








RESEARCH ARTICLE

Expression of p53 protein isoforms predicts survival in patients with multiple myeloma

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Abstract

Loss and/or mutation of the *TP53* gene are associated with short survival in multiple myeloma, but the p53 landscape goes far beyond. At least 12 p53 protein isoforms have been identified as a result of a combination of alternative splicing, alternative promoters and/or alternative transcription site starts, which are grouped as α , β , γ , from transactivation domain (TA), long, and short isoforms. Nowadays, there are no studies evaluating the expression of p53 isoforms and its clinical relevance in multiple myeloma (MM). We used capillary nanoimmunoassay to quantify the expression of p53 protein isoforms in CD138-purified samples from 156 patients with newly diagnosed MM who were treated as part of the PET-HEMA/GEM2012 clinical trial and investigated their prognostic impact.

Irena Misiewicz-Krzeminska and Norma C. Gutiérrez contributed equally to this study.

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Quantitative real-time polymerase chain reaction was used to corroborate the results at RNA levels. Low and high levels of expression of short and TAp53 β/γ isoforms, respectively, were associated with adverse prognosis in MM patients. Multivariate Cox models identified high levels of TAp53 β/γ (hazard ratio [HR], 4.49; $p < .001$) and high-risk cytogenetics (HR, 2.69; $p < .001$) as independent prognostic factors associated with shorter time to progression. The current cytogenetic-risk classification was notably improved when expression levels of p53 protein isoforms were incorporated, whereby high-risk MM expressing high levels of short isoforms had significantly longer survival than high-risk patients with low levels of these isoforms. This is the first study that demonstrates the prognostic value of p53 isoforms in MM patients, providing new insights on the role of p53 protein dysregulation in MM biology.

1 | INTRODUCTION

Loss and/or mutation of the *TP53* gene are associated with short survival in multiple myeloma (MM). *TP53* mutations are uncommon at diagnosis, being present in less than 8% of cases, and deletion of the 17p13 chromosomal region, del(17p), has been identified in approximately 10% of newly diagnosed MM patients.^{1,2} Although del(17p) has been routinely examined in the clinical setting for many years and *TP53* mutations are considered in present-day genetic studies, the p53 landscape covers a far wider range of phenomena than just *TP53* deletions and mutations.^{3,4} Therefore, the study of other deregulations of the p53 pathway, particularly those that trigger defective p53 activity, are also important.³⁻⁶

The discovery of an alternative promoter in the *TP53* gene in 2005 led to the identification and characterization of p53 isoforms.⁷ This finding has had a profound impact on our perspective on the p53 pathway and the ways of researching p53 tumor suppressor activity. The *TP53* gene expresses at least twelve p53 protein isoforms that are encoded by nine p53 mRNAs: TAp53 α , TAp53 β , TAp53 γ , $\Delta 40$ p53 α , $\Delta 40$ p53 β , $\Delta 40$ p53 γ , $\Delta 133$ p53 α , $\Delta 133$ p53 β , $\Delta 133$ p53 γ , $\Delta 160$ p53 α , $\Delta 160$ p53 β , and $\Delta 160$ p53 γ proteins. The p53 isoforms arise from the combination of alternative promoter usage (proximal and internal promoters), alternative initiation codons (ATG1, ATG40, ATG133 and ATG160), and/or alternative splicing of introns 2 ($\Delta 40$) and/or 9 (α , β , γ) (Figures 1A and S6A,B). They can be grouped into subclasses or variants, such as the α , β , γ , from transactivation domain (TA), $\Delta 40$, and $\Delta 133/\Delta 160$ variants, based on the molecular mechanisms that lead to their formation (Figures S1D and S6A,B). The 12 p53 protein isoforms share a common region of the DNA-binding domain (DBD) but have different transactivation and oligomerization domains that allow them to differentially regulate the expression of p53 target genes and, at the same time, to be distinctively modulated by their negative regulators, like the Mdm2 and Mdm4 proteins.⁷⁻¹¹

Several studies have demonstrated that p53 isoforms are differentially expressed in human cancers and that they affect the prognosis of some of these tumors. *TP53* deletions have been associated with low levels of p53 gene and protein expression in MM, using microarrays and immunohistochemistry, respectively.^{3,12,13} Moreover, haploinsufficiency of p53 has been functionally demonstrated in MM cell lines³ and a low level of expression of *TP53* gene has been associated with inferior outcome in MM patients. However, no studies have so far evaluated the expression of p53 isoforms and its clinical significance in MM.^{8,14-16}

In this study, we investigated for the first time the relative expression of p53 isoforms in MM at the protein and mRNA levels. Using samples from homogeneously treated MM patients, we found that the differential expression of short and TA p53 isoforms influenced survival. In addition, incorporating expression levels of p53 isoforms improved the current cytogenetic-risk classification.

2 | MATERIALS AND METHODS

2.1 | Primary samples

A total of 156 protein samples from newly diagnosed MM patients enrolled in the clinical trial GEM2012 (NCT01916252) were included in the study. Although 458 patients were evaluated in this trial, fewer than 40% of the purified MM samples had sufficient material available to enable proteins and nucleic acids to be extracted. Details of the GEM2012 trial and sample processing have been previously reported.^{17,18}

Baseline characteristics of patients for whom data were available are summarized in Table S1. No statistically significant differences between the present cohorts and the whole series from the GEM2012 trial ($N = 458$) were observed.

Fluorescence in situ hybridization (FISH) studies to detect *IGH* rearrangements, 17p and 1p deletions, and 1q gains were available for

all patients. Cytogenetic abnormalities were distributed as expected, based on previously published data. The high-risk cytogenetic group included those patients with t(4;14), t(14;16), and/or 17p deletion (del17p), according to International Myeloma Working Group criteria.¹⁹ The median follow-up of the GEM2012 patients in this study was 72 months (range, 32–88 months). At the end of their follow-up, 68 patients (47%) had progressed, and 34 patients (23%) had died.

2.2 | Protein extraction and capillary electrophoresis immunoassay

Proteins were extracted simultaneously with genomic DNA and RNA by ice-cold acetone precipitation, as previously described.²⁰ The total protein assay was used to quantify protein concentration using WES™ system (ProteinSimple). Capillary electrophoresis immunoassay (CNIA) analysis, also called Simple Western, was performed using a WES

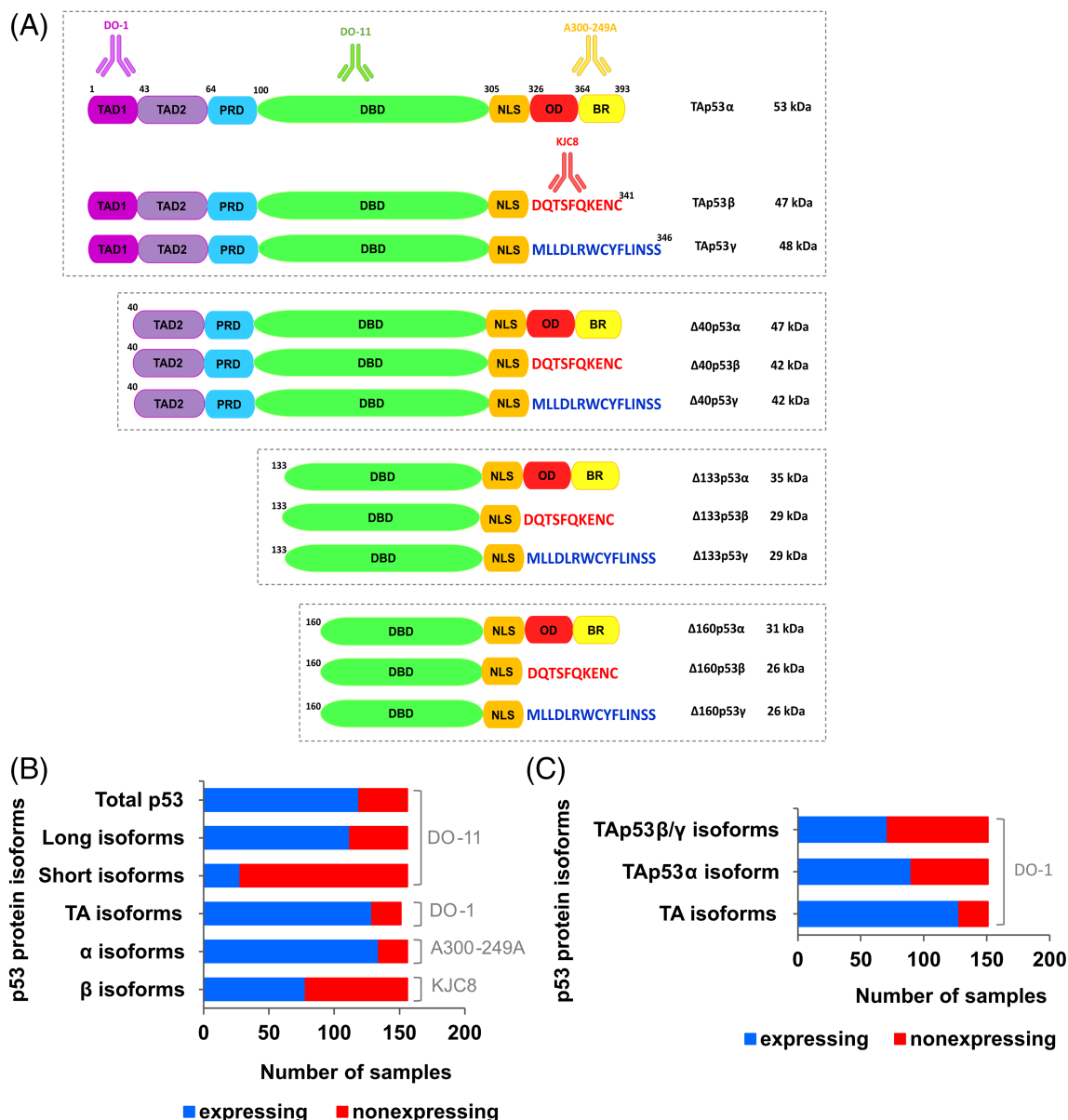


FIGURE 1 Schematic representation of human p53 protein isoforms, and the region containing the epitope for the p53 antibodies used in this study. (A) The main domains of p53 protein isoforms and their locations are represented by colors and amino acid (aa) numbering, respectively. The C-terminal sequences specific to the β (DQTSFQKENC) and γ (MLLDLRWCYFLINSS) variants are also shown. The molecular weight of each p53 isoform protein is indicated. The α , β , TA, long, and short protein isoforms are specifically recognized by A300-249A, KJC8, DO-1, and DO-11, respectively. (B) Number of MM patients with and without expression of each p53 isoform. (C) Number of MM patients with and without expression of TA isoforms, differentiating the two bands at 55–57 and 60–63 kDa that correspond to the TAp53 α and TAp53 β/γ isoforms, respectively. BR, basic region, aa 364–393 (yellow); DBD, DNA-binding domain, aa 101–292 (green); NLS, nuclear localization signal, aa 305–322 (orange); OD, oligomerization domain, aa 326–356 (red); PRD, proline-rich domain, aa 64–92 (blue); TAD1, transactivation domain 1, aa 1–42 (purple); TAD2: transactivation domain 2, aa 43–63 (violet) [Color figure can be viewed at wileyonlinelibrary.com]

machine (ProteinSimple, San Jose, CA) in accordance with the manufacturer's protocols and as previously described.^{20,21} The primary antibodies used in the study were: mouse monoclonal DO-11 (BioRad; MCA1704, aa 181–190), whose epitope is present in the common region DBD and allows detection of all p53 protein isoforms; mouse monoclonal DO-1 (Santa Cruz Biotechnology; sc-126, aa 11–25), whose epitope is situated in the transactivation domain 1 that is present only in the TAp53 α , TAp53 β , and TAp53 γ protein isoforms; rabbit polyclonal anti-p53 A300-249A-T (Bethyl Laboratories, Inc.; aa 375–393), which is specific to the α isoforms (TAp53 α , Δ 40p53 α , Δ 133p53 α , and Δ 160p53 α); and anti-GAPDH (Cell Signaling; rabbit mAb #2118), which was used as the endogenous control. The rabbit polyclonal KJC8 antibody, which is specific to the β isoforms (TAp53 β , Δ 40p53 β , Δ 133p53 β , and Δ 160p53 β), was provided by Prof. J.-C. Bourdon (aa 331–341).⁷

All protein data were analyzed with Compass™ software (ProteinSimple), qualitatively at first, using the virtual blots (analogous to the images of the long-established western blot) that show the protein band with the expected size and also quantitatively, measuring the chemiluminescence peaks (peak area) that correspond to the expression of a particular protein. The protein expression was reported as relative to the endogenous control, GAPDH.

A more detailed protocol of the relative quantification of protein by CNIA has been reported previously.^{18,20,21}

2.3 | Plasmids and transfection

We used the null-p53 JIN3 myeloma cells nucleotransfected with the specific expression vectors as positive controls for the p53 α , p53 β , p53 γ , and Δ 133p53 α protein isoforms based on their migration profiles (Figure S1A–C). The commercial expression plasmids containing each isoform were obtained from Origene: p53 β isoform (NM_001126114) SC322987, p53 γ isoform (NM_001126113) SC322990, and Δ 133p53 α isoform (NM_001126115) SC322927. The pcDNA3 p53 WT, which codes for the canonical p53 α , was a gift from David Meek (Addgene plasmid # 69003).²² For transient protein expression, the null-p53 JIN3 myeloma cell line was nucleotransfected with 1 μ g of each plasmid using the X005 program of the Amaxa II nucleofector (Lonza Bioscience). The proteins were collected 24 h post-transfection.

2.4 | Nucleic acid extraction and quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from all samples using the AllPrep DNA/RNA Mini Kit (Qiagen). RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies), and samples with an RNA integrity number (RIN) ≥ 6 were used. Total RNA (200 ng) was reverse-transcribed to cDNA using the SuperScript First-Strand Synthesis System, which uses oligo (dT) (Thermo Fisher). *TP53* isoforms (α , β , short [Δ 133/ Δ 160] and long [TA/ Δ 40] isoforms) (Figure S6A,B)

were examined by quantitative real-time polymerase chain reaction (qRT-PCR), as previously described.²³ We also measured the expression of the *TP53* gene, detected at a region of mRNA common to all isoforms. The *PGK1* gene was used as the endogenous control. Samples with a Ct value ≥ 35 were considered as not expressed. Values of Δ Ct, defined as Ct (housekeeping gene) – Ct (target gene), were also calculated.

2.5 | Next-generation sequencing

Genomic DNA samples were purified before sequencing using Genomic DNA Clean & Concentrator TM-10 (Zymo Research, Irvine, CA). Generation of libraries was carried out by target enrichment SeqCap EZ Choice gene panel (Roche NimbleGen Inc., Madison, WI) that included the coding sequence of *TP53* gene, which was designed by NimbleDesign platform. The resulting pool (48-plex) underwent high-throughput paired-end (101 bp) sequencing on an Illumina MiSeq System with 1500-fold coverage. Sequence alignment and variant calling was performed using MiSeq Reporter software (Illumina Inc.). Annotation of resulting variant call files (.vcfs) was performed with BaseSpace Variant Interpreter software, filtering out single nucleotide variants (SNVs) and small insertions/deletions with $<100\times$ reads and variant allele frequency (VAF) of $<10\%$.

2.6 | Statistical analysis

All statistical analyses were carried out with IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY), the Simfit package (W.G. Bardsley, University of Manchester, Manchester, UK; v7.0.9 Academic 32 bit), and R work packages. The Mann–Whitney *U* test was used to analyze the continuous variables.

For survival analyses, probabilities of overall survival (OS) and survival without progression (abbreviated to TTP) were assessed for each isoform using the Kaplan–Meier estimator. Patients who were not enrolled in the GEM2014 maintenance protocol after completing the GEM2012 clinical trial and who had not progressed, relapsed, or died were excluded from these analyses. The TTP was defined as the time from MM diagnosis to the day of disease progression. The survival curves of the isoform groups were compared using the log-rank test. The Cutoff Finder R package was used to determine the optimal cutoff for all survival analyses, which was defined as the most significant split discriminating between long and short survival when testing all possible cutoffs using the log-rank test.

After checking that the assumptions of proportional hazards held using Schoenfeld residuals and that there was no significant multicollinearity, multivariable Cox regression models were fitted considering a set of covariates with clinical and biological interest. Thus, the models included conventional covariates such as high-risk cytogenetics, age at diagnosis (years), International Staging System (ISS) stage (III vs. I/II), elevated lactate dehydrogenase (LDH), plasmacytoma occurrence, and protein isoforms expression previously

dichotomized by the Cutoff Finder R package. All these predictor covariates were entered into the regression equation together. These multivariable analyses were performed in R with the survival and car packages and associated forest plots were depicted using the survminer package. The relative contribution of the covariates to the Cox models was assessed by estimation of the proportion of the χ^2 statistic accruing from each covariate using the rms package; the higher the value of the statistic, the greater the contribution of the covariate to the model.

3 | RESULTS

3.1 | Expression patterns of p53 protein isoforms in MM

The expression of the p53 protein isoforms, α isoforms (TAp53 α , Δ 40p53 α , Δ 133p53 α , and Δ 160p53 α); β isoforms (TAp53 β , Δ 40p53 β , Δ 133p53 β , and Δ 160p53 β); TA isoforms (from transactivation domain: TAp53 α , TAp53 β , and TAp53 γ); long (includes TA and Δ 40: TAp53 α , TAp53 β , TAp53 γ , Δ 40p53 α , Δ 40p53 β , and Δ 40p53 γ) and short isoforms (Δ 133 and Δ 160: Δ 133/ Δ 160 α , β , and γ), as well as the total p53 protein, was assessed in the 156 MM samples by capillary nanoimmunoelectrophoresis using the specific antibodies (Figures 1A and S1A–D, Table S2).

The qualitative analysis of CNIA assays showed expression of the total p53 protein in 119/156 (76%) MM samples using the DO-11 antibody, which recognizes identical epitopes present in all the p53 protein isoforms. Based on the migration profile and the recognition pattern of this antibody, we observed that the major proportion of the total p53 protein corresponded to the long isoforms, which were detected in 112 of the 156 MM samples (72%), and the minor proportion corresponded to the short isoforms, which were only present in 28 of the 156 MM samples (18%) (Figure 1B).

The TA protein isoforms were detected in 128/151 MM samples (85%) using the DO-1 antibody, whose epitope is present in the transactivation domain 1 (TAD1, aa 20 to 25) (Figure 1A). Among the TA isoforms, we were able to distinguish the TAp53 α band at 60–63 kDa in 90/151 (60%) samples, and the bands corresponding to TAp53 β and TAp53 γ , which were observed merged as a single band at 55–57 kDa in 71/151 MM samples (47%). Twenty-three patients (15%) did not show any of those isoforms (Figure 1C). The α and β protein isoforms were identified in 132/156 (85%) and 81/156 (52%) samples, respectively, as measured by the antibodies A300-249A and KJC8, which were specific to each isoform (Figure 1B).

The quantitative analysis revealed a high level of variability in the expression of all the p53 protein isoforms analyzed (Figure S1E). The expression of long isoforms was the most homogeneous, while the TAp53 β / γ and α isoforms showed the greatest variability, as indicated by their higher coefficients of variation (Figure S1F).

3.2 | Relationship between p53 protein isoforms and cytogenetic abnormalities

We next analyzed the association between the expression of p53 protein isoforms and the cytogenetic features of MM patients. We found no statistically significant differences in the level of expression of total p53 protein between MM patients with and without del(17p). In contrast, we detected significant differences in the expression of long and TAp53 β / γ isoforms between the two groups (Figure 2). We also noticed that none of the 14 patients with del(17p) expressed short isoforms (Figure 2). The expression of α and β protein isoforms was similar in all patients, irrespective of the presence of del(17p). The analysis considering the mutation status of *TP53* showed comparable results. We found that the expression levels of TA isoforms were significantly higher in patients with mutated *TP53* gene than in those with nonmutated gene. No significant associations between the expression of the other p53 protein isoforms and the presence of *TP53* mutations were found (Figure S2).

On the other hand, no differences in the expression of any of the p53 protein isoforms analyzed and the total p53 protein were found to be associated with the presence of the gain of 1q, del(1p) or t(4;14). Since the limited number of patients with specific high-risk cytogenetic abnormalities may preclude the identification of significant differences, we also analyzed the relationship between the levels of p53 protein isoforms and cytogenetic alterations, grouping them into high- and standard-risk categories. We found statistically significant differences in the expression levels of TAp53 β / γ isoforms between the two cytogenetic groups. High-cytogenetic-risk patients expressed TAp53 β / γ isoforms significantly more strongly than did standard-risk patients (Figure 2). No differences in the expression of the other p53 isoforms and total p53 protein were found in either cytogenetic-risk group.

3.3 | Effect of the expression of p53 protein isoforms on MM patient survival

High levels of the short p53 isoforms were accompanied by a reduction of 75% (HR = 0.25, p = .004) in the risk of progression and an 88% (HR = 0.124, p = .014) drop in the risk of mortality (Figure 3A). Conversely, high levels of TA isoforms were associated with shorter TTP and OS (HR = 2.02, p = .008 and HR = 2.39, p = .018, respectively) (Figure S3A). Nevertheless, only the high level of expression of TAp53 β / γ isoforms, but not of TAp53 α , was associated with negative effects on TTP and OS (HR = 3.58, p < .001 and HR = 2.38, p = .022, respectively) (Figures 3B and S3B). The expression levels of α and β variants, as well as levels of total p53 protein had no influence on the survival of MM patients (Figure S3C).

We further explored the impact of expression levels of p53 protein isoforms on the survival of MM patients according to the cytogenetic abnormalities. In the present cohort, MM patients bearing at least one high-risk cytogenetic lesion, as well as those with the 17p deletion, had significantly shorter survival than those patients without these abnormalities, as described in previous studies (Figure S4A).

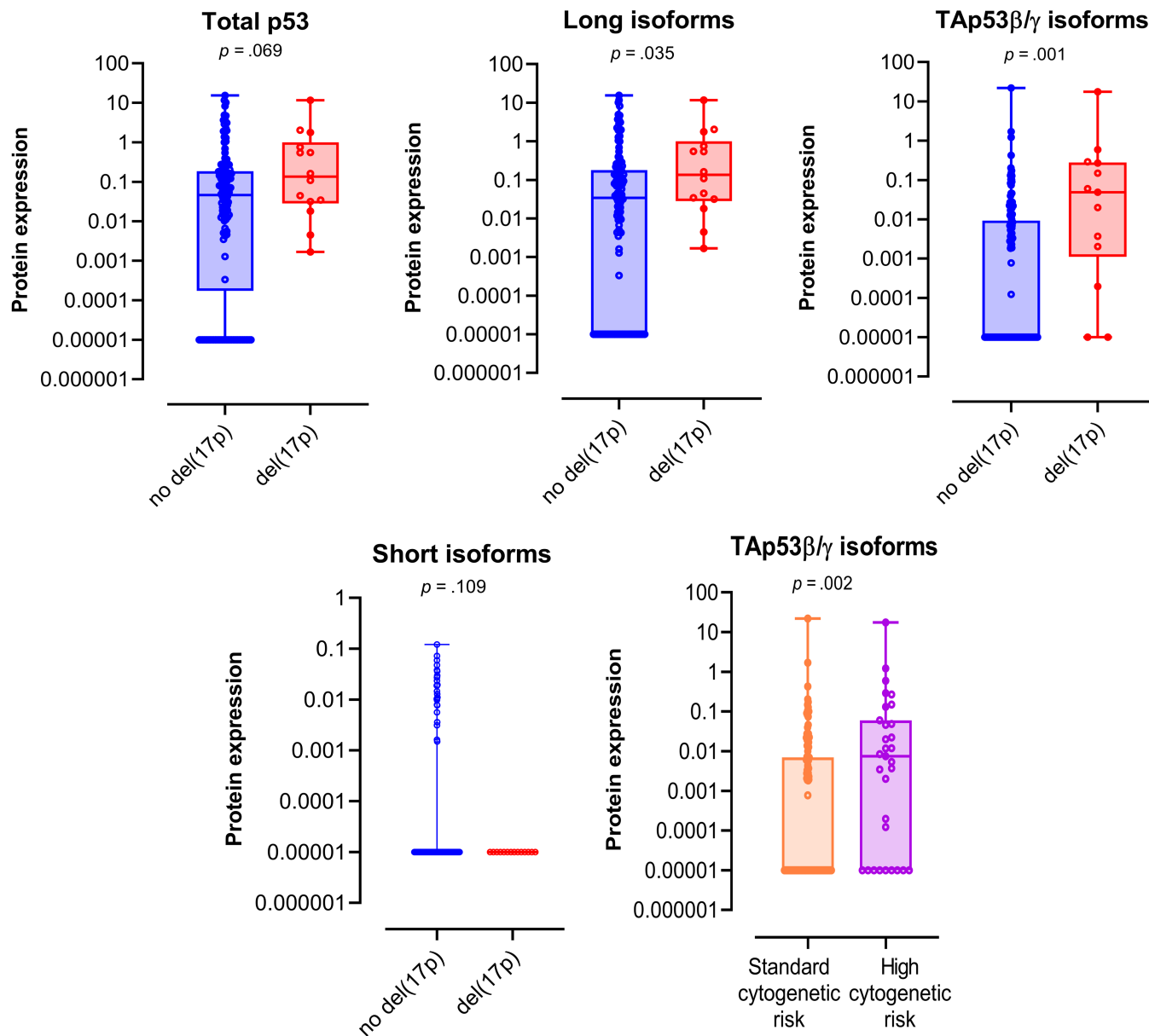


FIGURE 2 Association of p53 protein isoforms with deletion of 17p and with standard and high cytogenetic risk. Distribution of the expression of total p53 protein, long, TAp53 β/γ and short isoforms, based on the presence or absence of the 17p deletion. Expression levels of the TAp53 β/γ in MM patients with standard and high cytogenetic risk. The statistically significant differences between groups were determined by the Mann–Whitney *U* test (*p* values indicated) [Color figure can be viewed at wileyonlinelibrary.com]

When the cytogenetic-risk groups were analyzed with respect to the expression of p53 isoforms two different survival categories were identified within the high-risk group. Thus, MM patients with high-risk cytogenetics and a high level of expression of short p53 isoforms had significantly longer TTP and OS, which were comparable to those attained by standard-risk patients (Figure 4A). However, the effect of the TAp53 β/γ isoforms, and long isoforms were the opposite, in such way that MM patients with high-risk cytogenetics and low-level expression of TAp53 β/γ or long isoforms had longer TTP and OS, similar to those patients with standard risk as demonstrated by FISH (Figures 4B and S4B). These results indicate that high levels of short isoforms, and low levels of TAp53 β/γ and long protein isoforms allow

the identification of a subset of MM patients with high cytogenetic risk that showed a better prognosis than expected. Interestingly, a positive influence of the short p53 isoforms was also observed when t(4;14) or del(17p) were considered separately (Figure S5A). Furthermore, within the MM cases with t(4;14) the high levels of expression of TAp53 β/γ and long p53 isoforms distinguished a group of patients with significantly shorter TTP and OS (Figure S5B).

In the full multivariate Cox model for TTP, including p53 protein isoform expression and the conventional variables of proven prognostic impact on MM, we observed that high-risk cytogenetics and high expression levels of TAp53 β/γ isoforms remained independent prognostic factors (HR = 4.49, *p* < .001 and HR = 2.69, *p* < .001,

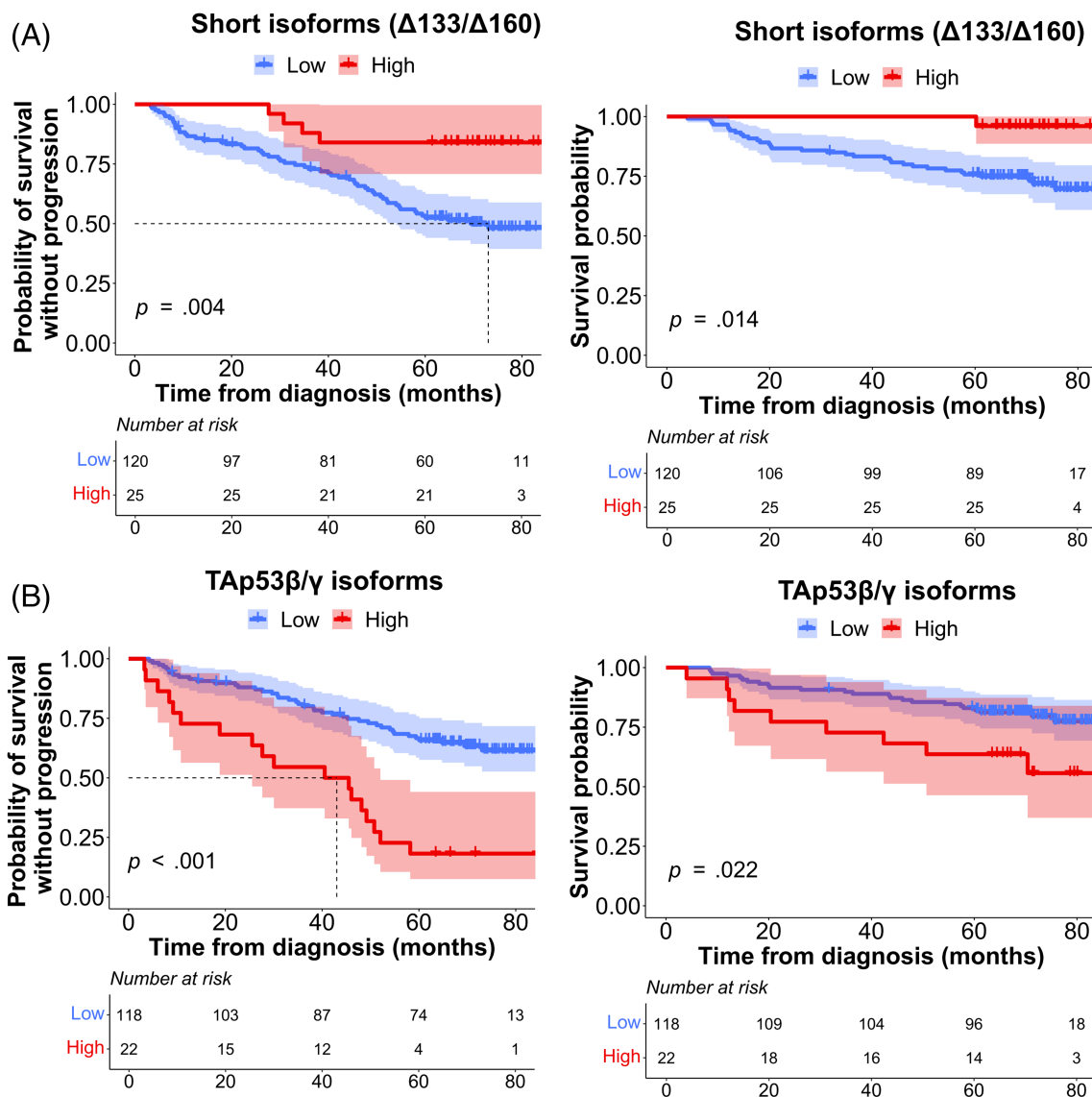


FIGURE 3 Probability of survival without progression and of overall survival of MM patients by level of p53 protein isoforms. (A) TTP and OS probabilities in patients with high levels of short isoforms. (B) TTP and OS probability according to the expression of TAp53 β/γ isoforms. The log-rank (Mantel-Cox) test p values are shown. MM, multiple myeloma; OS, overall survival; TA, transactivation domain; TTP, survival without progression [Color figure can be viewed at wileyonlinelibrary.com]

respectively). High-risk cytogenetics and the age at diagnosis retained their independent value in predicting adverse OS (Figure 4C). The analysis of the contribution of the variables showed that high-risk cytogenetics explained 28% and 44% of the variation in TTP and OS, respectively. Significantly, the expression levels of TAp53 β/γ isoforms explained 41% of the variation in TTP (Figure S5C).

3.4 | Expression patterns of p53 isoforms at the mRNA level in MM

mRNA was available from 109 of the 156 MM samples. We quantified the p53 isoforms by qRT-PCR using a well-established approach,²³ selecting the p53 mRNA variants that were homologous with those identified at the protein level by CNIA, that is to say, the α , β , short

($\Delta 133/\Delta 160$), and long (TA/ $\Delta 40$) isoforms (Figure S6A,B). The main limitation of this approach is the difficulty to quantify each specific isoform individually.²³ Therefore, our analysis of mRNA isoforms was limited to identifying the group of six long isoforms and the three TA isoforms were not distinguished. We also measured the expression of the TP53 gene, detected at a region of mRNA common to all isoforms. Samples with a Ct value ≥ 35 were considered as not expressed.

The TP53 gene and α isoforms were expressed in all the 109 MM samples. The long isoforms were only absent from one MM sample, while the short isoforms were the least frequently expressed, only in 72/109 (67%) MM samples, similar to the p53 protein isoforms levels (Figure S6C). We wondered whether the low expression of the short isoform detected in our cohort could be corroborated in another set of MM patients. We took advantage of the availability of RNA-Seq data from 780 MM patients included in the MMRF CoMMpass trial

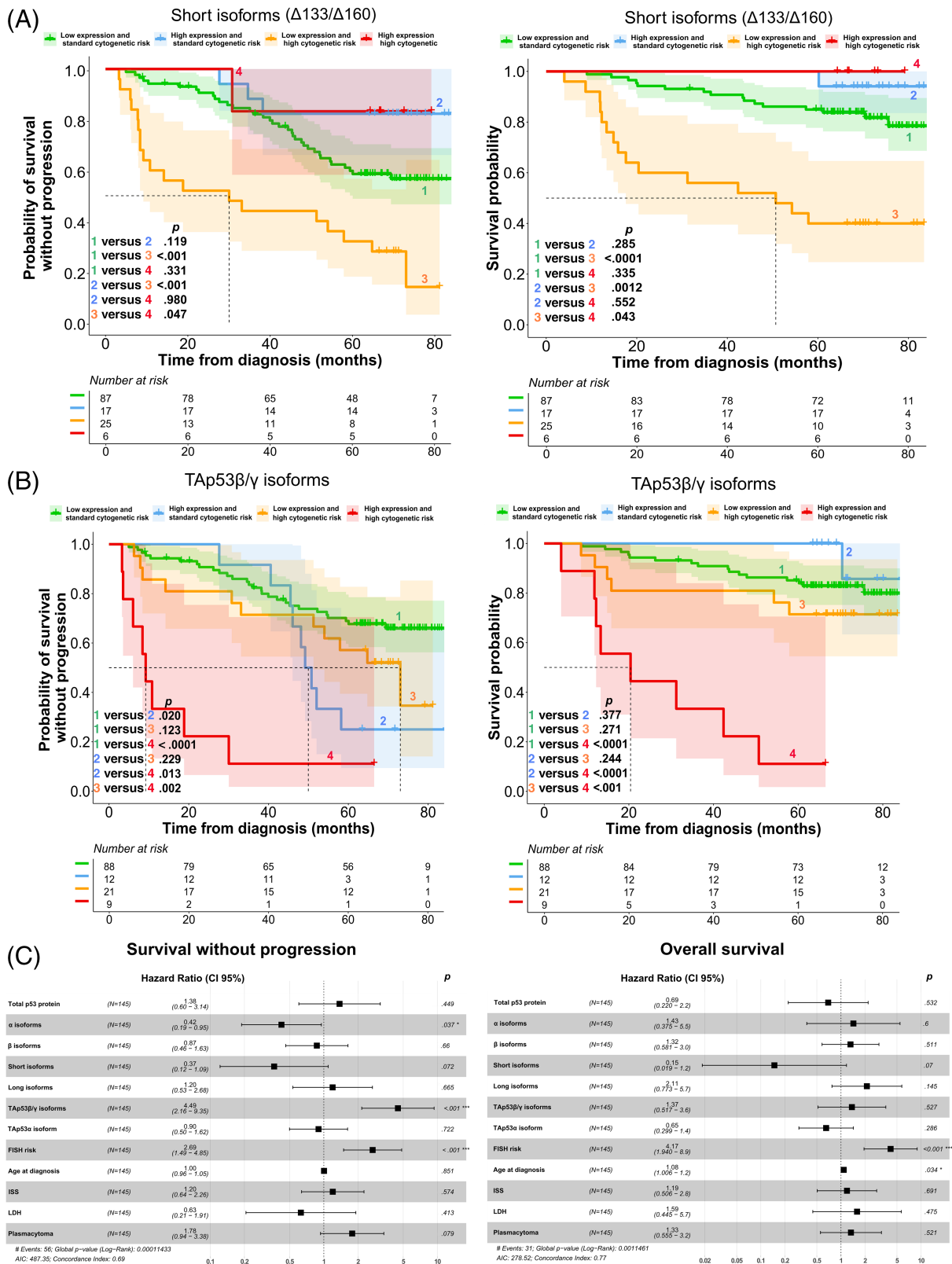


FIGURE 4 Probability of survival without progression and of overall survival of MM patients by cytogenetic risk and level of p53 protein isoforms, simultaneously. Analysis of MM patients with standard and high cytogenetic risk in combination with high and low expression levels of short (A) and TAp53 β/γ (B) protein isoforms. The log-rank (Mantel–Cox) test p values are shown. (C) Forest plot of multivariate models with probabilities for each factor associated with TTP and OS of patients, based on the expression level of the studied p53 protein isoforms and age at diagnosis (years), ISS III versus I/II, high-level LDH, plasmacytoma and high versus standard cytogenetic risk ($N = 145$). 95% Confidence intervals are indicated in parentheses. MM samples with missing values were excluded from the model. ISS, International Staging System; LDH, lactate dehydrogenase; MM, multiple myeloma; TA, transactivation domain; TTP, survival without progression [Color figure can be viewed at wileyonlinelibrary.com]

(NCT01454297) to address this question. Even using a different approach to quantify mRNA, such as RNA-Seq, we observed that the number of MM patients expressing short mRNA isoforms was significantly lower than that expressing long mRNA isoforms (Figure S6D).

The univariate survival analysis using qRT-PCR data from mRNA revealed similar results to those observed at the protein level. MM patients with high levels of short isoform expression had statistically significant longer TTP and OS (median not reached) compared with patients with lower levels (HR = 0.50, $p = .021$ and HR = 0.31, $p = .005$, respectively) (Figure S6E). However, unlike p53 protein, the low levels of the *TP53* gene were associated with a shorter OS (HR = 0.27, $p < .001$) but had no impact on the TTP of MM patients (Figure S6F). Nevertheless, the expression of the α and β isoforms did not affect the survival of patients, similarly to what was noted at the protein level.

4 | DISCUSSION

We have investigated for the first time the expression of p53 isoforms at the protein and mRNA levels and analyzed their putative impact on the outcome of a large cohort of homogeneously treated MM patients. Currently, most of the information regarding expression of p53 isoforms in tumors is based on mRNA levels quantified by reverse transcription PCR. In fact, the quantification of p53 isoforms using classical western blot assays would need a significant amount of protein, which is not available for most of the cases. Our previous implementation of the capillary nanoimmunoassay platform for protein quantification in the clinical setting^{18,20} allowed us to explore the hitherto unknown general landscape of p53 protein isoform expression in MM. Although this technology is highly effective analyzing low protein amounts, the genetic studies included in the evaluation of MM patients at diagnosis consume the entire sample in many cases.

We found total p53 protein to be expressed in more than 70% of MM samples, with the greater contribution of long than short isoforms, the latter being detected in very few samples. The TA protein isoforms were also expressed in most patients. Although qRT-PCR was able to detect expression of *TP53* gene in all the MM patients, and of p53 mRNA isoforms in the majority of cases, short mRNA isoforms were the least frequently expressed subclass, as observed at the protein level.

The short isoforms, also known as N-terminally truncated variants (the $\Delta 133$ and $\Delta 160$ variants), lack the first 132 amino acids, including a small part of the DBD. The integrity of the DBD is essential to the transcriptional factor functions of TAp53 α , through which it can bind to the p53 DNA consensus recognition element (RE) present in the promoters of its target genes.^{24,25} The absence of TAD1, TAD2, and a portion of the DBD from the short protein isoform structure entails the lack of sensitivity to proteasomal degradation and the absence of specific transactivation activity that characterize the TAp53 α isoform. The $\Delta 133$ p53 α isoform not only is defective in promoting apoptosis, but also acts dominant-negatively toward TAp53 α , inhibiting p53-mediated apoptosis.⁷ Some studies have shown abnormal expression of short mRNA isoforms in tumor cells relative to normal tissues. In addition, a negative impact of the short mRNA isoforms on the

outcome of oncological patients has also been reported.^{7,26–33} In contrast to these findings, we observed a significant positive influence on MM patient prognosis of a high level of expression of the short p53 isoforms, quantified at the protein or mRNA level. However, the impact of the short isoforms on prognosis differs depending on the tumor type. For example, the $\Delta 133$ p53 β expression has been associated with adverse prognosis in breast cancer,²⁷ while with better prognosis in mutant p53 ovarian cancer.^{28,31} Recently, the co-expression of the p53 isoforms has been shown to differentially regulate the p53-POL1-dependent DNA damage tolerance (DDT) pathway.³⁴ However, since only 12 MM patients with co-expression of TAp53 α and short isoforms were identified, we can speculate that, in this particular cancer, it is less likely that the short isoforms are affecting the DDT pathway. Accordingly, it can be said that there is little information about the quantification of short isoforms in hematological cancers, and their functions are not completely understood.

The TA isoforms (TAp53 α , TAp53 β , and TAp53 γ) possess the entire N-terminal domain, which contains the two independent transactivation domains (TAD1 and TAD2), but a different C-terminal. TAp53 α , which is the canonical p53 protein and the most abundant p53 isoform, contains the complete C-terminal region, including the oligomerization domain (aa 326–356) and the basic domain (aa 364–393), but the TAp53 β and TAp53 γ isoforms are C-terminally truncated variants. These structural differences determine their differential functions. Our cohort featured a higher level of expression of TAp53 α than of TAp53 β/γ proteins. We also noted a negative impact of the high level of expression of TAp53 β/γ proteins on the survival of MM patients. These data are consistent with another study of acute myeloid leukemia patients in which high levels of TAp53 α and low levels of TAp53 β/γ were associated with a greater sensitivity to valproic acid treatment.³⁵ Elevated TAp53 γ mRNA levels have also been associated with reduced progression-free survival in uterine serous carcinoma.³⁶ However, opposite results showing an association between low levels of TAp53 β and TAp53 γ mRNAs and worse prognosis have also been reported.^{14,37} In more than 10 years of intensive research, several studies have displayed the differential and aberrant expression of C-terminally truncated variants in various cancers. Nonetheless, their properties remain controversial, as shown in several apparently contradictory reports^{7,14–16,26,38–40} that do not provide enough and clear evidence for defining the TAp53 β/γ functions. For example, a significant increase in p21 and BAX promoter activities was found to be enhanced by overexpression of the TAp53 α isoform, but not by the overexpression of TAp53 β and TAp53 γ .⁴¹ Moreover, an *in vivo* study revealed that the TAp53 β and TAp53 γ isoforms significantly increased the tumor growth of H1299 cells.⁴² Although the discrepancies among these studies cannot easily be explained, it seems most plausible that the functions of the TAp53 β and TAp53 γ isoforms are dependent on the cellular and tissue context.

It was of particular note that considering the expression levels of p53 isoforms made it possible to refine the prognostic significance of the cytogenetic-risk classification. Thus, high-cytogenetic-risk patients who expressed low levels of short p53 isoforms or high levels of TA/long p53 isoforms had shorter survival than expected, while the survival of the group of high-cytogenetic-risk patients expressing high levels of short

p53 isoforms or low levels of TA/long p53 isoforms was comparable to that of patients with standard cytogenetic risk. These results were also observed when each of the high-risk cytogenetic alterations was analyzed in conjunction with the expression of p53 protein isoforms.

In conclusion, short and TAp53 β/γ protein isoform expression is associated with the clinical outcome of MM patients, and the prognostic stratification of MM patients is notably improved when cytogenetic risk is combined with their expression levels. These novel findings broaden the spectrum of the known actions of the p53 protein affecting MM outcome beyond the well-known unfavorable prognosis of deletions and/or mutations of the *TP53* gene.

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CONFLICT OF INTERESTS

Dr García-Sanz reports receiving personal fees from Amgen, Janssen, Takeda, and Pfizer, and receives honoraria from Pharmacyclics, research funding from Hospira, and travel accommodation from Celgene, unconnected with the submitted work. Dr Martínez-López has consulting and advisory roles with Novartis, Celgene, Janssen, and Bristol-Myers Squibb, receives speakers' bureau compensation from Novartis, Celgene, Janssen, and Bristol-Myers Squibb, and receives research funding from Novartis, Celgene, Janssen, and Bristol-Myers Squibb (institutional). Dr Oriol has consulting and advisory roles with Amgen and Janssen-Cilag, Amgen, and receives research funding from Janssen (institutional). Dr Bladé receives honoraria from Celgene, Janssen, and Amgen. Dr Lahuerta has consulting and advisory roles with Janssen-Cilag and Celgene. Dr San Miguel has consulting and advisory roles with Amgen, Bristol-Myers Squibb, Celgene, Janssen, MSD, Novartis, Takeda, and Roche. Dr Rosiñol receives honoraria from Janssen, Celgene, Amgen, Takeda, Sanofi, GSK, and Karyofarm. Dr Mateos receives honoraria and speakers' bureau compensation from Janssen and Celgene, Onyx, Takeda, Novartis, and Bristol-Myers Squibb, unconnected with the submitted work. Dr Gutiérrez receives honoraria from Janssen unconnected with the submitted work. The other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Elizabeta A. Rojas conceived the idea and designed the study, developed, and performed qRT-PCR and CNIA experiments, analyzed data, prepared the figures and wrote the manuscript; Luis A. Corchete analyzed the clinical data, supervised the statistical analysis and participated in the preparation of figures; Cristina De Ramón performed next-generation sequencing experiments, analyzed the clinical data, and participated in the preparation of tables; Patryk Krzeminski carried out the nucleofection assays for generation of positive controls; Dalia Quwaider assisted with laboratory experiments; Ramón García-Sanz, Joaquín Martínez-López, Albert Oriol, Laura Rosiñol, Joan Bladé, Juan José Lahuerta, Jesús F. San Miguel, Marcos González, María Victoria Mateos, and

Norma C. Gutiérrez provided patient samples and clinical data and were responsible for obtaining informed consent from patients. Jean-Christophe Bourdon provided antibodies, contributed to the data interpretation, and provided support with conceptual issues; Irena Misiewicz-Krzeminska designed the study, developed and performed CNIA experiments, analyzed protein data, prepared figures, and supervised the whole study; Norma C. Gutiérrez conceived the idea and designed the study, participated in the writing of the manuscript, supervised the whole study, and provided funding. All authors reviewed critically and approved the manuscript.

DATA AVAILABILITY STATEMENT

All data generated during this study are included in this published article and its supplementary information files. The raw data analyzed during the current study available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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