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Expression profile of telomere-associated genes in multiple myeloma

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Abstract

To further contribute to the understanding of multiple myeloma, we have focused our research interests on the mechanisms by which tumour plasma cells have a higher survival rate than normal plasma cells. In this article, we study the expression profile of genes involved in the regulation and protection of telomere length, telomerase activity and apoptosis in samples from patients with monoclonal gammopathy of undetermined significance, smouldering multiple myeloma, multiple myeloma (MM) and plasma cell leukaemia (PCL), as well as several human myeloma cell lines (HMCLs). Using conventional cytogenetic and fluorescence *in situ* hybridization studies, we identified a high number of telomeric associations (TAs). Moreover, telomere length measurements by terminal restriction fragment (TRF) assay showed a shorter mean TRF peak value, with a consistent correlation with the number of TAs. Using gene expression arrays and quantitative PCR we identified the *hTERT* gene together with 16 other genes directly involved in telomere length maintenance: *HSPA9*, *KRAS*, *RB1,* members of the Small nucleolar ribonucleoproteins family, A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins, and 14-3-3 family. The expression levels of these genes were even higher than those in human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), which have unlimited proliferation capacity. In conclusion, the gene signature suggests that MM tumour cells are able to maintain stable short telomere lengths without exceeding the short critical length, allowing cell divisions to continue. We propose that this could be a mechanism contributing to MM tumour cells expansion in the bone marrow (BM).

Keywords: cell survival • chromosomal instability • apoptosis • multiple myeloma • telomere length maintenance • telomeric associations

Introduction

Multiple myeloma (MM) is a clonal malignancy of terminally differentiated plasma cells that typically accumulate in the bone marrow (BM), producing lytic bone lesions and excessive amounts of M-protein. Progression of MM is considered a multistage and dynamic process of cell differentiation, survival, proliferation and dissemination [1–3]. Expansion of the neoplastic myeloma cell clone is the result of imbalances in proliferation and the induction or inhibition of apoptosis. Previous assumptions limited the problem of clinical progression of MM to the issue of cell proliferation, not taking into account the possibility that myeloma cells may have a low rate of apoptosis [4]. Some studies evaluated the contribution of the apoptotic process based on *in vitro* observations or animal models, and under normal conditions plasma cells survived for a variable period of time, but did

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not proliferate. It is thus not clear which of these mechanisms have an immediate and applicable implication for the control of the disease [5].

Telomeres are DNA and protein structures located at the ends of chromosomes, constituted by thousands of DNA tandem repeats of $(TTAGGG)_n$, and associated proteins in vertebrates [6]. Human telomerase is a ribonucleoprotein that consists of a RNA component (telomerase RNA, *hTR*), a reverse transcriptase subunit (telomerase reverse transcriptase, *hTERT*) and associated proteins [7, 8]. *hTR* anneals to the telomeric sequence TTAGGG and acts as a template for telomeric DNA synthesis, whereas *hTER*T catalyzes the addition of telomeric repeats onto the chromosome ends. Telomeres are needed to protect chromosome ends from degradation by capping them, to distinguish chromosome ends from double-stranded breaks [9]. It has also been shown that telomeres' length maintenance also contributes to cell immortalization [10, 11]. In normal human somatic cells, shortened telomeres are often associated with senescence. At some point, telomeres become so short that the genes at the end of the chromosome are in danger of being deleted. When this critical length reduction is reached, a signal is activated inside the cell to prevent further cell divisions and cells may die. Based on these observations, telomeres have been thought to be a mitotic clock that counts the number of times a cell can divide before eliciting the senescence programme [12–15].

The role of the telomeres in the mechanisms of ageing and carcinogenesis has generated a considerable interest. Most immortal cells, including germ line cells [16], some stem cells [17, 18], most cancer cells [19, 20] and the majority of *in vitro* immortalized cells, possess enzymatic activity of telomerase [19, 21], which probably catalyzes *de novo* synthesis of telomeric repeats at chromosome ends, suggesting that telomerase and telomere maintenance may be required for unlimited cell proliferation and tumorigenesis [22–25]. Thus, in MM cancer stem cells, an increase in telomerase levels/activity has been proposed to be the main mechanism of telomere maintenance that enables evasion of apoptosis and conversely, a telomerase activity inhibition may impact both by decreasing telomere length and by modulating self-renewal programmes [26, 27].

Telomerase activity is mainly regulated by *hTERT* transcription, but it can also be regulated positively or negatively by post-transcriptional and/or post-translational modification of the enzyme [28–30], *via* interaction of *hTERT* with accessory proteins that modulate telomerase activity [31, 32]. Thus, telomere length is important for tumour cell growth and survival. Increases in telomere length and the appearance of telomeric associations (TAs), defined as a cytogenetic phenomenon in which the telomeres of a single chromosome or two distinct chromosomes are associated to form dicentric, multicentric and ring chromosomes without visible loss of material from either chromosome end, are considered to be biomarkers in haematological malignancies and solid tumours [33]. When Cottliar *et al.* [34] studied telomere length measured by terminal restriction fragments (TRF) in patients with MM and monoclonal gammopathy of undetermined significance (MGUS) they observed a reduction in telomere length in MM patients, in correlation with other studies in MM [35] and other haematological malignancies [36, 37], as well as a significant increase in the occurrence of chromosome instability, a critical factor in the initiation and progression of human cancers [15, 38]. Telomere length reduction in mantle cell lymphoma is independent of the clinical characteristics, morphology and karyotype of the disease [39], but in MM high telomerase activity and short telomere length defined a subgroup of patients with poor prognosis and shorter mean survival [35].

To further contribute to the understanding of MM and the different molecular pathogenesis, we wanted to determine the expression levels of genes involved in telomerase activity in myeloma samples. We performed gene expression profiling analysis of samples from patients with MGUS, smouldering multiple myeloma (SMM), MM and plasma cell leukaemia (PCL), and human myeloma cell lines (HMCLs). We found a gene signature in that suggests that MM cells are able to maintain stable short telomere lengths without exceeding the short critical length, allowing cell divisions to continue, and thus contributing to MM cells expansion in the BM.

Materials and methods

Culture of human myeloma cell lines

We analysed human myeloma cell lines (HMCLs: JIM-1, KMS-11, KMS-12, KMS-26, KMS-28, MM1R, RPMI8226 and U266). All cell lines were acquired from either ATCC, DSMZ or JHSF, with exception of JIM-1, which was kindly provided by Birmingham University (Birmingham, UK). All HMCLs were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsband, CA, USA) supplemented with 10% FCS (Invitrogen Life Technologies). Cultures were maintained in exponential growth phase at 37°C in a humidified atmosphere with 5% carbon dioxide.

Culture of human embryonic stem cells lines and induced pluripotent stem cell lines

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) were maintained undifferentiated on allogeneic human feeders as described previously [40]. Briefly, two hESCs lines (AND1, H9) and one iPSCs line (AND4) were cultured in plates on a confluent layer of inactivated human foreskin fibroblasts (HFFs) or human mesenchymal stem cells (hMSCs) with mesenchymal stem cell-conditioned medium supplemented with 8 ng/ml basic fibroblast growth factor (Invitrogen, Burlington, Ontario, Canada). Mesenchymal stem cell-conditioned medium was changed daily, and the cells were split weekly by dissociation with 200 U/ml of collagenase IV (Invitrogen).

Patient samples

Bone marrow aspirates were obtained from 42 newly diagnosed patients (20 male patients and 22 female patients) with MGUS, and 46 newly diagnosed patients (14 male patients and 32 female patients) with MM,

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after informed consent from all patients (Table 1). The research was carried out according to the World Medical Association Declaration of Helsinki.

Plasma cells were selected as previously described [41], to a purity of >90% using CD138 microbeads and magnet-assisted cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany).

Conventional cytogenetics, fluorescence *in situ* hybridization and SKY analysis

Conventional cytogenetic, fluorescence *in situ* hybridization (FISH) and spectral karyotyping (SKY) analysis were performed in both HMCLs and patient samples. G-banding technique was done using standard approaches aimed at identifying TAs as previously described [42]. The human telomere sequences were detected using the telomeric probe (Telomere PNA FISH Kits) according to the manufacturer's instructions (DAKO, Glostrup, Denmark). SKY was prepared according to the manufacturer's protocol (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Images were acquired with a HiKSY system SD300-28B Spectra Cube (Applied Spectral Imaging) mounted on Leica DM 5500B microscope using a custom designed optical filter (SKY-1; Chroma technology, Brattleboro, VT, USA). Twenty metaphases were analysed for each sample, and normal BM cells were used as negative control.

Telomeric length evaluation

Telomere length was evaluated by TRF assay in HMCLs and MM patients as previously described [43]. Hybridization signals were evaluated in the autoradiographs by densitometric scanning in each lane with respect to a k/HindIII molecular weight standard, and analysed using the Image Master 1D Prime (Pharmacia-Biotech, Uppsala, Sweden) software. The median 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, respectively, at position i on the autoradiogram. In addition, peripheral blood mononuclear cell (PBMC) from normal control (NC), K-562 cell line as internal positive control of telomere shortening, and cord blood (CB) cells as negative control of telomere shortening were used.

Microarrays hybridization

RNA from HMCLs was prepared for hybridization to the U133 Plus 2.0 expression GeneChip (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions, as previously described [44–47].

Expression data analysis

The raw signal values were imported into GeneSpring GX 11.0. Data from individual microarrays were pre-processed using the MAS5 summarization algorithm and the baseline was done to the median of all samples. To analyse the especial regulation of telomere length in the haematological cancer MM, we determined the expression level of a list of 333 genes involved in regulation, inhibition, protection and maintenance of telomere length and apoptosis. We applied an ANOVA with Tukey's HSD and Benjamin & Hochberg FDR, $P \leq 0.05$ and Fold Change ≤ 2. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus [48] and are accessible through Gene Expression Omnibus Series accession number GSE15695.

Comparison with published expression data

We compared our results in an independent publicly available data set GSE6205 [49] from HMCLs. We extend the analysis to examine primary BM samples from patients with MGUS, SMM, MM and PCL from two additional data sets GSE16122 [50] and GSE6477 [51, 52].

RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

The genes with altered expression patterns were validated using quantitative real-time PCR (qPCR) in HMCLs and CD138^{+} malignant plasma cells from patients with MM. For comparative analysis, PBMC from five NC, CD138^{-} plasma cells from patients with MM, two hESCs and one iPSCs were used. For qPCR experiments, equal amounts of total RNA (3 μg) were reverse transcribed from each sample by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. qPCR amplification was performed with Brilliant III UltraFast SYBR green qPCR Master mix (Agilent Technologies, La Jolla, CA, USA) and detection of PCR products in real time with a M $\times$ 3005 Thermocycler (Agilent Technologies) in a final volume of 20 μl with 10 pmol of each primer (Table S1). The PCR program was initiated by 3 min. at 95°C before 40 thermal cycles, each of 10 sec. at 95°C and 20 sec. at 60°C. Data were analysed according to the $2^{-\Delta\Delta Ct}$ method [53] and were normalized to beta-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product, and were confirmed by a single peak and a single band in agarose gel. A no-template negative control was also included in each experiment. Analysis of all samples was carried out in duplicate, and the mean values were calculated.

Results

Conventional cytogenetics, FISH and SKY analysis

We analysed a minimum of 20 metaphases per patient in 88 patients (46 MM and 42 MGUS) and more than 50 metaphases per HMCL, in 8 HMCLs, by conventional cytogenetic analysis and telomere FISH. As shown in Table 2, the percentage of TAs was 4.97 times higher in patients with MGUS (median percentage of

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Table 1 Clinical data of patients with MM and MGUS

Groups	Gender						Median age (year) (range)	
	Female		Types of myeloma	Male		Types of myeloma	Female	Male
MGUS	22	21: 46,XX	16: MGUS 3: IgA/Kappa MGUS 3: IgG/Lambda MGUS	20	19: 46,XY	14: MGUS 1: IgM MGUS 2: IgA/Kappa MGUS 3: IgA/Lambda MGUS	75.3 (44–87)	64.3 (39–72)
		1: 46,XX/Hyperdiploid			1: 46,XY/45,XY,+12, −13,−20,cp[16]			
MM	32	27: 46,XX	30: MM 1: IgG MM 1: IgG/kappa MM	14	11: 46,XY	12: MM 1: IgG/Kappa MM 1: IgA MM	73.5 (42–85)	62 (34–70)
		1: 46,XX/47,XX,+3,+18, −13,*t*(10;13)			1: 46,XY(15)/46,XY, del1q(5)			
		1: 46,XX,del1q 46,XX.ish del1q,Tel10p,Tel10q, Tel15q,LSI PML(15q22)			1: 46,XY[16]/45,XY, −13[2]/45,XY,−21[6] [cp22]			
		1: 46, XX[17]/46, XX, −1, +i(1q)[3]			1: 52,XY,−1,+der(1), +der(1),+3,+5,6,+r(6), +11,+15,16,+19, +22,del7q[12]/46, XY[9]			
		1: 45~54,XX,−1[4],+2[3], +3[4],−4[4],+5[3], +6[5],+9[4],−9[3], +10[4],−11[5],−13[7], +13[6],+15[12],+16[4], +17[7],+18[8],+19[5],+ 21[7],+22[8],[cp 29].						
		1: 46,XX(6)/39–56,X(28)						

MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance.

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64.71% metaphases with TAs per MGUS patient, range, 50–81.8%); 5.68 times higher in patients with MM (median percentage of 73.86% metaphases with TAs per MM patient, range, 60–89.5%); and 2.47 times higher in HMCLs (median percentage of 32.13% metaphases with TAs per HMCL, range, 26.02–64.45%), when compared with the control group (normal BM cells), with a median percentage of 13% metaphases with TAs per healthy donor (range 12–16%) (Table 2). Different types of TAs were observed in HMCLs and patient samples (Fig. 1), affecting either one or two chromatids. The results obtained by FISH also identified TAs, deletions and translocations of specific telomere regions (Fig. 2). SKY analysis revealed multiple nonreciprocal translocations involving several chromosomes which, in most cases, were not fully characterized by G-banding or FISH (Fig. 3). Our data suggest that chromosomal translocations were common in chromosomes with loss of telomeric signals as a result of telomere shortening.

Table 2 Median percentage of metaphases with telomeric associations (TAs) by conventional cytogenetics and fluorescence *in situ* hybridization (FISH), and terminal restriction fragment (TRF) values

Cell lines, MGUS and MM patients	Median% of Metaphases with TAs	TRF (kb)
NC	13.00	8.12 ± 0.26
CB	–	14.20 ± 2.6
K-562	–	5.02 ± 0.30
JIM-1	26.02	5.30
KMS-11	28.89	5.08
KMS-12	28.37	5.30
KMS-26	64.45	4.99
KMS-28	27.75	5.43
MMIR	26.13	6.07
RPMI8226	29.03	4.78
U266	26.45	5.97
Mean HMCLs	32.13	5.36 ± 0.45
Mean 42 MGUS patients	64.71	6.55 ± 0.89 *R*: 7.14–5.16
Mean 46 MM patients	73.86	6.39 ± 0.67 *R*: 7.02–4.66

NC (normal BM cells and PBMC) as normal control, CB (cord blood) as negative control of telomere shortening, and K-562 (a human erythroleukemia cell line) as internal positive control of telomere shortening. R as telomere length range.

Telomeric length evaluation

Next we studied telomere length measured by TRF on eight HMCLs (Fig. 4). The analysis of telomere length revealed a mean TRF peak value (5.36 ± 0.45 kb) significantly shorter than those observed in NC (8.12 ± 0.26 kb) and CB (14.20 ± 2.60 kb), and in the same range of human erythroleukemia cell line K-562 (5.02 ± 0.30 kb), and MGUS and MM patients (6.55 ± 0.89 and 6.39 ± 0.67 kb) respectively (Table 2).

On the basis of these data, we conclude that there is a strong correlation between shorter telomeres in HMCLs correlates with the percentage of telomeric association and chromosome instability (R^2 = 0.9265) (Fig. 5A). Human myeloma cell lines with higher number of TAs had lower telomere length (Fig. 5C), with the exception of KMS26. This cell line is the only HMCL that shows an up-regulation of *KRAS* similar to CD138$^+$ malignant plasma cells from patients with MM (Figure S9). Interestingly, all the patients with MGUS, SMM, MM and PCL, also show up-regulation of *KRAS* (Fig. 6). These data also are in accord when comparing the telomere lengths with the percentage of telomeric association in mean of MGUS patients, mean of MM patients and NC, showing a R^2 = 0.9969 (Fig. 5B and D).

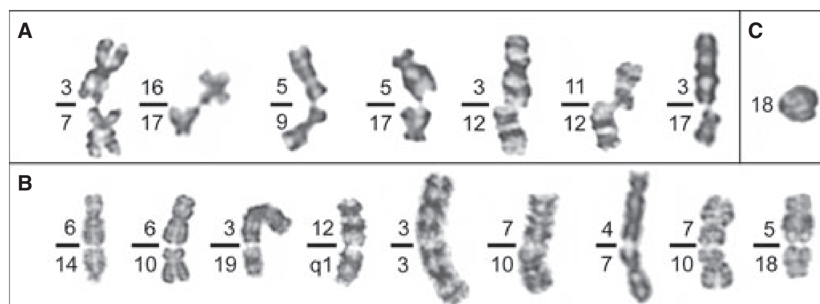

Fig. 1 Different types of telomeric associations (TAs) were found in human myeloma cell lines, and monoclonal gammopathy of undetermined significance and multiple myeloma patients. We have divided these TAs into three types: (**A**) TAs between single chromatids of nonhomologous chromosomes (**B**) TAs between double chromatids of nonhomologous chromosomes (**C**) TAs between the arms of the same chromatid on a single chromosome.

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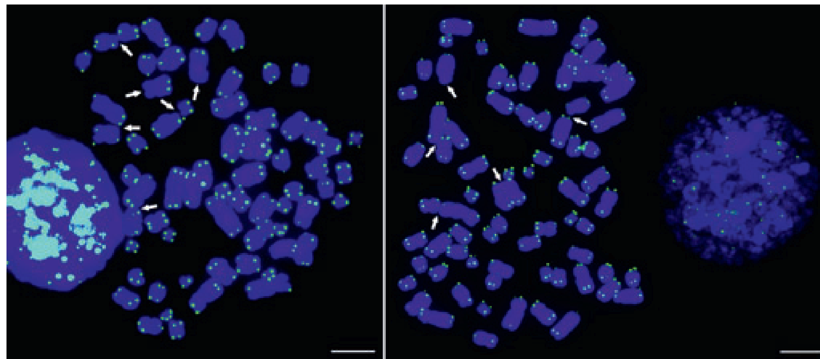

Fig. 2 Fluorescence *in situ* hybridization (FISH) analysis of two metaphase spread from the human myeloma cell lines KMS26, using a PNA FISH probe specific for the telomeric repeats. The arrows indicate multiple chromosome ends showing absence or reduction of FISH signals, including those involved in telomeric associations. There is an accumulation of telomeric associations, involving one or two chromatides, probably related to a reduction in telomeric signal. Scale bars 5 μm.

Fig. 3 Spectral karyotyping (SKY) analysis from the human myeloma cell line KMS26, a hyperdiploid myeloma cell line that reveals multiple non-reciprocal translocations involving several chromosomes.

Gene expression signature of MM cells

To get an insight on the molecular mechanisms underlying these changes in telomere length and the increase in telomeric associations, we performed microarray analysis using HMCLs. To analyse the especial regulation of telomere length in MM, we determined the expression level of a list of 333 genes involved in regulation, inhibition, protection and maintenance of telomere length and apoptosis, and found 203 genes that were differentially expressed in the HMCLs. Ninety of these genes were overexpressed and 113 were underexpressed. We confirmed our results on a large independent data set (GSE6205) consisting of 23 HMCLs [49]. The analysis of this list of 333 genes in patients with MGUS, SMM, MM and PCL showed 63 deregulated genes with 49 genes in common with HMCLs, including 16 genes directly involved in telomere maintenance (Table 3). These sixteen genes are *HSPA9*, *KRAS*, *RB1*, members of the small nucleolar ribonucleoproteins family (*FBL*, *NHP2*, *DKC1*, *NOP10*, *NOP56*, *IMP3* and *GAR1*), A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (*HNRNPA1*, and *HNRNPK*) and the 14-3-3 family (*YWHAQ*, *YWHAE*, and *YWHAZ*). Most importantly, we can see an obvious progressive increase in the expression levels of these genes from MGUS to PCL, with overexpression most notably predominating (Fig. 6).

Gene expression data analysis

To confirm the results from the microarray analysis we analysed gene expression by quantitative PCR. We used reverse transcribed RNA extracted from five controls of PBMC from healthy donors, eight HMCLs, six samples of $CD138^+$ malignant plasma cells and $CD138^-$ plasma cells from patients with MM, two hESCs and one iPSCs, and measured expression levels for all sixteen deregulated genes plus four additional genes involved in telomere maintenance (*TRF1*, *TRF2*, *TNKS* and *hTERT*) [43]. Log_2 of the normalized microarrays ratio values were compared with log_2 ratio values from qPCR experiments. The qPCR results confirmed that the relative RNA expression levels of the 16 deregulated genes were consistent with the microarray data and with public data sets, confirming the increased expression of these genes in the HMCLs and patients with respect to the hESCs, iPSCs and controls (Fig. 7).

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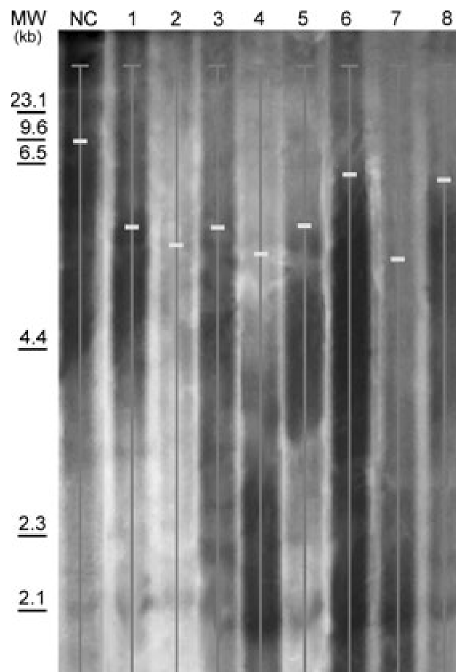

Fig. 4 Example of Southern blot analysis used for terminal restriction fragment quantification, where hybridization signals were evaluated in the autoradiographs by densitometric scanning. The median telomeric length ratio of a sample was calculated considering the peaks observed in 1-JIM1, 2-KMS11, 3-KMS12, 4-KMS26, 5-KMS28, 6-MM1R, 7-RPMI8226, 8-U266 and NC (normal controls of peripheral blood mononuclear cell from health donors). MW: molecular weight.

Discussion

Telomeres are maintained by telomerase, a specialized ribonucleoprotein complex that includes an RNA template (*hTR*) and a reverse transcriptase catalytic subunit (*hTERT*). Telomerase activity is low or absent in most human somatic tissues, although it is acting in tumoural, germ and stem cells. Telomeric DNA is progressively lost with each cell division, and when telomeres become critically short, the cells undergo senescence or apoptosis [54]. This process is irreversible in most human somatic cells, and they normally die by apoptosis. However, MM tumour cells maintain stable short telomere lengths without reaching the short critical length. This suggests that telomere length maintenance is necessary for continued cell division and immortalization of normal and malignant cells [55].

In the present work, we found a distribution of chromosome ends involved in TAs that was obviously non-random. We have demonstrated an increase in the percentage of TAs and chromosomal instability in MGUS and MM patients, and HMCLs respect to NC (normal BM cells) (Figs 1, 2 and 3). Both this instability and the increase in TAs numbers are obviously correlated with telomere length (Fig. 5), with an inverse correlation between telomeric association and telomeric length. Therefore, it is possible to establish an association between telomere length and the probability of TAs to occur as a cause of chromosomal instability, to ensure the stability of chromosome ends, suggesting some selective advantage. Moreover, an increase in telomere stability facilitated by the increased expression of telomere maintenance proteins may constitute a possible strategy to avoid senescence or apoptosis.

Theoretically, FISH should be able to provide information on the telomere length of individual chromosomes. The telomeric FISH signals in MGUS and MM patients and HMCLs indicated a dramatic reduction of telomeric signals in some chromosomes, as compared to NC, probably because of a reduction in the number of $(TTAGGG)_n$ repeats. The lost of telomeric signal can affect either isolated chromosomes or those implicated in TAs (Fig. 2). The analysis of telomere length revealed significantly shorter TRF values in the HMCLs, but in the same range of other tumoural cell lines, MGUS and MM patients (Fig. 4), with values strongly correlated with the percentage of telomeric association and chromosome instability (Fig. 5). This stability in telomere length maintenance may be a critical component that enables evasion of apoptosis, being necessary for telomere protection without reducing their size [56].

Telomerase and telomere maintenance is required for unlimited cell proliferation and tumorigenesis, and in tumoural cells an increase in telomerase levels/activity has been proposed to be the main mechanisms that enable evasion of apoptosis. We decided to establish the expression levels of genes involved in telomere length maintenance process in MM through microarray analysis, which were subsequently validated by qPCR (Fig. 7). In this study, we have found other genes importantly involved in telomere maintenance in MM. We have identified 16 deregulated genes implicated in mechanisms for the post-transcriptional and/or post-translational modification of telomerase, which modulates telomerase activity and telomere maintenance: *DKC1, FBL*, *NHP2*, *NOP10*, *NOP56*, *IMP3*, *GAR1, HNRNPC, HNRNPA1, HNRNPK, YWHAQ, YWHAE, YWHAZ, HSPA9, KRAS* and *RB* (Fig. 8). Our analysis showing alteration in the expression of these 16 human telomere maintenance genes in MM indicates that other genes in addition to *hTERT*, *TRF1*, *TRF2* and *TNKS* are involved in these mechanisms. The analysis also showed a significant difference in the expression levels from MGUS to PCL (Fig. 6), with overexpression most notably predominating and correlating with disease progression. These proteins seem to be mainly responsible for tumour cells survival and proliferation, both important factors in the progression of MM.

FBL, *NHP2*, *DKC1*, *NOP10*, *NOP56*, *IMP3* and *GAR1* are members of the H/ACA snoRNPs gene family. Mutations in the components of the H/ACA snoRNP result in a reduction in physiological *hTR* levels, which are strongly correlated with the poor telomere maintenance and the pseudouridylation of rRNAs and snRNAs [57]. *DKC1* gene (Dyskeratosis congenita 1, dyskerin) is required for ribosome biogenesis and telomere maintenance through the correct processing and intranuclear trafficking of *hTR* [58–60]. It has been reported in the literature that this gene is overexpressed in prostate cancers and is also necessary for extensive tumour growth [61]. In MM, their expression in patients and HMCLs is higher than in NC, hESCs and iPSCs, which have unlimited proliferation capacity, which could ensure the correct processing and intranuclear trafficking of *hTR*, and therefore telomere maintenance, explaining why tumour plasma cells have a higher survival rate than normal plasma cells.

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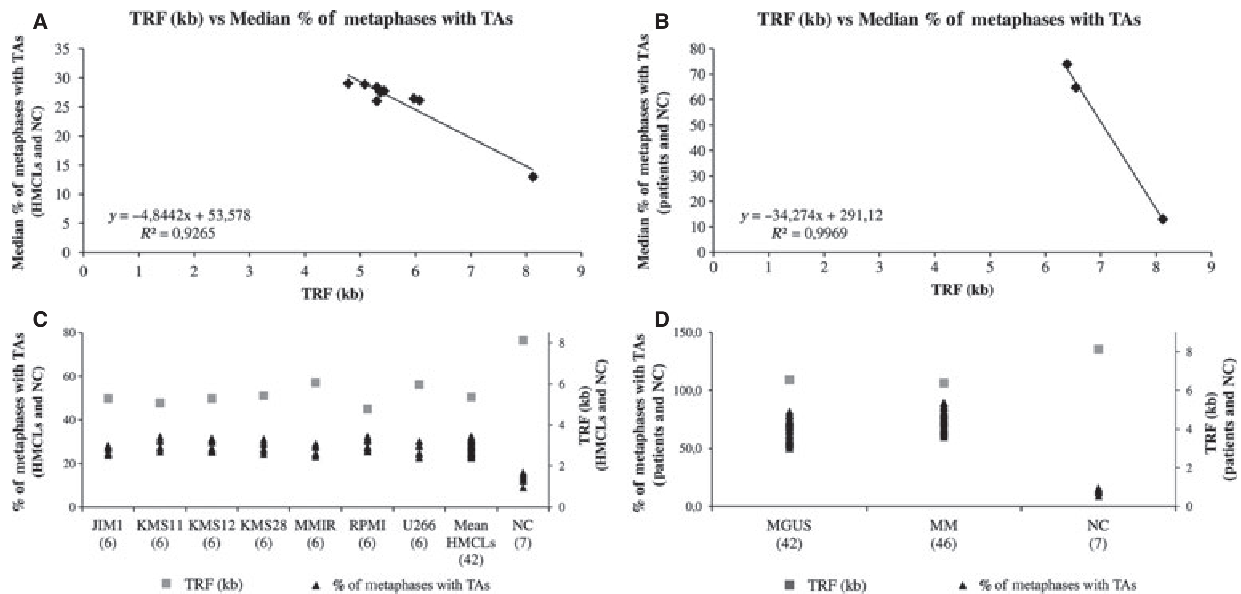

Fig. 5 Correlation between the median percentage of metaphases with telomeric associations (TAs) *versus* the terminal restriction fragment (TRF) values. (**A** and **C**) in seven human myeloma cell lines (HMCLs) (JIM1, KMS11, KMS12, KMS28, MM1R, RPMI8226, U266) (six cultures of each cell type) and seven normal control (NC) (healthy donors). (**B** and **D**) in 42 monoclonal gammopathy of undetermined significance (MGUS) patients, 46 multiple myeloma (MM) patients and NC (healthy donors). There is a strong correlation between shorter telomeres correlates with the percentage of telomeric association, showing a R^2 of 0.9265 (A) and 0.9969 (B) respectively. More number of TAs had lower telomere length.

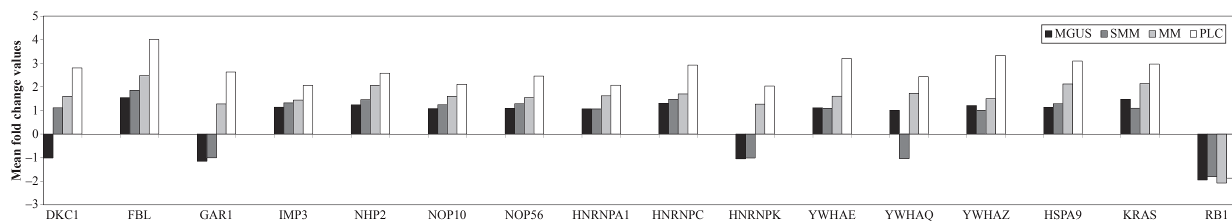

Fig. 6 Microarray expression levels of 16 selected genes, in monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM), multiple myeloma (MM) and plasma cell leukaemia (PCL). Test applied: ANOVA with Tukey's HSD and Benjamin & Hochberg FDR, $P \leq 0.05$.

HNRNPA1, HNRNPC and *HNRNPK* belong to the A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). This family of proteins are RNA- and DNA-binding proteins localized primarily in the nucleus where they bind telomerase RNA and telomeric DNA, and may thus be capable of recruiting telomerase to form part of the protective complex on telomere ends, which participates in telomere maintenance [62]. The hnRNPs family appears to be important for telomere biology because hnRNP A1 and hnRNP C1/C2 proteins are capable of interacting with the telomere and the telomerase, and may regulate telomere length [63–66]. Moreover, hnRNPs A1 can associate with the single-stranded telomeric repeat sequence *in vivo* [67–73], whereas short telomeres in hnRNP A1 deficient mouse CB3 cells are elongated after reconstituting hnRNP A1 expression [66]. In MM we have found that the hnRNPs gene family has a similar expression in patients and HMCLs with the same ratio than in hESCs and iPSCs, forming the protective complex of telomerase RNA and telomeric DNA at the telomere ends, regulating telomere length and increasing cell survival.

YWHAQ, *YWHAE* and *YWHAZ* genes (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta, epsilon and zeta respectively) belong to the 14-3-3 family that is involved in metabolism, protein trafficking, signal transduction, evasion of apoptosis, cell cycle regulation, cell death and mitogenesis [74, 75]. Members of the 14-3-3 protein family antagonize the activity of proteins that promote cell death and senescence, such as Bad, Bim, Bax and SFN [76]. This family of proteins are *hTERT*-binding partners and its

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Table 3 The 16 selected genes involved in telomere maintenance and telomerase activity

Gene Symbol	Gene Title
DKC1	Dyskeratosis congenita 1, dyskerin
FBL	Fibrillarin
GAR1	*GAR1* ribonucleoprotein homolog (yeast)
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
HNRNPK	Heterogeneous nuclear ribonucleoprotein K
HSPA9	Heat shock 70kDa protein 9 (mortalin)
IMP3	*IMP3*, U3 small nucleolar ribonucleoprotein, homolog (yeast)
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
NHP2	*NHP2* ribonucleoprotein homolog (yeast)
NOP10	*NOP10* ribonucleoprotein homolog (yeast)
NOP56	*NOP56* ribonucleoprotein homolog (yeast)
RB1	Retinoblastoma 1
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

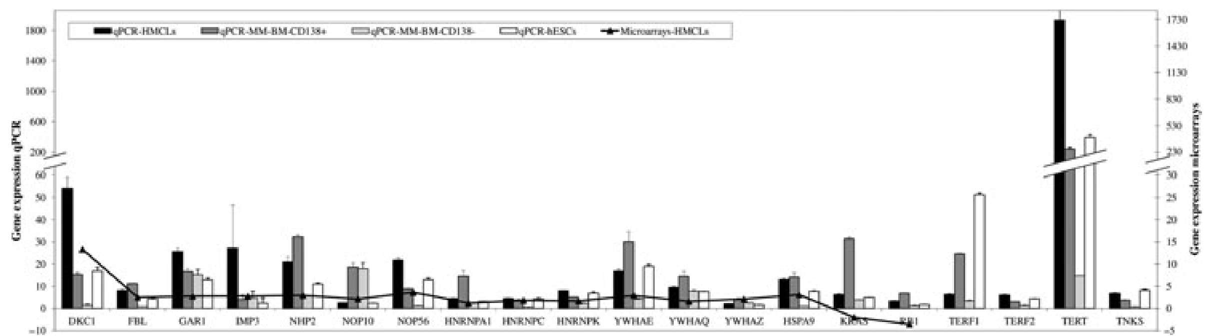

Fig. 7 Expression levels in the eight human myeloma cell lines, six samples of CD138$^+$ malignant plasma cells and CD138$^-$ plasma cells from patients with multiple myeloma, two human embryonic stem cells (hESCs) and one induced pluripotent stem cells (iPSCs) using qPCR, confirming the gene expression levels by microarray. Data were analysed according to the $2^{-\Delta\Delta Ct}$ method and normalized to beta-actin expression. Y values are fold change, and controls of peripheral blood mononuclear cell (PBMC) from healthy donors are fold change = 1.

interaction is required for efficient accumulation of *hTERT* in the nucleus. In MM tumour cells, the expression of these genes in patients is higher than in HMCLs, hESCs and iPSCs, and especially that in NC. These observations suggest that the 14-3-3 family is a post-translational modifier of telomerase that functions by controlling the intracellular localization and the antiapoptotic action of *hTERT*, suppressing the DNA damage-related signal, and enabling evasion of apoptosis.

Regarding the other differentially expressed genes, *HSPA9* (heat shock 70 kD protein 9, mortalin) is a gene that plays a role in the control of cell proliferation and may act as a chaperone. Mortalin is functioning in intracellular trafficking [77, 78], stress response [79–81], inactivation of p53 [82] and growth arrest in human immortalized cells that have compromised p53 and pRB functions [83]. Also, along with *hTERT*, in MM permitted bypass of senescence, a substantial extension of lifespan, and possibly immortalization.

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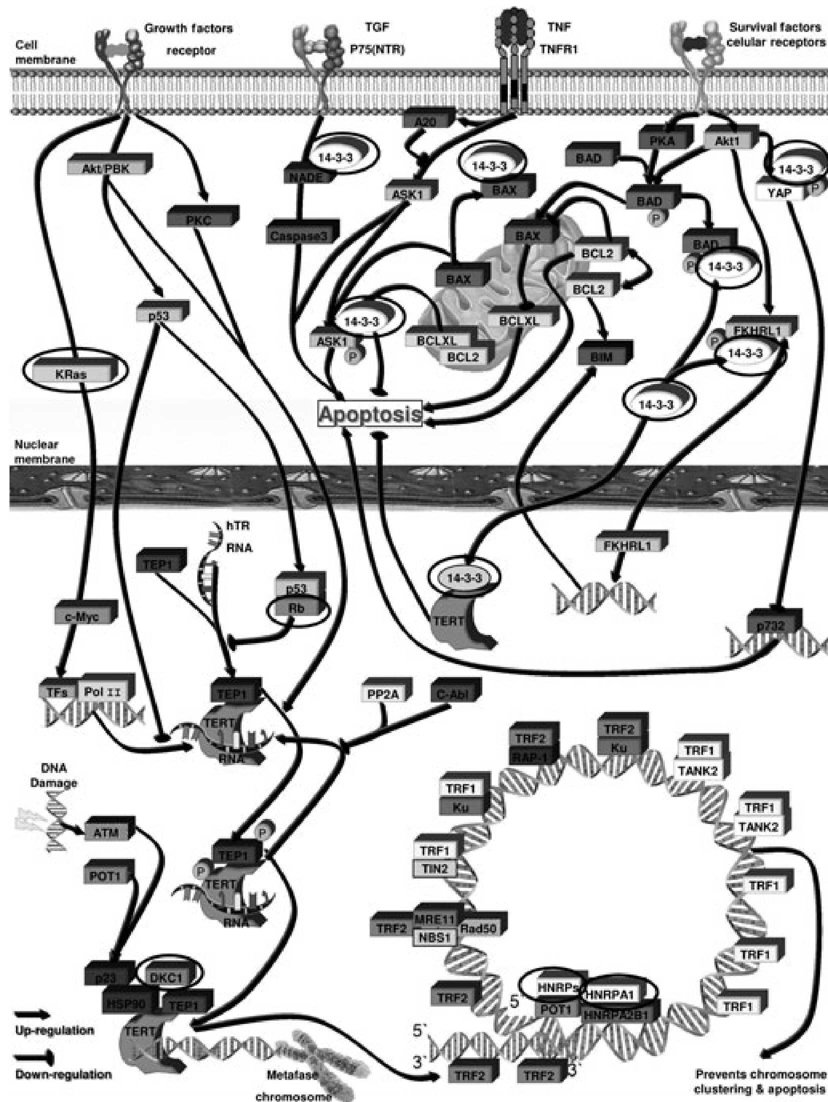

Fig. 8 Molecular pathways involved in telomere maintenance, where we can see the 16 deregulated genes implicated directly in telomere length maintenance activity in clonal plasma cells identified in this study highlighted with an oval shape. See Table 2 for the list of genes.

KRAS is an oncogene involved in various malignancies, including MM [84]. It is implicated in telomere maintenance by the stimulation of telomere-telomere fusions (T-TFs) [85], demonstrating a high degree of genomic instability [86]. Its high expression in KMS12 and patients could be the cause of the high increase in frequency of TAs. Retinoblastoma 1, *RB1* has an established role in the implementation of cellular senescence [87]. It has been demonstrated that the disruption of members of the Rb1 pathway results in rapid telomere elongation, which, in turn, may help to sustain immortal cells. The abnormally elongated telomeres retain their end-capping function, as shown by the normal frequency of chromosomal fusions. This deregulation of telomere length is not associated with increased telomerase activity [88]. These observations suggest that plasma tumour cells have other mechanisms of telomere maintenance more developed than hESCs and iPSCs, and not just the action of *hTERT*.

In conclusion, telomere length stabilization is required for cellular immortality in most cells, and in MM tumour plasma cells have a higher survival than normal plasma cells, which may be a possible mechanism for their accumulation in the BM. Our data suggest that in MM disease, tumour cells acquire telomere maintenance capability through the alteration of three gene families with well-defined functions in telomere maintenance, together with other genes directly involved in telomere length such as *hTERT* and the RNA component (*hTR*). These genes are involved in the process of regulation, inhibition, protection, telomere length maintenance, telomerase activity, cell signalling and regulation of apoptosis. These genes with altered expression levels could be used

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as targets for cancer therapy in MM. However, future studies to determine the protein expression patterns together with the use of siRNA that identify the function and the importance of these genes will be performed.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Real-time qPCR gene expression data in sixteen deregulated genes in multiple myeloma (MM), implicated in mechanisms for the post-transcriptional and/or post-translational modification of telomerase (*DKC1, FBL*, *NHP2*, *NOP10*, *NOP56*, *IMP3*, *GAR1, HNRNPC, HNRNPA1, HNRNPK, YWHAQ, YWHAE, YWHAZ, HSPA9, KRAS*, and *RB*) in addition to *hTERT*, *TRF1*, *TRF2* and *TNKS*, in eight human myeloma cell lines (JIM-1, KMS-11, KMS-12, KMS-26, KMS-28, MM1R, RPMI8226 and U266), $CD138^{+}$ malignant plasma cells and $CD138^{-}$ plasma cells from six patients with MM, 2 hESCs lines (AND1, H9), and 1 iPSCs line (AND4). PBMC from five healthy donors were used as normal control NC.

Table S1 Primers used for qPCR analysis.

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