Thymosin β4 has tumor suppressive effects and its decreased expression results in poor prognosis and decreased survival in multiple myeloma

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ABSTRACT

Thymosin β4 (Tβ4) is a polypeptide involved in cellular proliferation, differentiation, and migration, over-expressed in several tumor entities. We evaluated its expression and function in 298 newly diagnosed multiple myeloma patients and the murine 5TMM model. Mean *Tβ4* expression was significantly lower in myeloma cells compared to normal plasma cells ($P<0.001$). The same observation can be made in the 5TMM-mouse model by qRT-PCR and ELISA. Here, *Tβ4* overexpression by lentiviral transduction of 5T33MMvt-cells led to significantly decreased proliferative and migratory capacities and increased sensitivity to apoptosis-induction. Mice injected with *Tβ4* over-expressing myeloma cells showed a longer survival compared to mice injected with controls (88,9 *vs.* 65,9 days, $P<0.05$). In 209 MM patients treated with high-dose therapy and autologous stem cell transplantation, expression of *Tβ4* below the median was associated with a significantly shorter event free survival (37.6 *vs.* 26.2 months, $P<0.05$). In conclusion, our results indicate a possible tumor suppressive function of Tβ4.

Key words: thymosin β4, cellular proliferation, multiple myeloma.

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Introduction

β-thymosins are a family of small peptides that were originally proposed to be thymic hormones.[1] They were identified as actin monomer binding proteins, controlling the availability of actin for polymerization. They may, therefore, have a crucial role in regulating cellular functions involving actin polymerization/depolymerization cycles. Currently, 15 *β*-thymosins have been identified and characterized as highly conservative 5-kDa peptides containing 40 to 44 amino acid residues. In most mammalian tissues, thymosin-*β* 4 (T*β*4), the most abundant thymosin peptide, T*β*10 and T*β*15, have been studied as important members of the *β*-thymosin family.[2] Several studies reported that these genes are over-expressed in solid tumors, which could be correlated to the angiogenic and metastatic potential of the studied tumors.[3]

Multiple myeloma (MM) is a hematologic malignancy characterized by the accumulation of monoclonal plasma cells (PC) in the bone marrow (BM). MM cell biology can be dissected into the interactions of MM cells with their surrounding stroma (matrix proteins, cytokines and BM cells) and in the acquisition of essential changes in cell behavior, such as self-sufficiency in growth signals, evasion of apoptosis and acquisition of invasive and spreading capacities.[4] Earlier reports indicated that T*β*4 was down-regulated in RNA from primary human MM cells and cell lines.[5]

This observation is in contrast to the results obtained in most solid tumors where an upregulation is seen in malignant cells compared to their normal counterparts. Cha *et al.* showed that overexpression of T*β*4 resulted in an increased

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metastatic capacity of lung cancer cells and increased angiogenic response.[6]

Since migration, invasion and associated angiogenesis are key features in MM biology, we were interested in studying Tβ4 expression in a large panel of MM patients and its functionality in the murine 5TMM model.

Design and Methods

Gene expression analysis on human myeloma cells

Tβ4 expression was analyzed in purified PCs from BM samples obtained from 14 healthy donors, 11 patients with monoclonal gammopathy of unknown significance (MGUS) and 298 previously untreated multiple MM patients at the University Hospitals of Heidelberg or Montpellier.[7] Of these, 209 MM patients were treated by high-dose therapy and autologous stem cell transplantation (ASCT). Biotinylated complementary RNA (cRNA) was amplified according to the Affymetrix labeling protocol (Affymetrix, Santa Clara, CA, USA). cRNA from a first group of patients (7 normal donors, 7 MGUS and 65 MM patients) was hybridized to the human U133 A and B. This group will be referred to as the HM1-group. A second independent validation group of patients (7 normal donors, 16 MGUS and 233 MM patients) was named the HM2 group. For these patients, the U133 2.0 GeneChip was used. These micro-array data had been previously used for several analyses, but thymosin β4 expression had never been analyzed before.[8,9] HM2 data were corrected for batch effect due to the usage of different labeling kits according to Johnson *et al.*[10] Expression data were gcrma-normalized and analyzed by the bioconductor packages for R. For patients' characteristic see *Online Supplementary Table S1*.

The 5T2MM and 5T33MM murine models of myeloma

The 5TMM models originated in elderly C57Bl/KaLwRij mice.[11] The 5T33MM*vivo* (5T33MMvv) cells grow *in vitro* stroma-dependently with a limited survival while the 5T33MM*vitro* (5T33MMvt) cell line is a clonally identical variant that originated from an *in vitro* culture of 5T33MMvv cells, growing BM stroma-independently in RPMI-1640 supplemented with 10% bovine serum 1% natriumpyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (all from Biowhittaker, Verviers, Belgium).[12]

Quantification of intracellular protein levels of Tβ4 and F-Actin G-Actin

Enzyme-Linked Immunosorbent Assays (ELISA) for measuring Tβ4 concentrations were performed according to the manufacturer's instructions (Immundiagnostik, Bensheim, Germany). Cells (10^7) were lyzed in a phosphate buffer containing 0.14 M NaCl, 2.6 M KCl, 8 mM Na2HPO4, 1.4 M KH2PO4 and 1% Triton X100 and sonicated with an ultrasound finger. Protein levels and ratios between F-Actin and G-Actin were determined using the G-actin/F-actin *in vivo* assay kit (Cytoskeleton Inc, Denver, USA).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the ABI Prism 7700 Sequence Detection System. For the detection of both human and mouse Tβ4 mRNA and the endogenous reference gene *GUS*, Assays on Demand (Applied Biosystems) were used. To verify the results obtained with the microarrays studies, *Tβ4* expression was measured in 3 cell lines and in 3 patient samples and their correlations statistically verified using a Spearman correlation test.

Generation of 5T33MMvt cells over-expressing Tβ4

A lentiviral transferplasmid encoding mouse *Tβ4* (m *Tβ4*) was constructed. The *mTβ4* gene was obtained from HJ Cha (NIDCR, NIH, Bethesda, USA)[6] and inserted into the transferplasmid pHR'tripCMV-IRES-tNGFR-SIN.[13] *mTβ4*-encoding lentiviral vector particles were produced in 293T cells, collected, ultracentrifugated and their viral titer determined.[14] After transduction, 5T33MMvt cells were surface stained using an in-house biotinylated anti-tNGFR antibody and purified by FACS sorting into a 6-well plate (Becton Dickinson, FACSVantage). Next, they were analyzed for *Tβ4* expression by RT-PCR. The 5T33MMvt cells over-expressing *Tβ4* will be referred to as 5T33MMvt$^{T\beta4+}$.

In vitro *and* in vivo *effects of* Tβ4 *overexpression*

In vitro proliferation was assessed by measuring DNA synthesis using a ^{3}H-thymidine incorporation assay, as described earlier.[15] Apoptosis sensitivity of the MM cells was analyzed by staining with FITC labeled-annexin V and propidium iodide according to the manufacturers' instructions (BD Biosciences, Erembodegem, Belgium). *In vitro* migration studies were performed using Transwell chambers and 10% fetal calf serum as chemoattractant and were quantified through flow cytometry. To determine the effect of *Tβ4* overexpression on survival, groups of 10 C57BLKaLwRij mice were intravenously injected with either 5×10^5 5T33MMvt$^{T\beta4+}$ or wild-type 5T33MMvt cells. Animals were sacrificed when they showed signs of morbidity, namely hind limb paralysis. Kaplan-Meier analysis was used to determine a difference in the survival.

Results and Discussion

Different studies indicated a pivotal role of Tβ4 in the metastatic process of solid tumors.[16,17] An adenoviral-based overexpression of *Tβ4* was applied in a colon cancer and melanoma model showing increased growth, motility and invasive capacities *in vitro* and a larger tumor load *in vivo*.[18,19] Since proliferation, migration and invasion are part of the hallmarks of the biology of MM, we were interested in investigating an involvement of *Tβ4* in this disease. We first investigated the *Tβ4* expression pattern in 298 primary MM-cell samples and 14 normal plasma cell samples from healthy donors. *Tβ4* expression is significantly lower in MM cells of the HM1 group ($P<0.05$) and HM2 group ($P<0.001$) compared to normal plasma cells. This holds true for a significantly lower Tβ4 expression in its pre-malignant stage (MGUS), its early (Durie Salmon stage I) or late stage (Durie Salmon II and III) in both HM1 and HM2 groups ($P<0.001$) (Figure 1A). No relevant correlation could be found between *Tβ4* expression and percentage of plasma cell infiltration in the bone marrow smear. Gene expression assessed by DNA-microarray correlates well with qRT-PCR performed on MM patient samples (coefficient of correlation r=0.993, $P<0.001$). These data are in agreement with results from Gondo *et al.* showing a decrease in *Tβ4* expression in a small number of MM samples by Northern blot analysis.[5]

Given the differential *Tβ4* expression in MM patients, we subsequently investigated a possible prognostic value and influence on event free (EFS) and overall survival (OS) in our patient population. As *Tβ4* was expressed in all MM cells, we examined the survival of 209 patients by comparing patients with *Tβ4* expression above (*Tβ4*^{high}) and below

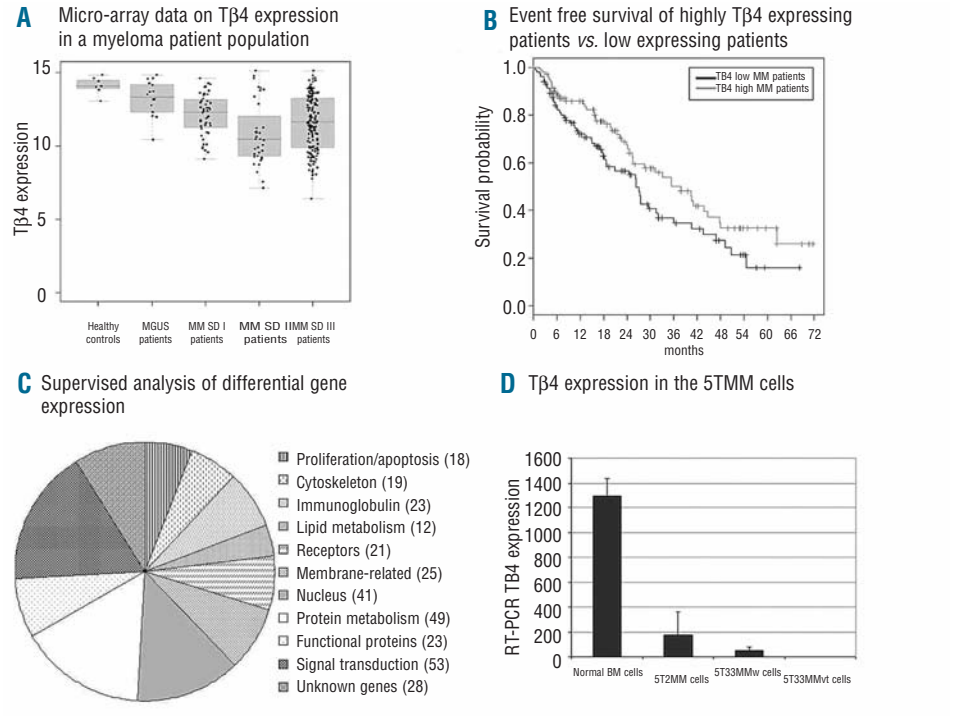

Figure 1. (**A**) **The micro-array data obtained for the *Tβ4* expression in CD138^{+} sorted BM plasma cells from healthy donors and MM patients. These results were validated by quantitative RT-PCR. MGUS: monoclonal gammopathy of undetermined significance, MM multiple MM, SD: Salmon and Durie Stage.** (**B**) **The event free survival of *Tβ4*^{high} and *Tβ4*^{low} patients. Patients with *Tβ4*^{low} MM had statistically significantly decreased event free survivals compared to patients with *Tβ4*^{high} MM (*P*<0.001), while also their overall survival tended to be shorter.** (**C**) **The differently expressed genes between *Tβ4*^{high} and *Tβ4*^{low} patient groups. After identification of the gene, these were grouped into similar biological function. A complete list of the genes can be found in *Online Supplementary Table* S2.** (**D**) **A similar gene expression pattern was observed in the murine 5TMM models where *Tβ4* mRNA expression in 5T33MM and 5T2MM invaded BM was lowered compared to normal BM cells.**

(*Tβ4*^{low}) the median (Figure 1B). Patients with *Tβ4*^{high} compared with *Tβ4*^{low} show a significantly longer median EFS (n=209, 37.6 months *vs.* 26,2, *P*<0.05), but only a trend regarding OS (*P*=0.1). Concerning EFS, multivariate analyses on *Tβ4* expression with either ISS or β2-microglobulin indicated an independent (*P*=0.04) prognostic value of *Tβ4* expression regarding ISS (*P*<0.001), but not (*P*=0.07) regarding β2-microglobulin (*P*=0.003) levels. In multi-variate analyses for OS, ISS and β2-microglobulin appear as significant (*P*=<0.001) variables, whereas *Tβ4* expression fails to reach independence (*P*=0.09 and *P*=0.01, respectively).

A supervized analysis of expression data comparing the *Tβ4*^{high} to the *Tβ4*^{low} group identified over 300 significantly differentially expressed genes. These genes are listed in *Online Supplementary Table S2*. Analysis of their biological function allowed them to be divided into main functional categories and this distribution is illustrated in Figure 1C. Signal transduction, protein metabolism and nuclear functions were the largest categories, but 19 genes were implicated in cytoskeletal organization, and 32 genes in lymphoid differentiation and immunoglobulin processing. In general, these gene clusters indicate a biological difference between MM cells of the two patient groups.

The data obtained in MM patients were also seen in the 5T33MM murine MM model by qRT-PCR demonstrating a decreased mRNA expression in 5TMM cells compared to normal BM cells (*P*<0.001, Figure 1D). Competitive ELISA confirmed these results on protein level (*results not shown*). To study functional effects of T*β*4, the gene was over-expressed using a lentiviral expression vector. The 5T33MMvt cell line was stably transduced and after subcloning, a 99% pure clone with strong t-NGFR expression was obtained. qRT-PCR confirmed the overexpression of *Tβ4* compared to control cells (Figure 2A).

To assess the functional involvement of differential *Tβ4* expression we used the 5T33MMvt and 5T33MMvt$^{Tβ4+}$ cell lines. In a ^{3}H thymidine assay, 5T33MMvt$^{Tβ4+}$ cells showed a significant decrease in DNA synthesis compared to control cells (*P*<0.05). 5T33MMvt$^{Tβ4+}$ cells showed a significantly increased sensitivity to vinca-alkaloids (vinblastin) and bortezomib (Figure 2B; *P*<0.001 for both bortezomib and vinblastin).

Likewise, bortezomib induced apoptosis was higher in 5T33MMvt$^{Tβ4+}$ compared with 5T33MMvt cells (*P*<0.05; Figure 2C). In addition to affect survival pathways, *Tβ4* overexpression reduced migratory capacities of 5T3MM cells; the percentages of cells that migrated in basal conditions and in 10%FCI was significantly lower in 5T33MMvt$^{Tβ4+}$ compared to control cells (*P*<0.05; Figure 2D). The relative increase after stimulation (compared to basal conditions) was, however, similar in both populations. We further examined the effects of *Tβ4* expression on tumor development and survival of diseased mice by injecting mice intravenously with 5T33MMvt$^{Tβ4+}$ or control cells. In this study, the mean survival of mice injected with control cells was significantly shorter 65.9 days (SD 6.6 days), compared to 88.9 days (SD 9.3 days) for mice injected with 5T33MMvt$^{Tβ4+}$ cells (*P*<0.05; Figure 2F). These *in vivo* results confirm data obtained using the *in vitro* proliferation and apoptosis assays.

In solid tumors, Tβ4 expression is frequently upregulated in malignant and metastatic cells. In these cancers, higher Tβ4 expression resulted in increased metastatic and invasive capacities of tumor cells, while proliferation remained unaffected.[6] In hematologic disorders, malignant plasma cell disorders, such as plasma cell leukemia and MM were the rare disorders that showed a decreased Tβ4 expression.[5,20] In contrast to solid tumors, publications on the function of Tβ4 in hematologic conditions are scanty

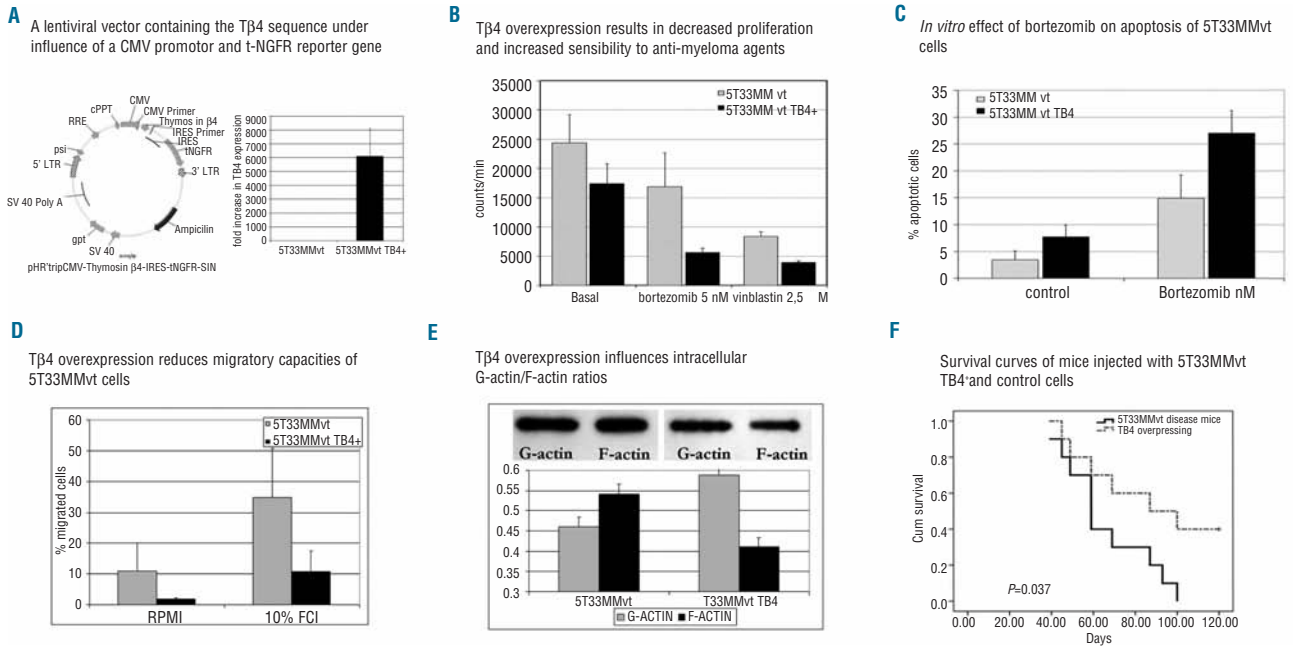

Figure 2. (A) **Schematic representation of the modified lentiviral transfer plasmid and results of RT-PCR and qRT-PCR indicating the presence of the inserted *Tβ4* gene in cultured 5T33MMvt$^{Tβ4+}$ cells.** (B) **^{3}H thymidine uptake revealed a decreased DNA synthesis rate in 5T33MMvt$^{Tβ4+}$ cells compared to wild-type cells. Incubation with the anti-MM agent bortezomib (5 nM) or the micro-tubuli inhibitor vinblastine (2,5 μM) had significantly (*P*<0.001) stronger effects on 5T33MMvt$^{Tβ4+}$ cells than on control cells. A similar observation was made in apoptosis studies (C), where 5nM of bortezomib resulted in a significantly (*P*<0.05) increased apoptotic cell population after 18h incubation. (D) The effects of *Tβ4* overexpression on migration of 5T33MMvt cells: using 10% fetal calf serum as chemo-attractant, only 10.8% (SD 6.6%) of 5T33MMvt$^{Tβ4+}$ cells migrated compared to 34.7% (SD 15.9%) of the control 5T33MMvt cells (*P*<0.05). (E) (Upper) The F-actin and G-actin bands of 5T33MMvt and 5T33MMvt$^{Tβ4+}$ cells. The graph illustrates the ratios of quantified F-actin and G-actin. In 5T33MMt cells actin is present in its polymerized form, whereas Tβ4 overexpression results in decreased F-actin formation and a greater pool of G-actin. (F) C57Bl/KaLwRij mice were injected with 5T33MMvt wild-type and 5T33MMvt$^{Tβ4+}$ cells. Kaplan-Meier analysis showed a significantly different survival between these 2 groups with a mean survival of mice injected with 5T33MMvt wild type of 65.9 days (SD 6.6 days), compared to 88.9 days (SD 9.3 days) for mice injected with 5T33MMvt$^{Tβ4+}$ cells. (*P*<0.05). LTR: long terminal repeat; gag: frame-shifted gag gene; RRE: rev-responsive element; CMV: cytomegalovirus promotor trip: central polypurine tract + termination sequence; Ires: internal ribosomal entry site; tNGFR: truncated form of the nerve growth factor receptor.**

but indicate some inhibitory activity. Tβ4 was initially isolated and purified from a thymic protein preparation, called thymosin fraction-5. Addition of this protein fraction to different leukemic cell lines resulted in a decrease in growth responses.[21] Similar inhibitory effects were recently described for Tβ4 on hematopoietic stem cells,[22] bone marrow derived mast cells[23] and human promyelocytic leukemia cells,[24] in agreement with the results presented here. Whereas a mechanistic explanation of this discrepancy is beyond the scope of this paper, further investigations are clearly merited.

Since T*β4* has been shown to bind G-actin in a 1:1 manner and thus affects the polymerization of G-Actin into F-Actin, we analyzed in a semi-quantitative way, intracellular G-actin and F-Actin. This quantification showed a lowered G-Actin-F-Actin ratio after T*β4* overexpression (Figure 2E). F-Actin is of particular importance in cytoskeleton changes involved in cellular migration and in microtubuli organization controlling the mitotic spindle.[25,26] In line with these results, vinca-alkaloids (e.g. vinblastine used here) with micro-tubulin (polymerization) inhibitory activity, had more affect on the proliferation capacities of 5T33MMvt$^{Tβ4+}$ cells than on control cells (Figure 2B). Since immunohistochemical studies also showed a nuclear staining of T*β4* in 5TMM cells (*results not shown*), involvement of other pathways might also be implicated. Supervized gene analysis comparing *Tβ4*^{high} with *Tβ4*^{low} found different groups of genes differently expressed, including genes involved in cytoskeleton organization, nuclear homeostasis, lymphocyte differentiation and protein metabolism, which might indicate that the role of T*β4* is more complicated than initially supposed.

In conclusion, our results propose a tumor suppressive function of *Tβ4* expression in MM with impact on survival. *Tβ4* was down-regulated in MM cells of patients compared to the normal BM plasma cells and studies with the murine 5T33MM model show a decreased *in vitro* and *in vivo* tumor growth for cells over-expressing the *Tβ4* gene.

Authorship and Disclosures

JC and DH were the principal investigators and took primary responsibility for the paper. JC, DH, IK, TJB, EDB and EM participated in the laboratory work for this study. BVC, EVV, BK and KV coordinated the research. HG and BK were responsible for patient recruitment and patient data. JC, DH, TJB, BK and KV wrote the paper.

The authors reported no potential conflicts of interest.

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