distribution of hTERT mRNA levels in multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS).

Figure 3: Significant correlation between TRF2, TANK1 and hTERT mRNA expression in multiple myeloma ($p < 0.0001$) (A) and monoclonal gammopathy of undetermined significance ($p \leq 0.01$) (B).

Figure 4: Evaluation of clinical parameters in MM. A) positive correlation between the percentage of bone marrow plasma cell infiltration (BMPCI) and TRF2, TANK1 and hTERT mRNA levels ($p \leq 0.005$) and negative correlation with telomere length (TL) ($p = 0.02$); B) positive correlation between TRF2 expression and lactate dehydrogenase (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 bone marrow sample from a patient with multiple myeloma (H&E, 400x); B and C) Immunohistochemical determination of proliferative index with Ki-67 in two samples of patients with multiple myeloma (B: 75% of positive nuclei and, C: 15% of positive nuclei) (DAB, 400x).

Figure 6: Distribution of MM patients according to hTERT expression groups. Significant differences in cases from GII/GIII with respect to GI for: A) the percentage of bone marrow plasma cell infiltration ($p = 0.04$) and B) albumin (Alb) levels ($p = 0.03$).

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

Characteristics	MM	MGUS
No Cases	68	64
Sex (F/M)	35/33	36/28
Age (years) Mean (Range)	68.5 (30-87)	69.3 (39-88)
DS stage (%)		
I	27.6	-
II	12.1	-
III	60.3	-
ISS stage (%)		
1	31.1	-
2	33.4	-
3	35.5	-
BMPCI (%)		
0-10	-	100
>10-30	42.0	-
>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
	Mean-Range	
β_2 microglobulin ($\mu\text{g/ml}$)	0.7 (0.11-1.79)	0.3 (0.11-0.77)
LDH (U/l)	193.6 (84-1265)	167.3 (94-395)
Albumin (g/dl)	3.3 (1.7-4.6)	3.7 (3-4.44)
Calcium (mg/dl)	9.2 (7.3-14.6)	9.2 (7.8-10.6)
Creatinine (mg/dl)	1.7 (0.58-11.3)	0.9 (0.5-1.82)
Hemoglobin (g/dl)	10.9 (5.8-15.8)	12.7 (9-15.6)
M Band (g)	3.1 (0.08-9.48)	0.7 (0.16-1.73)
Ki-67 (% positive cells)	14.8 (0-40)	4.7 (0-25)

MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance; F: female; M: male; DS: Durie-Salmon; ISS: International Staging System; BMPCI: bone marrow plasma cell infiltration; LDH: lactate dehydrogenase.

Table 2. Analysis of telomeric genes 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)
	hTERT	TRF1	TRF2	TANK1	
Monoclonal gammopathy of undetermined significance					
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 [#]	0.39±0.11	4.93±1.01 ^{**}
Multiple myeloma					
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 [#]	0.79±0.16 [#]	4.14±0.63 [*]

-Significant differences of GIII respect to GI and GII in MM and respect to GI in MGUS: [#]p<0.01

-Significant differences of GIII between MGUS and MM: ^op=0.01

-Significant differences of GIII respect to GI and GII: ^{*}p=0.04, and respect to GII: ^{**}p=0.03

Figure 1.

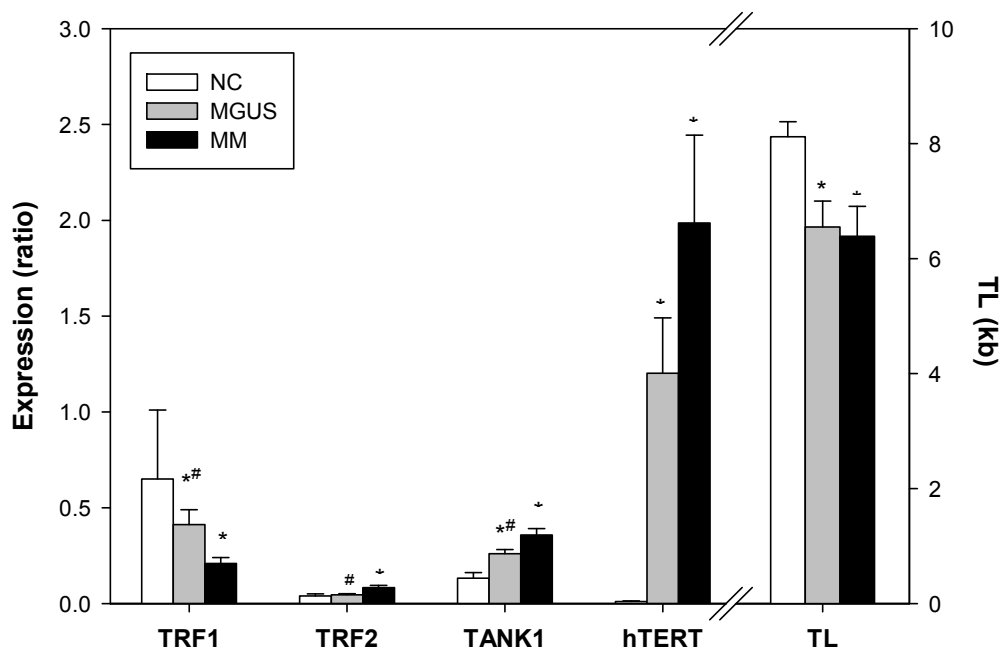
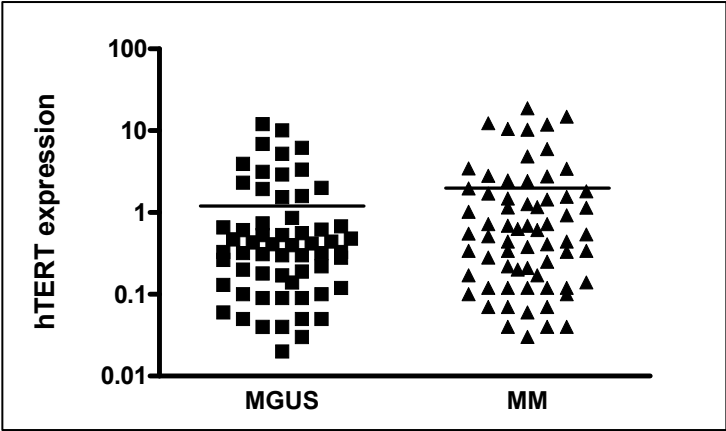
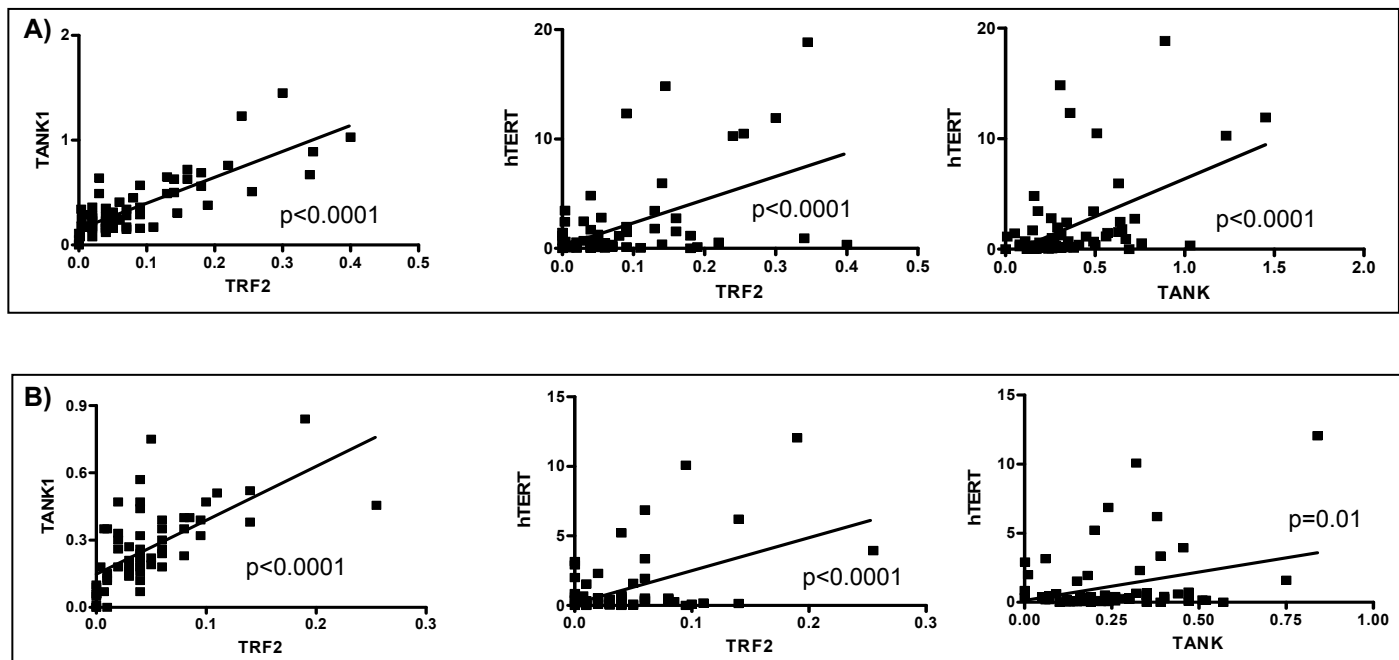


Figure 2.



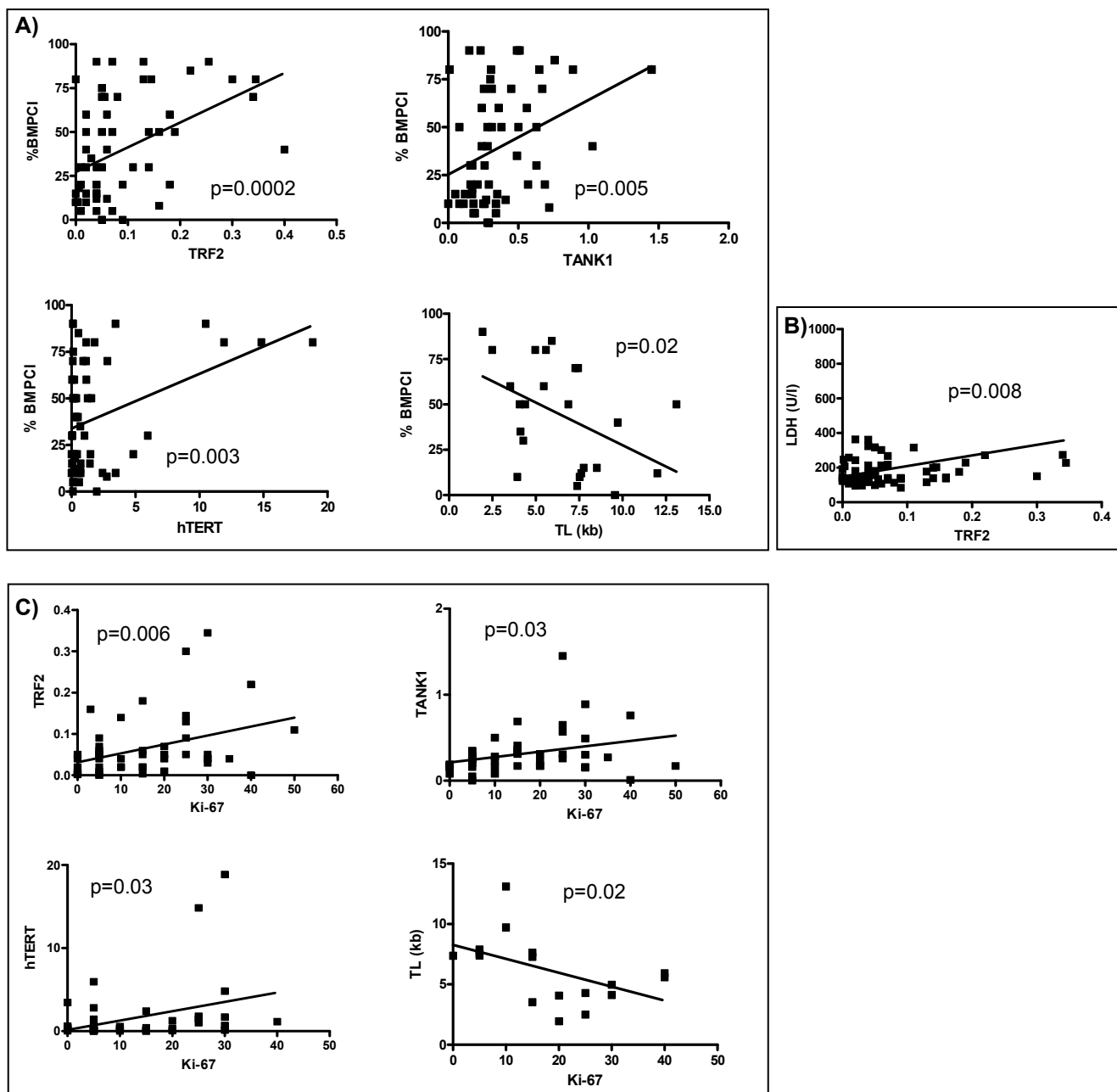
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Figure 3.



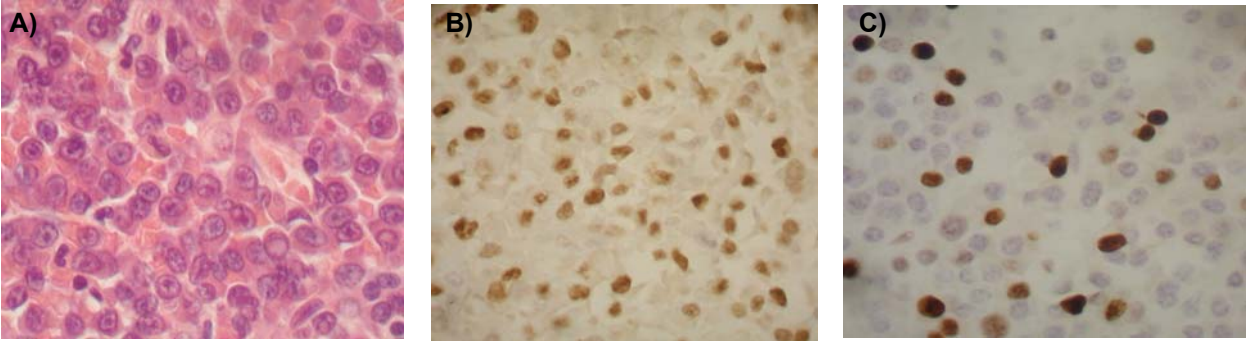
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Figure 4.



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Figure 5



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Figure 6.

