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Expression profile of shelterin components in plasma cell disorders. Clinical significance of *POT1* overexpression

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ABSTRACT

The core complex of telomere-associated proteins, named the shelterin complex, plays a critical role in telomere protection and telomere length (TL) homeostasis. In this study, we have explored changes in the expression of telomere-associated genes *POT1*, *TIN2*, *RAP1* and *TPP1*, in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). A total of 154 patients: 70 with MGUS and 84 with MM were studied. Real-time quantitative PCR was used to quantify gene expression. TL was evaluated by Terminal Restriction Fragments. Our data showed increased expression of *POT1*, *TPP1*, *TIN2* and *RAP1* in MM with respect to MGUS patients, with significant differences for *POT1* gene ($p = 0.002$). In MM, the correlation of gene expression profiles with clinical characteristics highlighted *POT1* for its significant association with advanced clinical stages, high calcium and β2-microglobulin levels ($p = 0.02$) and bone lesions ($p = 0.009$). In multivariate analysis, *POT1* expression ($p = 0.04$) was a significant independent prognostic factor for overall survival as well as the staging system (ISS) ($p < 0.02$). Our findings suggest for the first time the participation of *POT1* in the transformation process from MGUS to MM, and provide evidence of this gene as a useful prognostic factor in MM as well as a possible molecular target to design new therapeutic strategies.

Introduction

Plasma cell disorders are characterized by the proliferation of a single clone of plasma cells in the bone marrow (BM) and by the production of monoclonal immunoglobulins (Igs). These entities may range from a benign entity, named monoclonal gammopathy of undetermined significance (MGUS), to symptomatic multiple myeloma (MM) [1]. MGUS is one of the most common premalignant disorders in Western countries, characterized by the presence of Ig (M-protein) levels less than 3 mg/dl, BM plasma cell infiltration (BMPCI) less than 10%, no clinical manifestations related to their monoclonal gammopathy and an average rate of progression to MM of 1% per year. Recent studies show that almost every MM case is preceded by an MGUS stage [2]. The signals that lead to the transition of MGUS to MM require changes in the stromal compartment that enhanced plasma cell survival and proliferation [3,4]. The interaction between stromal cells and plasma cells induces the secretion of multiple factors, which mediates MM initiation and progression. Among them, galectin-1 (Gal-1) is a multifunctional member of a growing family of β-galactoside-binding animal lectins that has recently emerged as a critical regulator of plasma cell differentiation, and a potent inducer of the survival and proliferation of primary myeloma cells *in vitro* [5].

Telomeres are distinctive DNA-protein structures that cap the ends of linear chromosomes. They are essential to maintain chromosomal integrity and genome stability. Failure of telomere protection can have deleterious effects that result in chromosomal end-to-end fusions, breakage and rearrangements [6]. Telomere nucleotide repeats progressively shorten with each cell division, and it has implications in oncogenesis and cellular aging due to limited cellular proliferation below a certain critical telomere length (TL). Therefore, mammalian cells employ several mechanisms that sense and regulate TL to ensure proper telomere function.

TL is maintained by telomerase, a ribonucleoprotein complex that consist of a catalytic reverse transcriptase protein (hTERT), a RNA template (hTR), dyskerin and other accessory proteins [7,8]. An increase in telomerase activity has been observed in myeloma cells of about 90% of newly diagnosed and relapsed patients [9] and also associated to poor prognosis [10,11]. Telomerase activity is mainly regulated by *hTERT* transcription, which expression can be regulated at different levels.

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Studies including those of our group have shown a wide heterogeneity in telomerase activity and *hTERT* mRNA levels in both MM and MGUS patients [10,12,13]. In addition, due to its promoter region located in a CpG island, it is a potential target for repression through DNA methylation. This mechanism has been shown, at least in part, in several tissues [14,15].

The core complex of telomere-associated proteins, named the shelterin complex, is composed of six proteins: TRF1, TRF2, TIN2, POT1, TPP1 and RAP1, that plays a critical role in telomere protection and TL homeostasis [16]. In addition, the shelterin complex modulates telomerase activity at chromosome ends, recognizes telomeric DNA and remodels it into a t-loop, which protects the 3′ overhang from being recognized as DNA damage. Particularly, POT1 possesses high specificity for the single-strand DNA (ssDNA) overhangs, influencing the maintenance of telomeric DNA by telomerase and protecting the 5′ end of the chromosome [17]. A POT1 mutant that does not bind ssDNA results in complete loss of TL control, suggesting that the ssDNA-binding activity of POT1 could block telomerase from gaining access to the 3′ terminus [18]. In addition, the TPP1-TIN2 interaction regulates the bridge between TRF1 and TRF2 which promotes as well as stabilizes the assembly of high-order telomeric complexes. TRF1 and TRF2 bind the double-strand DNA (dsDNA) through their myb domains and act as negative length regulators in human cells [19]. Finally, RAP1 is critical for inhibiting non-homologous end-joining of mammalian telomeres [20]. The mammalian RAP1 does not bind DNA on its own, but is brought to the telomere by binding to TRF2. This association is essential for RAP1 binding to telomeres [21].

Recent studies have reported modifications in the expression of *TRF1*, *TRF2* and *TANK1* genes in patients with plasma cell disorders and human myeloma cell lines [13,22], but no study has as yet addressed the analysis of the remaining shelterin genes in these pathologies. Here, we report for the first time, the expression profile of *POT1*, *TIN2*, *RAP1* and *TPP1* shelterin genes and their clinical significance in patients with MM and MGUS. Furthermore, the relationship between telomerase and *LGALS1* (encoding galectin-1) expression was also explored.

Materials and methods

Patients

One hundred and fifty-four newly diagnosed patients with plasma cell disorders: 70 with MGUS and 84 with MM were analyzed. The diagnosis was based on the International Myeloma Working Group Criteria [23]. MM staging was made according to the classification proposed by Durie and Salmon (DS) [24] and the International Staging System (ISS) [25]. Clinico-pathological characteristics of all patients are summarized in Supplementary Table 1S. Patients under the age of 65 years and fit were treated with an induction therapy with thalidomide or bortezomib plus hematopoietic stem-cell transplantation. Conventional therapy combined with thalidomide or bortezomib were administered in patients older than 65 years or unfit for high dose treatment. A small number of cases received VAD chemotherapy alone. The median of follow up was 30.1 month (range 2–60 months). All individuals provided their informed consent according to institutional guidelines. The study was approved by the Ethics Committee of our Institution.

RNA extraction and expression analysis of shelterin components

Total RNA was extracted from mononuclear cells isolated from BM samples of patients and K-562 cell line, as previously reported [13]. The cDNA synthesis was performed in a final 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 inactive the enzyme; cDNA was stored at −20 °C until use. The mRNA expression of *TRF1*, *TRF2*, *TIN2*, *POT1*, *TPP1*, *RAP1* and *hTERT* was determined using real-time quantitative PCR (qPCR) in a LightCycler system (Roche Diagnostics, Mannheim, Germany), based on TaqMan methodology. Primer and probe sequences for *TRF1*, *TRF2* and *hTERT* were previously described by Klapper et al. [26], whereas primers for *TIN2*, *POT1*, *TPP1* and *RAP1* were described by Poncet et al. [27]. Probes for these genes were specifically designed for this work:

TIN2: 5′ CTGTTGCCCCTGGCTTGGTTCGCTAC 3′
POT1: 5′ ACTAGAAGCCTATCTCATGGATTCTGAC 3′
TPP1: 5′ CCCGGCTACGGGTGCCTGGTT 3′
RAP1: 5′ CTTGTGGAAGCCACCCGGGAGTT 3′.

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. [28]. The PCR reaction was done using 4 μl of each RT reaction, 1× TaqMan master mix (Roche Diagnostics, Mannheim, Germany), 200 nM of the probe and 500 nM of *TRF1*, *TRF2*, *TIN2*, *POT1*, *TPP1*, *RAP1*, *hTERT* and *GAPDH* primers in a 20 μl final volume. For all targets, the PCR conditions were 10 min at 95 °C, 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 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 K-562 cell line.

Telomere length evaluation

TL was evaluated on BM samples from patients by terminal restriction fragment (TRF) assay as previously described [29]. Hybridization signals were evaluated in the autoradiographs by densitometric scanning in each lane with respect to a λ/HindIII molecular weight standard, and analyzed by the Image Master 1D Prime (Pharmacia-Biotech, Uppsala, Sweden) software. The mean telomeric length of a sample was evaluated over the range of 2–23 kb, and calculated by integrating the signal intensity over the entire TRF distribution as a function of TRF length using the formula: $\sum(ODi)/\sum(ODi/Li)$, where ODi and Li are the signal intensity and length, respectively, at position "i" on the autoradiogram. In addition, peripheral blood mononuclear cell (PBMC) from healthy controls, K-562 cell line as internal positive control of telomere shortening, and cord blood cells as negative control of telomere shortening, were used.

Methylation analysis

Genomic DNA was modified by sodium bisulfite as previously described [30]. PCRs on the *hTERT* gene were performed using three sets of primers described by Guilleret et al. [14]: P2 (−441/-217), P1 (−200/-13) and G1 (−31/+169), according to the ATG start site, and the enzyme Go Taq DNA polymerase (Promega, Madison, WI, USA). PCR cycling conditions were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, Tm (54 °C for P2, 51 °C for P1 and 61 °C for G1) for 45 s and 72 °C for 45 s, and a final extension of 10 min at 72 °C. PCR products were separated on a 2% agarose gel, purified using the Illustra™ GFX™ Gel Band Purification Kit (GE Healthcare), sequenced bi-directionally and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE Biosystem, Foster City, CA). Bisulfite conversion was verified using CpGenome™ Universal Methylated DNA (Intergen, New York, USA) as positive control. The sequence of methylated control DNA was aligned and compared with sequence of the wild-type DNA. Methylated cytosine residues in CpG dinucleotide remained as C, whereas unmethylated cytosines were changed to T after bisulfite conversion.

Immunohistochemical (IHC) and expression analysis of galectin-1

For IHC analysis, paraffin-embedded human tumor sections were stained with rabbit anti-Gal1 IgG as described [31] using the Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). Tissue sections were

counterstained using hematoxylin. Specificity was assessed by omission of the specific antibody and incubation with non-immune mouse serum, generating negative results.

Galectin-1 gene (*LGALS1*) mRNA expression was evaluated by qPCR with SYBR Green methodology. Primers were previously described [32]. The PCR reaction was performed using 4 μl of each RT reaction, 1 × SYBR Green master mix (Roche Diagnostics, Mannheim, Germany), 4.5 mM $MgCl_2$, 250 nM of *LGALS1* and 750 nM of *GAPDH* primers, in a 20 μl final volume. For all targets, the PCR conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 5 s, 60 °C for 3 s and 72 °C for 10 s. Standard curves were constructed as mentioned above and all measurements included the determination of the negative control.

Statistical evaluation

Statistical analyses were performed using GraphPad Prism Version 5.0 (2008). The analysis of data was performed using the Mann–Whitney test. Kendall's coefficient was used to correlate gene expression with TL and clinical variables. 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). Receiver operating characteristic (ROC) curves analysis was applied to calculate the expression cut-off value for each telomere-associated gene, with the highest sensitivity and specificity. The distribution of patients according to the cut-off value is shown in the Supplementary Table 2S. Overall survival (OS) was estimated by the Kaplan-Meier method and compared with the log-rank test. Cox proportional hazards regression analysis was used to assess the association of several prognostic factors and survival. For all tests, $p < 0.05$ was regarded as statistically significant.

Results

Patients

This study was performed in a cohort of MM and MGUS patients that harbors previously published features of telomere and telomerase changes. Thus, they showed a general decrease in TL (MM: 6.29 ± 0.3 kb; MGUS: 6.55 ± 0.45 kb) as well as a significant increase in *hTERT* mRNA expression (MM: 2.33 ± 0.47; MGUS: 1.18 ± 0.27) compared with age and sex matched controls (TL: 8.12 ± 0.26 kb; *hTERT*: 0.01 ± 0.003) ($p = 0.003$ and $p < 0.0001$, respectively).

Expression profile of shelterin components

As referred to above, we have recently reported imbalances of *TRF1* and *TRF2* gene expression in plasma cell disorders and human myeloma cell lines [13,21], but there are no studies about the expression profile of the remaining shelterin components in MM and MGUS patients. Thus, to get an insight on the global behavior of genes encoding the telomere-binding proteins of the shelterin complex, we have determined the expression levels of *POT1*, *TPP1*, *TIN2* and *RAP1* in both pathologies. Our data showed increased expression of *POT1*, *TPP1*, *TIN2* and *RAP1* in MM with respect to MGUS patients, with significant differences for *POT1* between both entities ($p = 0.002$) (Table 1). In addition, we have confirmed the significant increase in *TRF2* ($p = 0.001$). Besides, we have analyzed the correlation among all shelterin genes. In both pathologies, a positive association between *TRF2-POT1* (MM: $p < 0.0001$ and MGUS: $p = 0.01$) and *TRF2-RAP1* (MM: $p < 0.0001$ and MGUS: $p = 0.0008$) was observed. In MM patients, positive correlations between the expression of *TIN2-TPP1* ($p = 0.0006$), *TIN2-RAP1* ($p = 0.03$) and *TPP1-RAP1* ($p = 0.02$) were also detected. From all these correlations, *TRF2-POT1*, *TIN2-RAP1* and *TPP1-RAP1*, have not been detected previously in the literature, while the remaining ones were described in mammalian cells [20,33,34].

Furthermore, we would like to determine if the expression profile of shelterin genes had clinical significance. Previously, we have shown that the percentage of bone marrow plasma cell infiltration (BMPCI) and lactate dehydrogenase (LDH) levels correlated positively with *TRF2* mRNA expression in MM patients, but no association with clinical outcome was observed [13]. In this study, the correlation of gene expression with clinical characteristics highlighted *POT1* for its significant association with advanced DS stages ($p = 0.02$), high calcium levels ($p = 0.02$), bone lesions ($p = 0.009$) and β2-microglobulin (β2M) ($p = 0.02$) (Fig. 1). In addition, by multivariate analysis, *POT1* expression ($p = 0.04$) was the only significant independent prognostic factor for OS as well as the ISS ($p < 0.02$) (Fig. 2). In MGUS, no correlation among gene expression and clinical parameters was found.

hTERT promoter methylation analysis

In our cohort, *hTERT* was significantly upregulated in MM compared to MGUS patients ($p = 0.03$) (Table 1). As previously reported [13], a great heterogeneity in *hTERT* expression in both MM and MGUS was detected, with cases that over-expressed *hTERT* and others with low mRNA transcript levels. As known, a CpG island is located at the 5′ end of the *hTERT* gene, overlapping with the hTERT promoter, being a potential target for repression through DNA methylation. Thus, in order to evaluate the origin of this heterogeneity, methylation analysis of hTERT promoter was performed in 13 MM cases with high (6) and low (7) expression. The study showed undetectable methylation at the P2 and P1 promoter regions in all patients, and in three cases (2 with high and 1 with low *hTERT* expression) partial methylation (from +76 to +123) or full methylation (from +129 to +144) at the proximal exonic G1 region were found. However, no correlation with *hTERT* mRNA levels was observed, suggesting that other mechanisms than DNA methylation may be involved in the regulation of *hTERT* expression in this pathology (Supplementary Figs. 1Sa and b).

IHC and molecular analysis of Gal-1

Gal-1 was shown as a potent inducer of proliferation of primary myeloma cells *in vitro* [5]. However, the role of Gal-1 in patients with plasma cell disorders is poorly understood. Thus, we were interested in exploring its expression profile at the protein and mRNA levels in MM and MGUS patients, and evaluate if it was related with the expression of telomere-associated genes. By IHC analysis, plasma cells from both MM and MGUS patients expressed Gal-1 at a similar intensity (Supplementary Fig. 2S), whereas plasma cells from normal individuals were negative. Unlike, the analysis by qPCR showed a global tendency towards an upregulation of *LGALS1* transcripts in MM compared to MGUS (0.39 ± 0.08 and 0.18 ± 0.03, respectively). Interestingly, in MM patients we found an association between *hTERT* and *LGALS1* expression. Thereby, patients with high *hTERT* mRNA levels (≥5.08) showed a significant upregulation of *LGALS1* (0.62 ± 0.18) with respect to those with low telomerase expression (0.23 ± 0.10) ($p = 0.005$) (Fig. 3). These results would support our previous findings in which

Table 1
Gene expression profiles in patients with plasma cell disorders.

Telomere-associated genes	MGUS (X ± ES)	MM (X ± ES)	p value
TRF1	0.43 ± 0.07	0.32 ± 0.09	0.29
TRF2	0.04 ± 0.005	0.09 ± 0.01	**0.001**
TIN2	1.80 ± 0.19	2.37 ± 0.18	0.052
POT1	0.02 ± 0.002	0.05 ± 0.008	**0.002**
TPP1	0.49 ± 0.04	0.64 ± 0.04	0.73
RAP1	0.20 ± 0.03	0.28 ± 0.03	0.054
hTERT	1.18 ± 0.27	2.33 ± 0.47	**0.03**

Significant differences between MGUS and MM patients were highlighted using bold text.

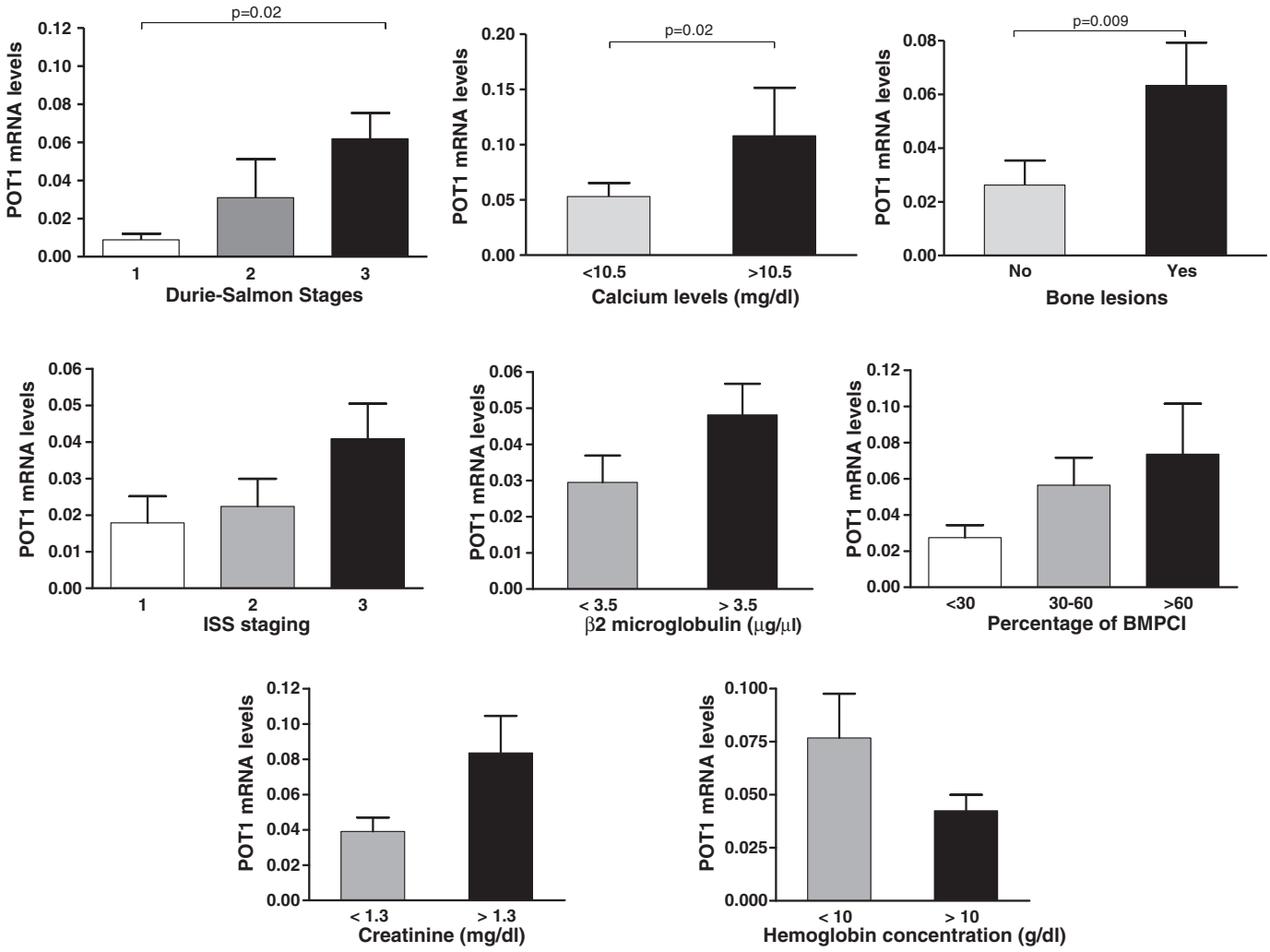

Fig. 1. Associations between *POT1* expression and clinical parameters in MM patients. Significant upregulation of *POT1* in patients with advanced DS stages, high calcium levels and bone lesions. No significant differences were observed according to ISS staging ($p = 0.2$); β2-microglobulin ($p = 0.09$); percentage of BMPCI ($p = 0.12$); creatinine ($p = 0.07$) and hemoglobin ($p = 0.07$). MM: multiple myeloma; DS: Durie and Salmon; ISS: International Staging System; BMPCI: bone marrow plasma cell infiltration.

hTERT expression was found associated with increased proliferation, evidenced by Ki-67 index and the percentage of BMPCI ($p \leq 0.03$) [13]. Conversely, no association between *LGALS1* expression and shelterin genes was observed.

Discussion

Dysfunctional telomeres play a critical role in the development and progression of human cancer [35]. These types of telomeres could arise either from progressive telomere shortening or by disruption of the shelterin complex, resulting in end-to-end fusions and activation of DNA repair pathways. In the present study, we have evaluated for the first time the expression profile of *POT1*, *TPP1*, *TIN2* and *RAP1* genes in MM and MGUS. Overexpression of all genes, with the exception of *TRF1*, with significant differences for *POT1* and *TRF2* in MM compared to MGUS was found. Our findings would indicate that the global modification of shelterin components might affect telomere structure and function in myeloma cells contributing to the progression from MGUS to MM. In agreement with our results, gastric cancer tissues has been shown to express higher levels of *TRF2* and *TIN2* compared to precancerous lesions [36] and a similar pattern was observed in adult T-cell leukemia cells by Bellon et al. [37]. A more recent report [38] provided evidence of telomere deprotection in link with an alteration of the shelterin complex in the early stage of chronic lymphocytic leukemia. In this context, different studies in both myeloid and lymphoid non-neoplastic cells from patients with hematological malignancies and solid tumors showed telomere dysfunction as an early event in tumorigenesis [39,40] contributing to malignant transformation.

Simultaneously, it is important to highlight the clinical impact of *POT1* overexpression in our MM cohort, which was associated with adverse prognostic factors and poor clinical outcome, suggesting *POT1* as another possible molecular marker in plasma cell disorders. POT1 is the only shelterin component with high specificity for the telomeric ssDNA. It interacts presumably indirectly with telomeric dsDNA through binding of TRF2, and directly with telomeric ssDNA in association with TPP1. In line with our findings, Wan et al. [41] showed that *POT1* interference RNA inhibits proliferation and invasion, and induces apoptosis of gastric cancer cells, supporting that increased expression of *POT1* may play an important role in the process of multistage tumorigenesis in this pathology. Conversely, Poncet et al. [27] found down-regulation of telomere-associated genes, including *POT1*, in CLL, and more recently, Ramsay et al. [42] showed numerous telomeric and cytogenetic abnormalities in *POT1*-mutated CLL cells, providing evidences that *POT1* mutations favor the acquisition of the malignant features of CLL cells. Thus, our findings reflect the importance of the tumor expression profile in the development and progression of plasma cell disorders. Nevertheless, further studies are needed to evaluate these results in relation to different cytogenetic risk groups and as part of a clinical trial.

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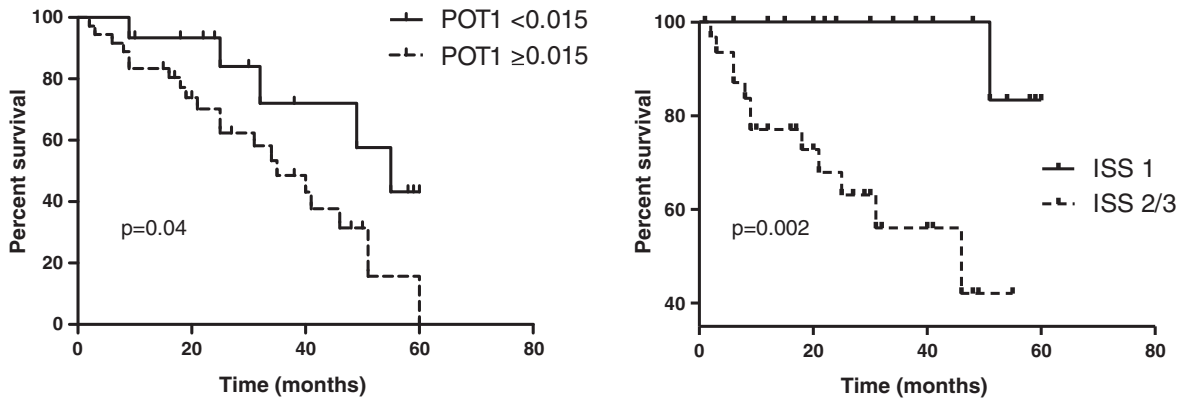

Fig. 2. Kaplan–Meier analysis of overall survival (OS) in multiple myeloma patients stratified by *POT1* expression ($p = 0.04$) and ISS ($p < 0.002$). ROC curve analysis was used to determine the cut-off value for *POT1* expression (0.015). Low *POT1* mRNA: <0.015 (continue line) (mean OS: 55 months); high *POT1* mRNA: >0.015 (dotted line) (mean OS: 35 months). ISS: International Staging System.

The presence of a CpG island in the hTERT promoter suggests that methylation might play an important role in the transcriptional regulation of the *hTERT* gene [43]. Unlike the general association between promoter methylation and gene silencing [44] the hTERT promoter is hypermethylated in most telomerase-positive tumors and hypo-methylated in telomerase-negative normal tissues [45,46]. Thus, hTERT methylation prevents the binding of negatively acting transcription factors such as CTCF inhibitor, whereas a partial hypomethylation of the hTERT promoter region can result in some level of transcriptional activity [47]. Nevertheless, complete hTERT promoter methylation was associated with full transcription repression [14,47]. However, several studies failed to demonstrate a close relationship between promoter CpG island methylation and telomerase activity. In our MM series, only isolated sites of methylation in the proximal exonic region were found and no correlation with *hTERT* mRNA levels was detected. Recently, Auchter et al. [48] studied *hTERT* methylation in CLL patients with no *hTERT* transcript showing complete unmethylation in some cases and disseminated methylation profile in the promoter and proximal exonic region in others. Although the number of cases that we have evaluated is limited, our results suggest that CpG methylation would not be the main mechanism involved in the regulation of telomerase expression in myeloma cells.

Recently, Gal-1 has emerged as a regulator of inflammatory responses, angiogenesis, and tumor progression, contributing to the carcinogenesis process [49]. A wide variety of tumors up-regulate this lectin as a mean of evading T-cell responses [50] and promoting angiogenesis [51]. Moreover, the presence of this lectin was associated with poor prognosis and metastasis, indicating that Gal-1 plays key roles in tumor cell migration [52]. In this study, we found a significant up-regulation of *LGALS1* associated to telomerase expression, supporting a role for this gene in the survival and proliferation of myeloma cells. In agreement with our findings, Abroun et al. [5] found that in myeloma cells Gal-1 binds to β1 integrins resulting in enhanced cell viability, supporting the survival/proliferation of primary MM cells. More studies will be necessary to understand the precise role of this gene in MM cells.

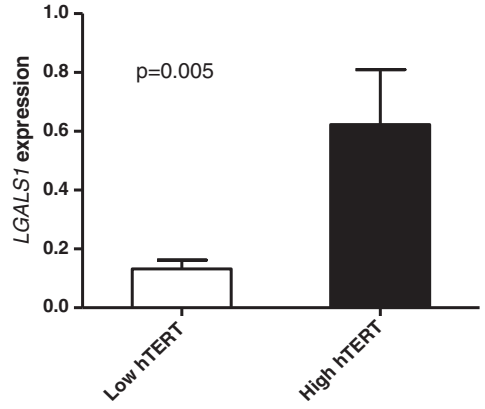

Fig. 3. Association between *LGALS1* and *hTERT* expression in MM patients. Significant *LGALS1* overexpression in patients with high telomerase mRNA levels ($p = 0.005$). MM: multiple myeloma.

In conclusion, this study shows a global modification of the six shelterin genes in plasma cell disorders as well as a strong association of *POT1* expression with different parameters of adverse clinical outcome in MM patients, suggesting *POT1* as another possible molecular target to design new therapeutic strategies. A number of telomerase inhibitors have been evaluated in a variety of cancer types [53,54] and have provided arguments to indicate that the enzyme is a well-validated cancer target [55]. In addition, increasing evidences suggest that directly targeting telomeric DNA has acceptable specificity for cancer cells. Therefore, agents against the shelterin complex may also have anti-tumor activity [56]. In this context, the expression profile of telomere-associated genes might be important to identify patients who may be specifically sensitive to these new approaches.

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2013.10.002.

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