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Unique Pattern of Overexpression of Raf-1 Kinase Inhibitory Protein in Its Inactivated Phosphorylated Form in Human Multiple Myeloma

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Abstract

Multiple myeloma (MM) is the second most common hematological and incurable malignancy of plasma cells with low proliferative activity in the bone marrow. MM patients initially respond to conventional therapy, however, many develop resistance and recurrences occur. We have identified RKIP as a novel gene product that is differentially overexpressed in MM cell lines and MM tissues compared to other studied tumors and normal bone marrow. This overexpression consisted, in large part, of a phosphorylated inactive form of RKIP at Ser153 (p-Ser153 RKIP). In contrast to RKIP, p-Ser153 RKIP lacks its ability to inhibit the MAPK signaling pathway. The overexpression of p-Ser153 RKIP in MM cell lines and MM tissues was further validated in a mouse model carrying a human MM xenograft, namely, LAG -1B. Bioinformatic analyses from databases support the presence of increased RKIP mRNA expression in MM compared to normal plasma cells. In these databases, high RKIP levels in MM are also correlated with the nonhyperdiploid status and the presence of IgH translocations, parameters that generally display more aggressive clinical features and shorter patients' survival irrespective of the treatment. Since RKIP expression regulates both the NF- κ B and MAPK survival pathways, the overexpression of "inactive" p-Ser153 RKIP in MM might contribute positively to the overall cell survival/antiapoptotic phenotype and drug resistance of MM through the constitutive activation of survival pathways and downstream the transcription of anti-apoptotic gene products. The overexpression of RKIP and p-Ser153 RKIP in MM is the first demonstration in the literature, since in most tumor tissues the expression of RKIP is very low and the expression of p-Ser153 RKIP is much lower. The relationship between the levels of active RKIP and inactive p-Ser153 RKIP in MM may be of

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prognostic significance, and the regulation of RKIP activity may be a target for therapeutic intervention.

Keywords

NF- κ B; Raf-1 kinase inhibitor protein (RKIP); apoptosis; multiple myeloma

I. INTRODUCTION

Multiple myeloma (MM) is an incurable hematological malignancy that accounts for 10% of deaths caused by blood cancers and has a median survival of approximately five years. MM is a malignancy of plasma cells with low proliferative activity in the bone marrow. Uncontrolled growth of MM within the bone marrow can lead to bone marrow failure that eventually causes anemia and, less commonly, thrombocytopenia and leukopenia. Interactions between MM cells and nonmalignant cells in the bone marrow microenvironment lead to enhanced bone loss, often resulting in the formation of osteolytic bone lesions, generalized osteoporosis, pathological fractures, hypercalcemia, and bone pain. MM is also associated with renal dysfunction most commonly caused by deposition of monoclonal proteins in the distal tubules of the collecting system of the kidney, hypercalcemia, or myeloma cell infiltration of the kidney. Most patients also show reduced production of other types of antibodies, and may also present with or develop recurrent infections.^{1,2}

In the past decade, there have been major advances in the treatment of MM. Thalidomide, bortezomib, and lenalidomide have emerged as highly active agents in the treatment of MM. Response rates with relapsed diseases are approximately 50% with the combination of thalidomide and steroids, and 65% with a three-drug combination of thalidomide, steroids, and cyclophosphamide. Patients who are not transplant candidates are treated with standard alkylating agents therapy, namely, melphalan, prednisone, and thalidomide (MPT); melphalan, prednisone, and bortezomib (MPB); and melphalan, prednisone, and lenalidomide (MPR).² Although initial clinical responses to drug therapy are achieved, a significant percentage of MM patients relapse and no longer respond to single or combined treatments.^{3,4} Hence, the resistance of MM to current therapeutic regimens remains an unsolved problem in the management of patients with MM.

Research studies on MM have provided new insights into many signaling pathways and proteins whose activation are associated with the pathogenesis of MM, including the PI3K/AKT,⁵ JAK/STAT3,⁶ transforming growth factor (TGF)- β 1/catenin,⁷ Wnt,⁸ Notch,^{9,10} insulin growth factor (IGF),¹¹ pleiotrophin,¹² and IKK/NF- κ B signaling pathways.^{6,13} These pathways and gene products also regulate several antiapoptotic gene products implicated in the resistance of MM to a variety of cytotoxic therapies.

The objective of our current study is to delineate potential underlying mechanisms that regulate the resistance of MM to apoptosis induced by drugs, and thus develop new means to override resistance through manipulation of the identified resistance factors. The rationale of these studies is based on our recent reports that have identified the Raf-1 kinase inhibitor protein (RKIP) as a gene product that can regulate tumor cell resistance to both chemotherapy and immunotherapy.^{14,15} RKIP is a member of the phosphatidylethanolamine-binding protein (PEBP) family that functions as a potent inhibitor of different kinases in the Raf/MAPK (Raf-1 kinase) and NF- κ B (TAK1, NIK) activation pathways, thereby antagonizing both cell survival and the expression of antiapoptotic gene products.^{14,16–18} RKIP also binds to the centrosomal and kinetochore regions of metaphase

chromosomes, in which it may be involved in regulating the partitioning of chromosomes and the progression through mitosis so that its loss can result in chromosomal abnormalities.¹⁹ RKIP expression was shown to be regulated at the transcription level by Snail,²⁰ while RKIP activity was found to be under the control of a post-translational modification, which involves a PKC-mediated phosphorylation at Ser 153.²¹ The phosphorylation was observed in response to growth factor stimuli and resulted in the derepression of the MAPK pathway. These results indicate that PKC regulates MAP kinase activation under physiological conditions through the phosphorylation of RKIP.

RKIP expression is significantly reduced in different cancer types, and its levels seem to regulate the tumor metastatic potential and resistance to apoptotic stimuli.^{22–26} Thus, we hypothesized that low RKIP expression in MM might be directly associated with the pathogenesis of MM and its resistance to therapy. To test this hypothesis, we investigated (i) the expressions of RKIP and phosphorylated RKIP (p-Ser153 RKIP) in MM cell lines and bone marrow–derived tissues from MM patients by immunohistochemistry, (ii) the expression of RKIP and p-Ser153 RKIP in vivo in mice bearing a human MM tumor xenograft, and (iii) the RKIP transcript levels in publicly available data sets containing gene expression profiles of MM and normal B cells. We further correlated our findings with other clinical parameters and patients' outcomes.

II. OVEREXPRESSION OF RKIP IN MM

II.A. Overexpression of RKIP and p-Ser153 RKIP in MM Cell Lines and Primary MM Tumors

Given the reported role of RKIP as a positive regulator of apoptosis in a plethora of tumors, we hypothesized that MM resistance to cytotoxic drugs might be partially attributed to low RKIP levels. We tested this hypothesis by examining the expression levels, using immunohistochemistry, of RKIP in a number of MM cell lines and bone marrow–derived MM tissues (BMMCs) from MM patients. The RKIP expression in MM cell lines was compared with that detected in other tumor cell lines such as the prostate cancer cell line PC-3 and the Non-Hodgkin's B-cell lymphoma (B-NHL) line Ramos. Interestingly, all of the tested MM cell lines, MM1.S, U266, IM9, and RPMI 8826, expressed significantly higher levels of RKIP compared to other tumor cell lines studied (Fig. 1A). These findings were further validated in MM patients–derived BMMCs whereby the majority of the MM samples were characterized by increased expression of RKIP compared to that detected in normal bone marrow (Fig. 1B). These unexpected findings suggested that MM is distinguished from the majority of other reported tumors in terms of levels of RKIP expression.^{22–26} Furthermore, the high level of RKIP expression in MM did not coincide with the resistant phenotype of MM. Hence, we postulated that the high RKIP expression in MM may be in its inactivated phosphorylated form.

Previous reports by Corbit et al.²¹ showed that the functional activities of RKIP are regulated by its phosphorylation status on Ser 153. In contrast to the unphosphorylated form, p-Ser153 RKIP lacks effective binding to Raf-1, and therefore does not inhibit the MAPK signaling pathway. In addition, p-Ser153 RKIP binds to the G protein–coupled receptor kinase-2 (GRK-2) and dissociates it from G protein–coupled receptors (GPCRs), thus preventing receptor internalization and inactivation.²⁷ The RKIP phosphorylation at Ser153 was shown to be mediated specifically by activated classic and atypical protein kinase C (PKC) isoforms such as the PKC δ . Thus, p-Ser153 RKIP antagonizes unphosphorylated RKIP in inhibiting survival signals and promoting apoptosis.²⁷

Using a monospecific antibody directed against p-Ser153 RKIP, which recognizes only the phosphorylated form, we performed immunohistochemical analysis for p-Ser153 RKIP staining in the same MM cell lines and patient-derived BMMCs used for the determination

of total RKIP expression. The findings revealed high p-Ser153 RKIP expressions with the same intensity range observed for total RKIP expression (Figs. 2A and 2B). In contrast, the PC-3 and Ramos cell lines as well as normal BM did not present any significant RKIP or p-Ser153 RKIP staining. It is noteworthy that the RKIP and p-Ser153 RKIP staining assessed in MM cell lines and MM-derived BMMCs was detected in both the cytoplasm and nucleus.

A quantitative overall analysis of RKIP and p-Ser153 RKIP expression levels in MM tissues revealed a significant difference ($p = 0.001$) among MM samples in terms of the mean percentage of RKIP and p-Ser153 RKIP positive cells (Fig. 2C). In addition, the average percentage of p-Ser153 RKIP positive cells corresponded to ~50% of the average percentage of RKIP positive cells, indicating that for the majority of RKIP positive MM samples, a significant proportion of RKIP expression is in the p-Ser153 RKIP form.

In summary, the above findings reveal that in MM, the aberrant expression of RKIP, which should have been correlated with a good prognosis, is in its inactive phosphorylated form and may be of poor prognosis. Preliminary findings were reported at the 2008 ASH Meeting.²⁸

II.B. Validation of High RKIP and p-Ser153 RKIP Expression in an MM Xenograft in SCID Mice

We have developed and successfully used in preclinical drug studies a murine model of human multiple myeloma derived from an MM patient, namely, LAG -1B.^{12,29,30} This stable tumor shows evidence of maintaining the same chemosensitivity and resistance to anti-MM agents as in the original patient's derived tissue. It also displays the same human para-protein in mouse blood. The above findings for the high RKIP and p-Ser153 RKIP expression levels in MM cell lines and MM tissues were validated in the LAG -1B tumor xenograft. IHC analysis in biopsies of LAG -1B-palpable tumors 20 days postimplantation revealed high RKIP and p-Ser153 RKIP expression at almost equal levels (Fig. 3). This finding validates our in vitro findings and our hypothesis for the dominance of p-Ser153 RKIP form in MM. Furthermore, the findings suggest that this in vivo model can be used for analysis of the correlation between the expression levels of RKIP and p-Ser153 RKIP, and the response to various cytotoxic agents. It also suggests that p-Ser153 RKIP overexpression might regulate, in part, MM survival and resistance to apoptotic stimuli, and could be a potential candidate target for reversal of MM resistance to conventional drug therapy.

II.C. Computational Analysis

A special focus in the field of MM prognostication during the last few years has provided novel approaches to analyze the pharmacogenomic databases and their correlations with prognosis. The negative prognostic impact of various genetic aberrations, including specific IgH translocations and chromosome 13 deletions and, mainly, the prognostic importance of the expression profiles of individual genes or groups of genes, have been proved to be valuable tools in guiding anti-MM therapeutic decisions and determining the clinical outcome.^{31,32} For that reason, computational analysis of RKIP mRNA levels in different groups of MM patients was performed as described in Figure 4.

RKIP overexpression was found to be a general event in MM cells and primary MM tumors compared to normal bone marrow and other studied tumors. The overexpression of RKIP in MM compared to normal plasma cells samples at the transcriptional level was confirmed in a publicly available gene expression data set³³ ($p < 0.0001$) (Fig. 4A). In the perspective to identify in MM tumors the existence of a possible association between RKIP overexpression and commonly used prognostic parameters for determining MM outcome, we explored two independent gene expression data sets that include a collection of molecularly characterized

and outcome-annotated clinical MM specimens.^{34,35} In the Agnelli et al.³⁴ data set, RKIP expression was stratified among different TC groups classified according to the presence of IgH chromosomal translocations and hyperdiploid status (H-MM) (Fig. 4B). RKIP overexpression was found significantly associated with non-H-MM (groups TC1, TC4, and TC5). These latter MM subgroups are also composed of cases harboring IgH translocations, generally displaying more aggressive clinical features and shorter survival, irrespective of the treatment modality.³⁶ The three main IgH translocations found in non-H-MM patients analyzed in the data set were the t(11;14), t(4;14), and t(14;16). The positive correlation of high RKIP levels with nH-MM status was further identified in the data set of Carrasco et al.³⁵ data set, where MM patients were classified in subgroups of H-MM (which harbors numerous chromosomal trisomies and a low prevalence of IgH translocations) and non-H-MM (Fig. 4C). Similar results were also obtained by separately analyzing the three RKIP probesets (210825_s_at, 211941_s_at, and 205353_s_at) or their normalized log2 mean expression values (data not shown).

The patients belonging to the nH-MM subgroup in the data set of Carrasco et al.³⁵ were shown to have a tendency toward a less favorable outcome. When these patients were stratified according to RKIP expression (above and below median values of expression in the data set) and correlated with the clinical outcome, no significant association with survival was observed (Fig. 4D). This finding suggests that the biological significance of RKIP in MM could not be determined only by its transcript and protein levels; but as shown in this study, could also be dictated by post-translational modifications such as the phosphorylation at Ser153, which represses RKIP activity as an antisurvival factor.

III. REMARKS

In contrast to other reported studies on the expression of RKIP in various cancer types, our findings demonstrate high levels of RKIP in MM cell lines and patients samples. The overexpression mainly concerns a p-Ser153 RKIP form. The overexpression of RKIP and p-Ser153 RKIP in MM is the first demonstration in the literature among many tumors studied. There have been no published reports (except studies performed in lung tumors, see p. 171 in this issue) to date on the analysis of p-Ser153 RKIP expression in any tumor and its possible role in the interpretation of the data. In contrast to MM, most tumors express low levels of total RKIP, and they are expected to have equal or lower levels of p-Ser153 RKIP. Given the role of RKIP as a negative regulator of survival signals induced by the activation of MAPK and NF- κ B pathways, the post-translational modification of RKIP (the phosphorylation at Ser153 residue) is important to assess. Thus, p-Ser153 RKIP might contribute positively to the overall cell survival and drug resistance of MM through the constitutive activation of survival pathways and downstream the transcription of antiapoptotic genes. Furthermore, our bioinformatic analyses in reported databases support the presence of increased mRNA levels of RKIP in MM compared to normal B cells and correlate the levels with different prognostic factors (such as IgH translocations) that are responsible for more aggressive clinical features and shorter patients' survival. However, we propose that further studies of larger cohorts are necessary to confirm our preliminary findings and to establish the relevance of the RKIP/p-Ser153 RKIP ratios as a prognostic biomarker that might be used in individualized anti-MM treatment approaches.

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ABBREVIATIONS

BMMCs	bone marrow mononuclear cells
IgH	immunoglobulin heavy (chain)
IKK	I kappa B kinase
JAK	janus kinases
MAPK	mitogen-activated protein kinases
NF- B	nuclear factor- B
NIK	NF-kappa B–inducing kinase
PI3K/Akt	phosphatidylinositol 3-kinase/Akt
PKC	protein kinase C
STAT	signal transducers and activator of transcription
TAK1	transforming growth factor beta–activated kinase 1

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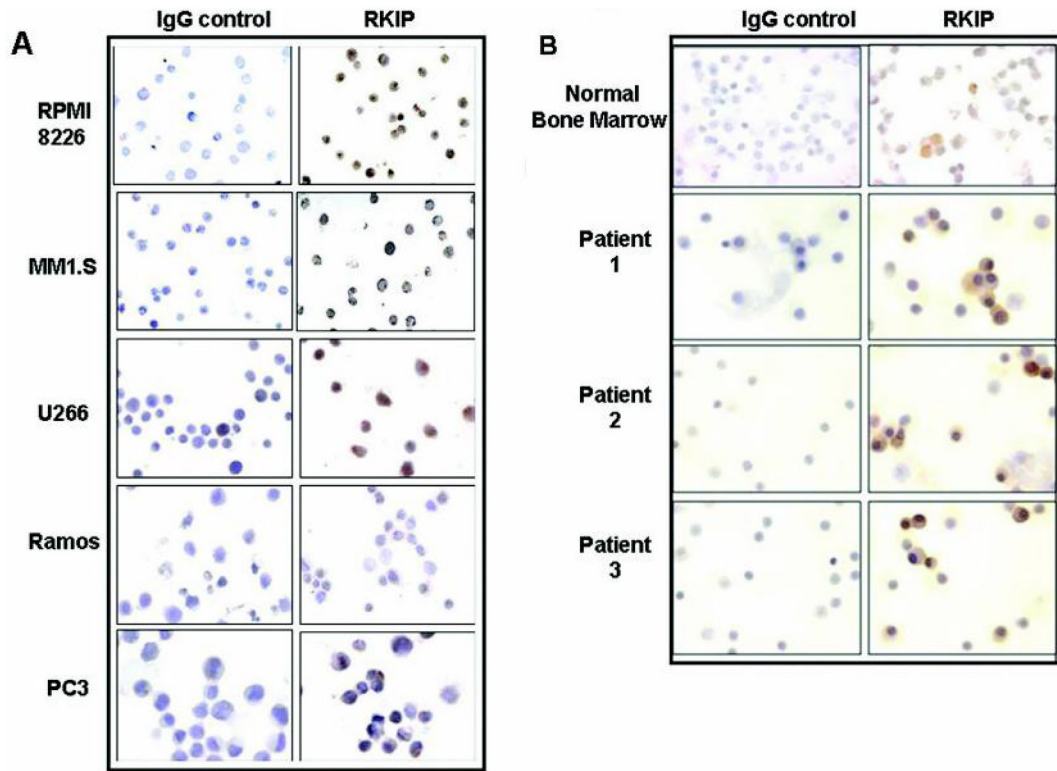
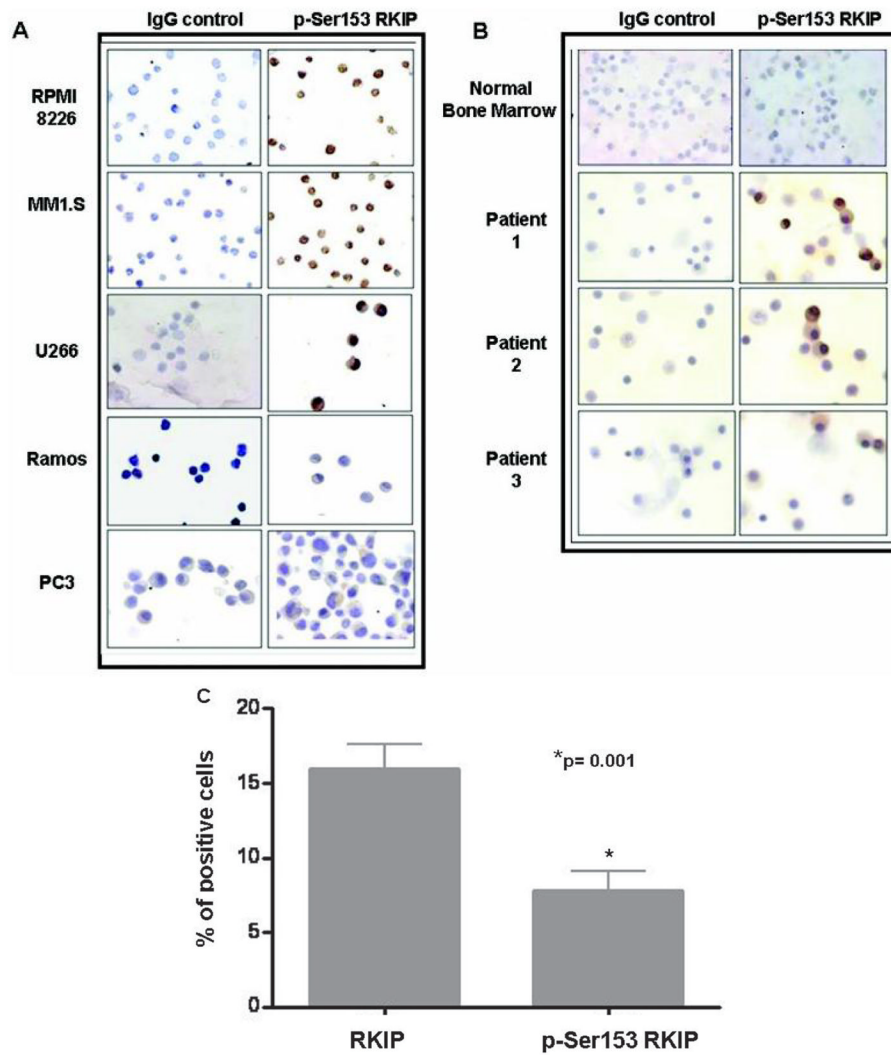


FIGURE 1.

Overexpression of RKIP in MM cell lines and primary MM tumors. (A) Immunohistochemical (IHC) analysis of RKIP expression in the MM cell lines RPMI 8226, MM1.S and U266, as well as in Ramos and PC-3 cell lines. Following inhibition of endogenous peroxidase activity and nonspecific binding, the slides were incubated overnight at room temperature with an anti-hRKIP (1/500) antibody. The slides were further incubated with a biotinylated antirabbit secondary antibody [Universal LSAB kit (DAKO corporation, Carpinteria, California)] and then with a streptavidin-HRP conjugate as previously described.³⁷ The color was detected using a 3, 30-diaminobenzidine tetrahydrochloride solution (DAB) according to manufacturer's instructions (DAKO corporation, Carpinteria, California) (B) Overexpression of RKIP in representative primary MM tumors and normal bone marrow as assessed by IHC. In all cases, rabbit IgG was used as antibody specificity control. In order to reduce the variability, all samples were processed at the same time in a single experiment using a single batch of antibody diluted in PBS supplemented with normal goat serum.

**FIGURE 2.**

MM exhibits overexpression of the inactive p-Ser153 RKIP form. (A) Expression of p-Ser153 RKIP in MM cell lines and other non-MM cell lines, as assessed by IHC. The slides were incubated overnight at room temperature with an anti-p-Ser153 RKIP antibody at 1/250 dilution in 1% BSA in PBS, and the staining was analyzed as described above. (B) IHC analysis of p-Ser153 RKIP expression in representative primary MM samples and normal bone marrow. (C) Quantitative analysis of RKIP and p-Ser153 RKIP expression in primary MM samples. The graph demonstrates the mean percentage \pm SD of RKIP and p-Ser153 RKIP-positive cells counted in five random fields of each of the 20 MM samples. $p = 0.001$ RKIP versus p-Ser153 RKIP positive staining (Mann Whitney *U*-test).

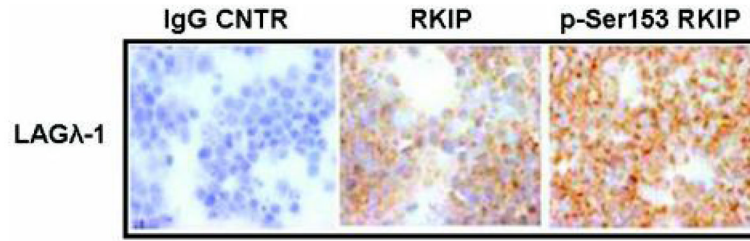
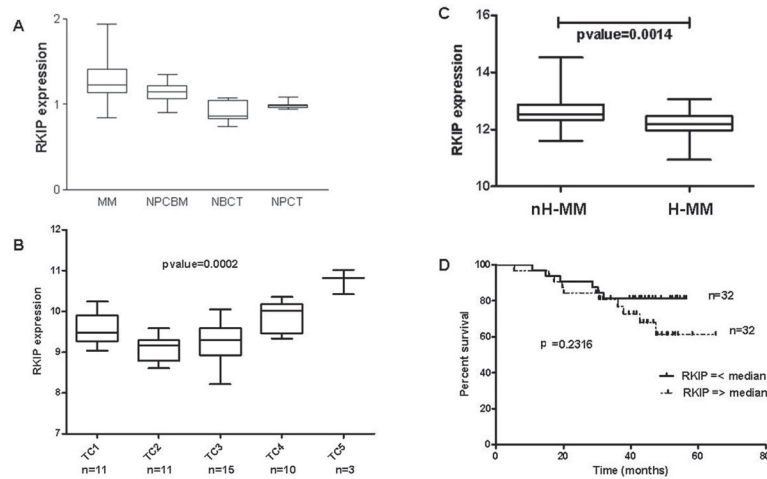


FIGURE 3.

In vivo validation of high RKIP and p-Ser153 RKIP expression. Expression profiles of RKIP and p-Ser153 RKIP in MM biopsies derived from an LAG λ -1B MM human xenograft in SCID mice. The expression of both RKIP forms was assessed by IHC using the specific antibodies. All animal studies were conducted according to the University of California at Los Angeles (UCLA) Animal Research Committee protocols. Tumors were measured weekly using calipers, and their volumes calculated with the following formula: $\frac{4}{3} \times (\text{width}/2)^2 \times (\text{length}/2)$, portraying the 3D volume of an ellipsoid.

**FIGURE 4.**

RKIP gene expression levels in MM data sets. The expression of RKIP was examined in independent, publicly available data sets of MM gene expression using HG-U133 series microarrays (Affymetrix, Santa Clara, CA, USA). The selection of the patients was based on the presence of recurrent IgH chromosomal translocations, hyperdiploidy, and cyclin D expression. The analyses were performed in: (a) 50 MM samples that were stratified according to the TC classification: TC1 = tumors with t(11;14) or t(6;14) translocations, overexpression of CCND1 or CCND3, and a nonhyperdiploid status; TC2 = tumors with low to moderate levels of the CCND1 gene in the absence of any primary IGH translocation but associated with a hyperdiploid status; TC3 = tumors that do not fall into any of the other groups and most of which express CCND2; TC4 = tumors showing high CCND2 levels and the presence of the t(4;14) translocation; and TC5 = tumors expressing the highest levels of CCND2 in association with either the t(14;16) or t(14;20) translocation (HG-U133A),³⁴ and (b) 67 MM samples classified into a hyperdiploid and a nonhyperdiploid subgroup characterized by loss of chromosomes 1p, 8p, 13, and 16q and by amplification of chromosome 1q. Clinical outcome of MM patients whose neoplastic tissues were analyzed was also available (HG-U133 Plus 2.0).³⁵ A data set obtained using Affymetrix HuGene FL microarrays was also used.³³ This data set reports the gene expression data obtained from 74 MM samples and 31 cell preparations from healthy donors. Normalized expression values of RKIP were extracted from the NCBI Gene Expression Omnibus database (GEO series: GSE2912³⁴ and GSE4452³⁵) or from one author's Web site (<http://lambertlab.uams.edu>).³³ For HG-U133A microarrays, the mean RMA log₂ intensity values of the three Affymetrix probe sets specific for RKIP (PEBP1: 210825_s_at; 211941_s_at and 205353_s_at in HG-U11-A and HG-U133 Plus2 platforms) were considered. (A) Zhan database³³: comparison of mean RKIP transcript levels among MM plasma cells, normal plasma cells derived from bone marrow (NPCBM), normal B cell from tonsils (NBCT), and normal plasma cells from tonsils (NPCT). $p < 0.01$ RKIP expression in MM plasma cells versus each of the other three groups (Mann Whitney *U*-test). (B) Agnelli data set³⁴: MM patients were classified into the five TC subgroups described above. RKIP expression in the five subgroups is different with $p < 0.01$, as assessed by Kruskal-Wallis statistics. Samples harboring IgH translocations TC1, TC4, and TC8 display significantly higher RKIP expression when compared to TC2 and TC3 samples without IgH translocations ($p < 0.01$ for TC2 versus TC4 and TC2 versus TC5), ($p < 0.05$ for TC2 versus TC1, TC3 versus TC4, and TC3 versus TC5) as assessed by Dunn's multiple comparison post test. The number of informative patients (*n*) is reported. (C) Carrasco data set³⁵: nH-MM (nonhyperdiploid) samples display increased expression of RKIP when compared to H-MM samples ($p < 0.01$,

Mann Whitney *U*-test). (D) Kaplan Meier overall survival curves for 64 patients indicating no significant difference in survival ($p = 0.2316$) in patients divided according to values above or below median of RKIP gene expression values.