A novel nano-immunoassay method for quantification of proteins from CD138-purified myeloma cells: biological and clinical utility

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Haematologica 2018
Volume 103(5):880-889

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Received: September 27, 2017.
Accepted: January 31, 2018.
Pre-published: March 15, 2018.

doi:10.3324/haematol.2017.181628

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/5/880

ABSTRACT

Protein analysis in bone marrow samples from patients with multiple myeloma has been limited by the low concentration of proteins obtained after CD138+ cell selection. A novel approach based on capillary nano-immunoassay could make it possible to quantify dozens of proteins from each myeloma sample in an automated manner. Here we present a method for the accurate and robust quantification of the expression of multiple proteins extracted from CD138-purified multiple myeloma samples frozen in RLT Plus buffer, which is commonly used for nucleic acid preservation and isolation. Additionally, the biological and clinical value of this analysis for a panel of 12 proteins essential to the pathogenesis of multiple myeloma was evaluated in 63 patients with newly diagnosed multiple myeloma. The analysis of the prognostic impact of *CRBN*/Cereblon and *IKZF1*/Ikaros mRNA/protein showed that only the protein levels were able to predict progression-free survival of patients; mRNA levels were not associated with prognosis. Interestingly, high levels of Cereblon and Ikaros proteins were associated with longer progression-free survival only in patients who received immunomodulatory drugs and not in those treated with other drugs. In conclusion, the capillary nano-immunoassay platform provides a novel opportunity for automated quantification of the expression of more than 20 proteins in CD138+ primary multiple myeloma samples.

Introduction

Genomics has come to dominate biomedical research in recent years. For example, high-throughput genomic technologies have been used for the comprehensive analysis of multiple myeloma (MM). In particular, gene expression profiling has enabled the molecular classification of MM, which is widely used in biological research.[1] However, a knowledge of protein expression is essential for identifying therapeutic targets, since proteins are the molecules through which most new drugs achieve their efficacy. The limited amount of sample remaining after plasma cell purification means that messenger RNA (mRNA) quantification is still used as an indirect measure of protein expression in most cases. However, several studies have shown that protein levels cannot be predicted from mRNA measurements.[2]

Immunohistochemistry and flow cytometry have been used to analyze expression at the protein level, although to a limited extent. These methods are of great value and are of proven clinical utility, but they have some limitations that make them less useful for studying intracellular protein levels. They mostly use directly

marked antibodies that reduce the sensitivity of detection, and fewer antibodies are available for these techniques, even when used in an indirect assay.[3] Immunohistochemistry allows only semiquantitative analysis of protein expression, and requires a well-trained pathologist to interpret the results. Moreover, neither technique is able to identify non-specific antibody binding to other proteins.[3]

Western blotting (WB) remains the "gold standard" technique for protein characterization in most laboratories. However, WB consumes large quantities of reagents, has a low throughput, and requires a great deal of time and effort involving many laborious manual processing steps. Moreover, WB only yields semiquantitative data of poor repeatability, making it a challenge to go beyond using the assay in discovery research to apply it reliably in the clinical setting.[4-6] A further drawback is that it is not always possible to obtain the quantity of protein extract required for WB from primary cancer samples.

MM is a clear prototype of a bone marrow-infiltrating tumor for which a relatively small quantity of sample is available after the diagnostic procedure, which involves morphological evaluation, immunophenotypic characterization by flow cytometry, and CD138+ plasma cell separation for routine fluorescence *in situ* hybridization analysis. The recent development of a method based on the combination of capillary nano-electrophoresis with immunoassay (CNIA), also known as 'simple western', requires only very small amounts of sample to be able to measure protein expression.[3,7] This technical advance makes it possible to analyze the expression of 50-100 proteins in a single MM sample. Here we present the results of a pilot study using this platform in MM patients. The main goal was to quantify accurately and robustly the proteins extracted from CD138-purified MM samples frozen in RLT Plus buffer, which is commonly used as a method for RNA and DNA preservation. Additionally, we attempted to establish the clinical value of this analysis using a panel of proteins essential to MM pathogenesis, comparing it with that of the corresponding RNA expression.

Methods

For more specific information see the *Online Supplementary File.*

Patients and multiple myeloma cell lines

Sixty-three samples from patients diagnosed with MM between October 2013 and November 2015 were included in the study (*Online Supplementary Table S1*). Forty-three had been enrolled in two Spanish Myeloma Group clinical trials: GEM2010 [bortezomib/melphalan/prednisone and lenalidomide/dexamethasone in a sequential or alternating manner; (n=24)] and BenVelPres [bendamustine/bortezomib/prednisone; (n=19)]. The other 20 patients were not treated as part of a clinical trial.

The impact of RNA and protein expression on patients' survival was evaluated only in the group of patients that took part in the clinical trials (*Online Supplementary Table S1*). The scheme of the study is presented in Figure 1.

Protein extraction from RLT Plus buffer

Proteins were extracted by ice-cold acetone precipitation from RNA-column flow-through liquid. To increase the rate of protein precipitation 10 mM NaCl was added to the acetone at 80% (v/v). For technical reasons, each sample was divided into two tubes and extracted separately. After overnight incubation at -20°C, the proteins were centrifuged at 13,000 x g for 30 min at 4°C, and washed twice with 70% ice-cold ethanol followed by centrifugation for a further 10 min. The protein precipitate was dried at 39°C and dissolved with 50 µL 0.2 M NaOH for 10 min at room temperature and 4x WB sample buffer for at least 15 min at room temperature. Samples were then denatured at 95°C for 5 min, cooled to room temperature and stored in aliquots at -80°C. Before any assay, samples were heated to room temperature, then kept at 37°C for 30 min in order to re-dissolve any protein that had precipitated during freezing.

Capillary electrophoresis immunoassay

Capillary electrophoresis immunoassay or simple western analysis was performed using the WES™ machine (ProteinSimple, San Jose, CA, USA) in accordance with the manufacturer's protocols. The Total Protein Assay (ProteinSimple) was used to quantify the protein concentration. In brief, 5 µL of proteins were loaded on the plate, separated by size, labeled with a biotin reagent and detected by chemiluminescence using streptavidin-horseradish peroxidase. At the end of the run, the proportion of the protein of interest in the total protein in the sample was measured, in comparison to a standard curve previously generated using JJN3 cell line extracts of known protein concentrations.

Primary antibodies used in the study and the optimized conditions for each antibody are presented in Table 1. Data were analyzed using Compass™ software. Each protein peak was measured automatically and normalized with respect to the GAPDH median area under the peak. Expression of each protein is presented as its abundance relative to GAPDH.

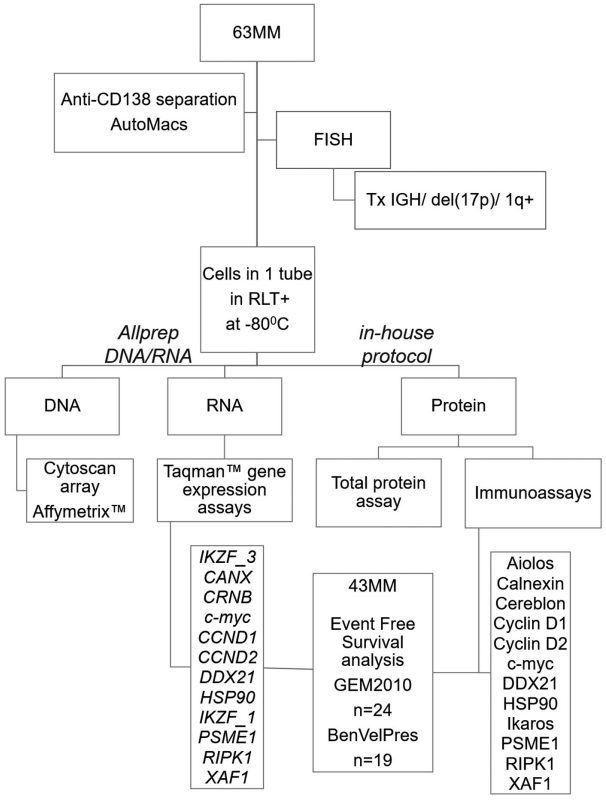

Figure 1. Scheme of the study.

DNA/RNA extraction and quantitative real-time polymerase chain reaction analysis

mRNA expression was evaluated by Taqman assay quantitative real-time polymerase chain reaction (qRT-PCR) analysis using the respective GAPDH Taqman assay as a control, by the $2^{-\Delta Ct}$ method.

Statistical analysis

Spearman correlations were calculated. Progression-free survival (PFS) was calculated for each gene and protein. Survival curves were plotted using the Kaplan–Meier method and statistical significance was evaluated with the log-rank test.

Results

Protein extraction from RLT Plus buffer results in optimal quality and quantity

Firstly, we evaluated the amount and quality of the protein extracted with our protocol. The data generated by the WES™ system were visualized as virtual blots (Figure 2A) or peaks (Figure 2B) that were quantified as the area under the curve using the inbuilt algorithm of the Compass™ software. Using JJN3 myeloma cell line lysates of known concentration, we generated a protein standard curve that proved to be linear over the evaluated range of concentrations (Figure 2C). The amount of protein obtained from each sample ranged between 0.00 and 0.36 mg protein, with a median quantity of 0.06 mg per sample. Three of the 63 samples had insufficient material to analyze protein expression (Figure 2D). We compared the expression of the various proteins extracted from MM cells stored in RLT Plus buffer with that obtained using the standard RIPA protocol and found the signals to be similar for the two protocols (Figure 2E,F).

Optimization of protein quantification by capillary nano-electrophoresis with immunoassay

For each analyzed protein, we first searched in the ProteinSimple antibody database for the optimized conditions (http://www.proteinsimple.com/antibody/antibodies.html). If the antibody was present, we re-evaluated it in our system, using the antibody at the indicated concentration and at double and half the indicated concentration. In the event that the protein evaluated was not present in the database, we performed a full optimization, which consisted of running the assays in the cell line samples at two concentrations (0.1 mg/mL and 0.2 mg/mL) with at least five antibody dilutions in order to determine the optimal concentration at which the antigen-antibody binding was saturated and no change in antibody concentration influenced the result. The optimized concentrations for each antibody, the molecular weights at which the peaks were observed, and the coefficients of variation arising from the validation of each protein are shown in Table 1.

Standard curves were produced for each protein to evaluate the range of linearity over which the expression of each protein could be quantified. Briefly, each capillary contained the sample at a different dilution, and the protein detection was visualized as virtual blots, as exemplified by the use of Aiolos in Figure 3A. The peaks obtained for each dilution, which were obtained automatically by the program, have a distinct height and width, depending on the sample dilution (Figure 3B). Once they had been quantified the standard curve was generated (Figure 3C). After protein quantification, we compared the value obtained for each sample and each protein with the respective standard curve to ensure correct measurement. The limit of quantitation was set as signal-to-noise ratio of 10:1 in accordance with the guidelines from the European Directorate for the Quality of Medicine set out in the European Union Pharmacopoeia.[8]

The results of Aiolos quantification in six samples are shown in Figure 3D, in which virtual blots for both Aiolos and GAPDH are presented.

Analysis of mRNA and protein expression

We analyzed the expression of 12 genes and their encoded proteins, together with GAPDH as a control (Figure 1). We decided to select proteins involved in MM or cancer pathogenesis: Cyclin D1 and Cyclin D2, whose overexpression is a unifying event for most MM;[9–11] c-myc, which is consistently found to be involved in the transformation of monoclonal gammopathy of undetermined significance into MM;[12,13] HSP90, which is upregulated in

Table 1. Summary of proteins and antibodies used in the study.

	Target protein	Company	Cat number	Species	Clonality	Dilution Ab	MW peak	Intra-assay CV (%)
1	Aiolos	Cell Signaling	12720	Rabbit	Polyclonal	1:100	85	10.9
2	Calnexin	Enzolifesciences	ADI-SPA-860	Rabbit	Polyclonal	1:250	119	4.9
3	Cereblon	NovusBio	NBP1-91810	Rabbit	Polyclonal	1:80	59	9.7
4	c-myc	Cell Signalling	5605	Rabbit	Monoclonal	1:50	75	8.3
5	Cyclin D1	Abcam	Ab134175	Rabbit	Monoclonal	1:50	40	9.9
6	Cyclin D2	Cell Signaling	3741	Rabbit	Monoclonal	1:100	38	7.8
7	DDX21	Abcam	Ab182156	Rabbit	Monoclonal	1:100	110	11.4
8	HSP90	Cell Signaling	4877	Rabbit	Monoclonal	1:50	95	7.0
9	Ikaros	Santa Cruz	Sc-13039	Rabbit	Polyclonal	1:50	70	10.8
10	PSME1	NovusBio	NBP1-83121	Rabbit	Polyclonal	1:50	37	10.2
11	RIPK1	Cell Signaling	3493	Rabbit	Monoclonal	1:50	79	5.8
12	XAF1	Cell Signaling	13805	Rabbit	Monoclonal	1:25	46, 110	15.5
13	GAPDH	Cell Signaling	2118	Rabbit	Monoclonal	1:50	42	8.6

Ab: antibody; MW: molecular weight; CV: coefficient of variation.

many solid and hematologic malignancies, including MM;[14] Calnexin, which forms endoplasmic reticulum and is upregulated in MM relative to normal plasma cells in genetically identical twin samples;[15] and DDX21 or RIPK1, with known involvement in several tumors.[16–18] In addition, proteins involved in the mechanism of action of antimyeloma drugs were included: Cereblon, Ikaros, Aiolos for immunomodulatory drugs; XAF1 for melphalan; and PSME1 for bortezomib.[19–22]

At the protein level, PSME1 and Calnexin showed the highest median level of expression, while HSP90 was the most strongly expressed mRNA (Figure 4A,B). Conversely, proteins Cyclin D1, Cyclin D2 and c-myc had the lowest median level of expression, and *CRBN*, *RIPK1* and *XAF1* were the least expressed mRNA.

In general, the expression of mRNA was more homogenous than that of proteins, as indicated by the higher coefficients of variation for the latter (Figure 4C). In fact, the coefficients of variation were significantly lower than those for *c-myc*, *DDX21*, *HSP90*, *IKZF1* and *PSME1* mRNA than for their respective encoded proteins. The highest variability in expression, both at the mRNA and protein levels, was observed for *CCND2*/Cyclin D2 and *CCND1*/Cyclin D1, as well as for c-myc protein.

Next, we analyzed the correlation between the two levels of gene expression, mRNA and protein. Interestingly,

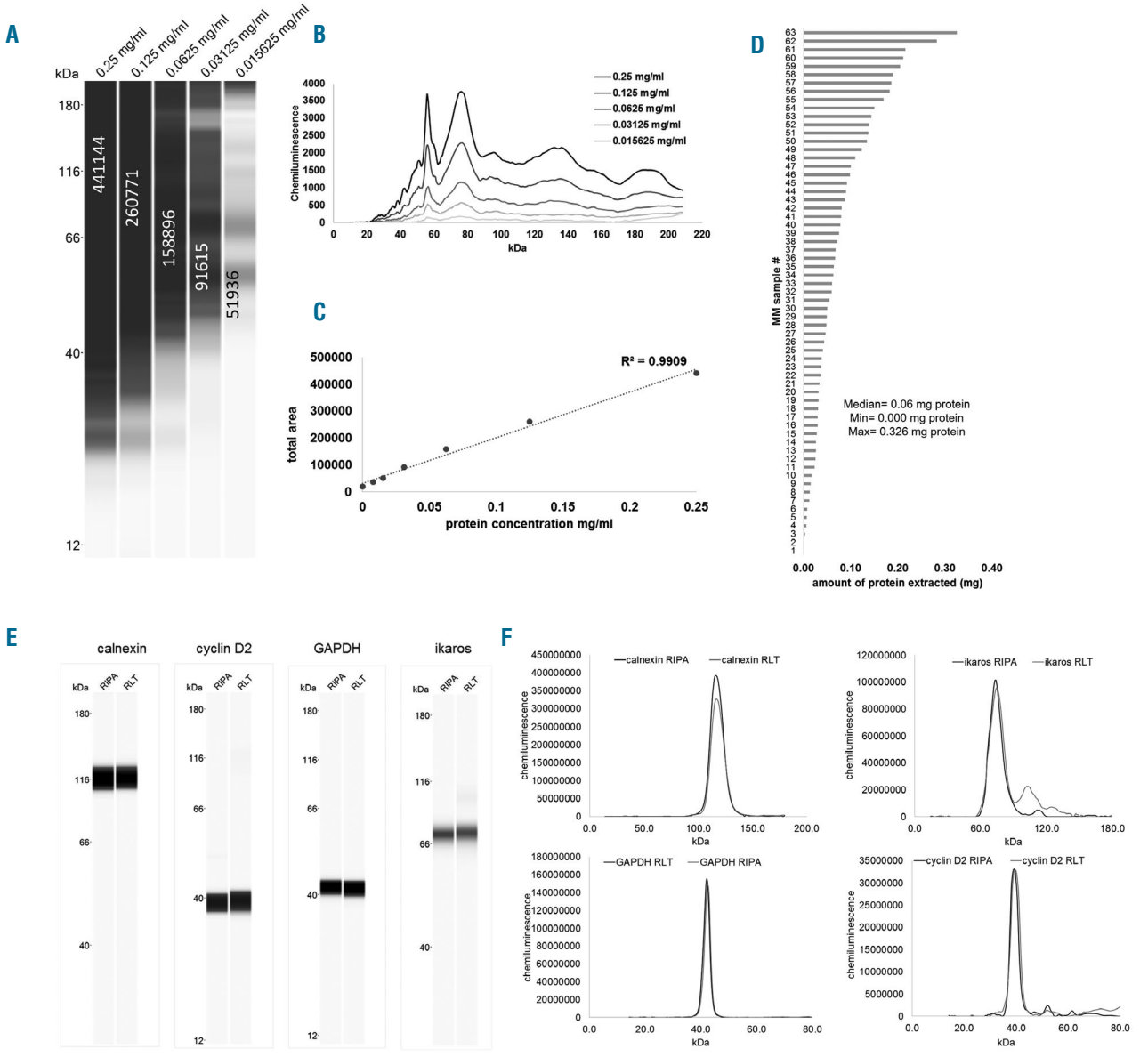

Figure 2. Optimization of protein extraction from RLT Plus samples. Due to the various additives in the sample buffer there is marked incompatibility with most of the normally used protein quantification methods. The Total Protein assay was therefore used, as it is insensitive to high SDS concentrations. The standard curve was generated using JJN3 cell line extracts at 0.25 mg/mL concentration, and serial dilutions thereof. Each capillary contained one sample of a known concentration. Results were visualized as virtual gels (A) and the numbers correspond to the areas under the curves of the peaks (B). A standard curve was generated, plotting the result for each capillary quantification (C). Amount of protein obtained from each sample (D). Comparison of results from Calnexin, Cyclin D2, GAPDH and Ikaros quantification in the U266 cell line, from which protein extracts were obtained by RLT Plus and RIPA extraction, and visualized as virtual blots or two distinct dilutions of the sample (E), and one dilution extracted with both protocols visualized as peaks (F).

only Cyclin D1 and Cyclin D2 protein levels were strongly correlated with the respective *CCND1* and *CCND2* mRNA levels (Figure 4D). We observed a modest correlation for Aiolos, Calnexin and DDX21 proteins with their respective mRNA.

Although the number of proteins analyzed was limited, we examined the correlation between the levels of the different proteins. A positive correlation was observed between most of the protein pairs (*Online Supplementary Figure S2*). We confirmed the previously described relationship between Ikaros/Aiolos and c-myc.[19] Additionally, c-myc protein expression was positively correlated with Cereblon, Calnexin, and RIPK1, and negatively correlated with DDX21 (*Online Supplementary Figure S2*). We found that protein levels of Cereblon, Ikaros and Aiolos, all of which are required for the activity of immunomodulatory drugs, were correlated with each other (*Online Supplementary Figure S2*).

The potential association between the expression of proteins and mRNA tested in the study and chromosomal abnormalities was also explored. We confirmed the well-established pattern of *CCND1*/Cyclin D1 and *CCND2*/Cyclin D2 expression in t(11;14) and t(4;14) (*Online Supplementary Figure S3*). A lower level of expression of PSME1 and RIPK1 proteins in MM with 1q gains, and a higher level of *IKZF1* mRNA expression in MM with t(11;14) were also observed.

Influence of mRNA and protein levels on survival of myeloma patients

Since clinical data were available for 43 MM patients, 24 enrolled in GEM 2010 and 19 in BenVelPres clinical trials, we also performed survival analysis for proteins and mRNA using PFS as the endpoint (Table 2). Cereblon and Ikaros were the only proteins able to predict PFS. Interestingly, mRNA levels of *CRBN* and *IKZF1* were not associated with prognosis (Figure 5). Accordingly, patients with a high level of Cereblon protein had a longer PFS than those with a low level (50.4 *versus* 16.3 months, $P<0.001$). Similarly, high levels of Ikaros protein were associated with longer PFS (45.1 *versus* 17.8 months, $P<0.01$). The levels of two mRNA were associated with longer PFS: a high level of *PSME1* (50.4 *versus* 23.5 months, $P<0.05$) and a low level of *XAF1* (20.3 *versus* 45.1 months, $P<0.05$).

Since Cereblon, Ikaros and Aiolos are involved in the mechanism of action of immunomodulatory drugs, and only GEM2010 patients were treated with lenalidomide, we examined whether the prognostic value of these proteins was influenced by the type of treatment. Indeed, high levels of Cereblon and Ikaros were both associated with longer PFS only in patients who received immunomodulatory drugs and not in those treated with other drugs (Figure 6).

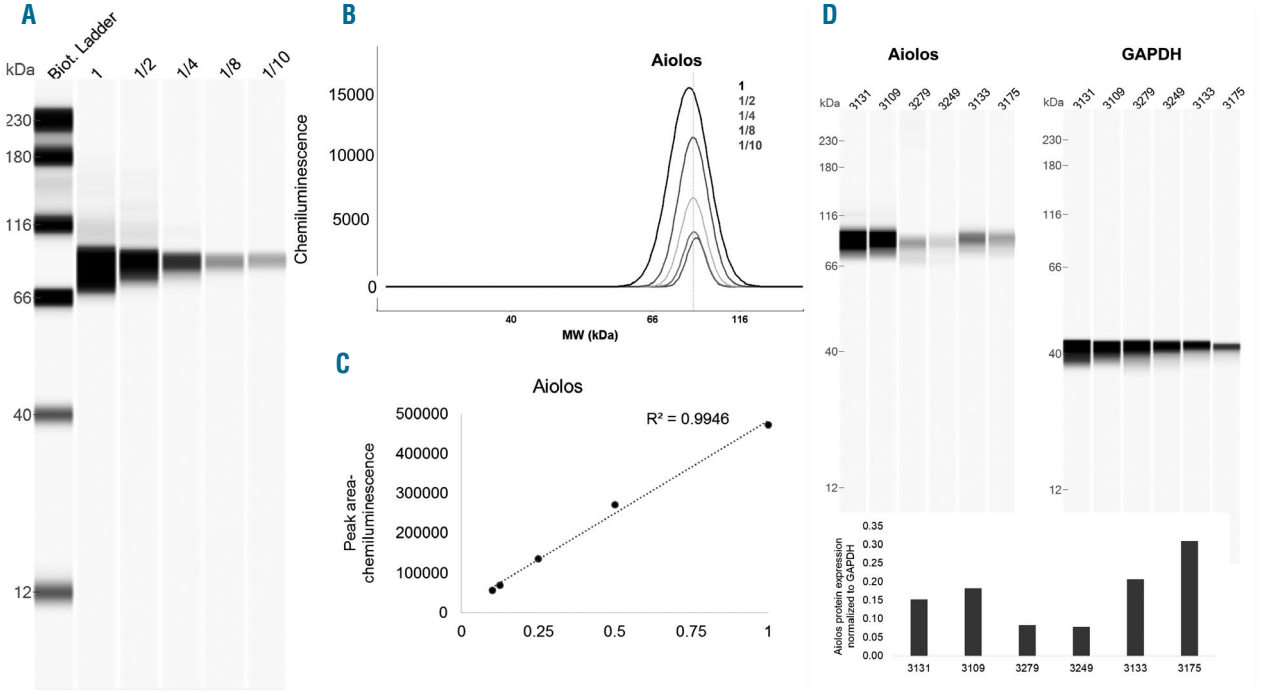

Figure 3. Optimization of protein expression quantification. For each protein the standard curve was generated using the sample with the strongest signal to prepare the serial dilutions. Each dilution was run in a separate capillary. The sample result for Aiolos is visualized as a virtual blot (A) or as peaks (B). The standard curve established the linear range for each protein (C). The sample result for Aiolos together with the respective GAPDH for each sample was run in separate capillaries (D). Each peak area was quantified and Aiolos was normalized with respective to GAPDH, as shown in the example.

Discussion

MM has been comprehensively studied at the DNA and RNA levels using high-throughput technologies such as microarrays for detecting copy number abnormalities, and gene expression profiling, and, more recently, next-generation sequencing for DNA mutation analysis. MM samples after CD138+ separation are usually stored in buffers such as TRIZOL or RLT Plus, which preserve nucleic acids for subsequent use in genomic studies. While it is technically possible to extract proteins from these buffers,[23–25] the quantity of protein would not be sufficient for multiple WB to be carried out. In the classic WB, the amount of the purified plasma cells, even if all of it were available for the

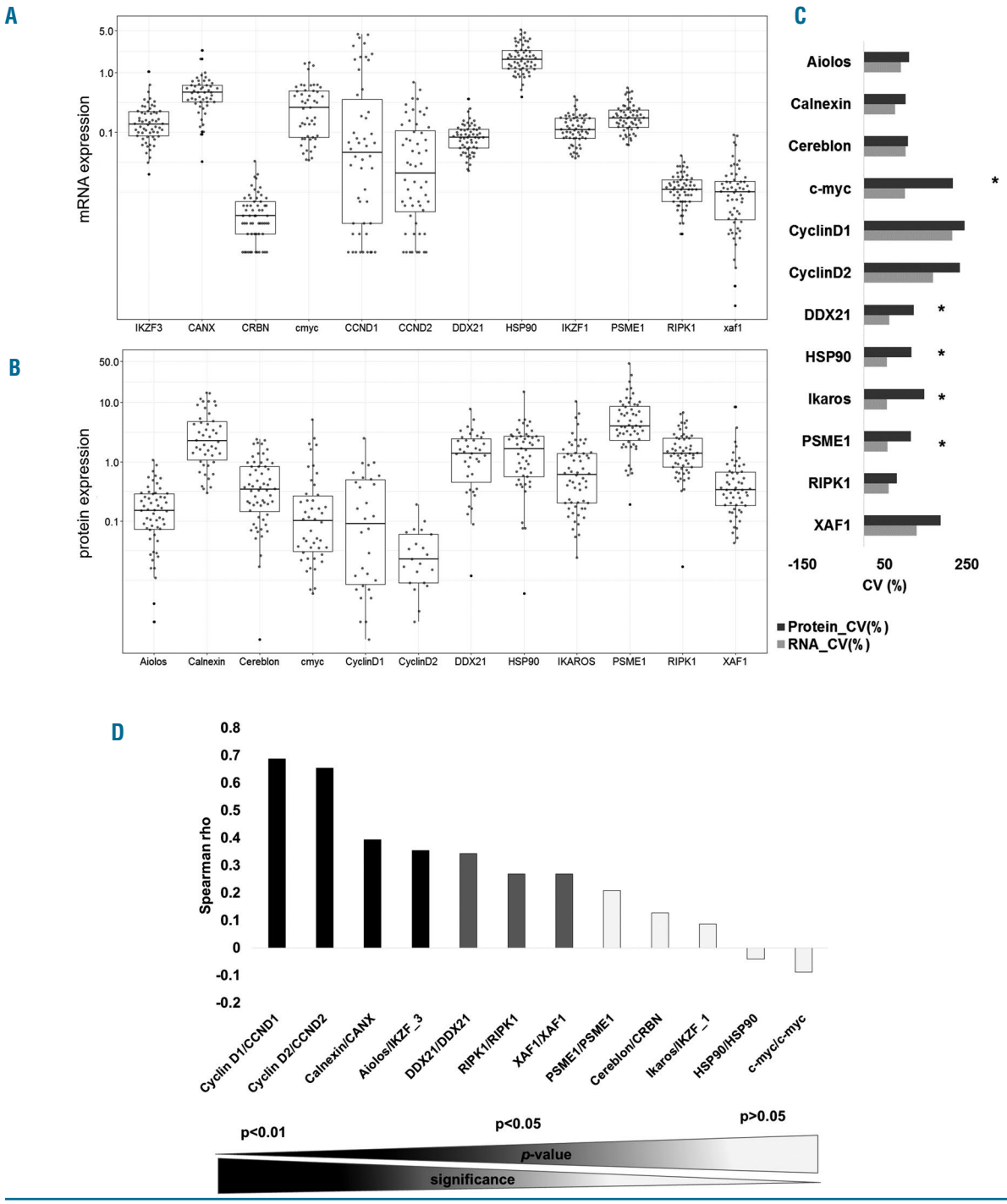

Figure 4. Two levels of analysis of each gene RNA and protein. mRNA expression of each gene was assessed by qRT-PCR and normalized relative to GAPDH and expressed as $2^{-\Delta Ct}$ (A). Abundance of each protein was assessed by CNIA and normalized relative to GAPDH abundance in each case (B). The Y axis of graphs (A) and (B) are expressed on a log scale. The variability of each mRNA and protein measurement in the analyzed population of patients with MM, measured as percentage coeffcient of variation (CV%). The threshold of statistical significance (*P<0.05) was determined as described in the Methods section (C). Spearman correlation coefficient for each mRNA/protein pair ranked by increasing P value (D).

Table 2. Univariate analysis of progression-free survival.

mRNA	HR (CI)	Group	n	Median PFS (months)	*P*-value	Protein	HR (CI)	Group	n	Median PFS (months)	*P*-value
IKZF3	1.84	H	10	21.7	0.19	Aiolos	0.51	H	33	45.1	0.16
	(0.74-4.6)	L	33	45.1			(0.19-1.34)	L	10	23.5	
CANX1	0.44	H	21	50.4	0.074	Calnexin	1.82	H	22	30	0.2
	(0.18-1.11)	L	22	23.5			(0.72-4.6)	L	18	43.8	
CRBN	0.49	H	10	50.4	0.24	Cereblon	0.23	H	31	50.4	**0.00034**
	(0.14-1.67)	L	33	29.8			(0.09-0.55)	L	12	16.3	
CCND1	2.55	H	27	29.8	0.066	CyclinD1	1.55	H	23	30	0.33
	(0.91-7.14)	L	16	NR			(0.64-3.78)	L	20	NR	
CCND2	0.38	H	17	NR	0.053	CyclinD2	0.24	H	11	NR	0.051
	(0.13-1.05)	L	26	29.8			(0.05-1.03)	L	32	29.8	
MYC	1.54	H	33	30	0.44	c-myc	0.63	H	27	45.1	0.31
	(0.51-4.64)	L	10	45.1			(0.26-1.56)	L	13	29.8	
DDX21	0.49	H	29	43.8	0.1	Ddx21	0.47	H	14	50.4	0.18
	(0.2-1.17)	L	14	23.5			(0.15-1.46)	L	23	30	
HSP90	0.52	H	24	50.4	0.14	Hsp90	1.5	H	12	29.8	0.44
	(0.22-1.25)	L	19	23.5			(0.53-4.27)	L	25	50.4	
IKZF1	1.93	H	25	29.8	0.15	Ikaros	0.31	H	32	45.1	**0.0075**
	(0.77-4.83)	L	18	43.8			(0.12-0.77)	L	11	17.8	
PSME1	0.33	H	18	50.4	**0.018**	Psme1	0.48	H	30	45.1	0.13
	(0.12-0.86)	L	25	23.5			(0.19-1.26)	L	10	16.3	
RIPK1	0.52	H	28	50.4	0.13	Ripk1	1.41	H	11	30	0.49
	(0.22-1.24)	L	15	23.5			(0.53-3.73)	L	29	45.1	
XAF1	2.39	H	11	20.3	**0.048**	Xaf1	1.56	H	15	30	0.34
	(0.98-5.8)	L	32	45.1			(0.63-3.85)	L	25	45.1	

HR: hazard ratio; CI: confidence interval; PFS: progression-free survival; H: high level; L: low level.

protein studies, would have allowed at most six proteins to be evaluated, the median amount of sample being sufficient to analyze two proteins.

Here we present for the first time a method for quantifying the expression of multiple proteins from myeloma cells stored in the buffer commonly used for nucleic acid preservation and isolation. We report a protocol for protein extraction from MM cells stored in RLT Plus buffer based on a well-known acetone precipitation procedure.[26,27] We decided to add NaCl to the sample before precipitation, since a greater inorganic salt content is known to improve protein yield.[28] After testing several types of salts and concentrations, we chose the one with the best performance. We also assessed several methods of protein pellet dissolution, finally settling for a 0.2 M NaOH and 4x WB sample buffer, since slight changes in the pH of the environment change protein solubility.[26,29]

In contrast to the classic WB, which provides only semi-quantitative (blot-based) results, CNIA quantifies the area under the curve of the signal in each capillary, enabling expression relative to the control protein to be calculated.[3,30] To determine whether the CNIA method is suitable for evaluating the expression of multiple proteins, using various antibodies, we tested the performance of each protein in the WES™ system. We first optimized the concentration of the antibody to be employed using RIPA-extracted proteins from MM cell lines, as suggested by the system provider. The antibody dilution used has to be the one that saturates the epitope-antibody binding, so that the additional increase in antibody concentration would not have caused the increase in the signal. Although the concentration of antibodies used by the CNIA platform is higher than that used in WB, lower amounts of antibody are required because of the small volume of antibody. Comparing the signal detected by each antibody when the sample was extracted by the RIPA method with that obtained using our protocol revealed no significant differences for any of the proteins evaluated, which supports the suitability of the present protocol for extracting proteins from RLT Plus buffer. We observed differences between the predicted molecular weight and that detected by the CNIA system for some proteins, regardless of the extraction method. The most probable explanation for this phenomenon is that migration depends on the mobility in the matrix.[31] In fact, each system provides a unique molecular weight value that depends on the particular interactions between the matrix and protein, and the true molecular weight can only be determined by mass spectrometry or sequencing.[32] In our assay, we analyzed only the data obtained from the antibodies that detected peaks whose signal intensity was linear in the serial dilutions, enabling the frequent biases in WB to be eliminated. Although such standard curves are not usually calculated as part of the WB method, this is highly recommendable.[33] Even though, at first glance, the optimization step required for each antibody seems laborious, one CNIA run allows 24 samples to be analyzed, so it would be possible to optimize, for example, four antibodies in 3 hours. Therefore, bearing in mind the subsequent possibility of quantifying protein abundances, it is not such a time-consuming process.

After demonstrating that our approach accurately quan-

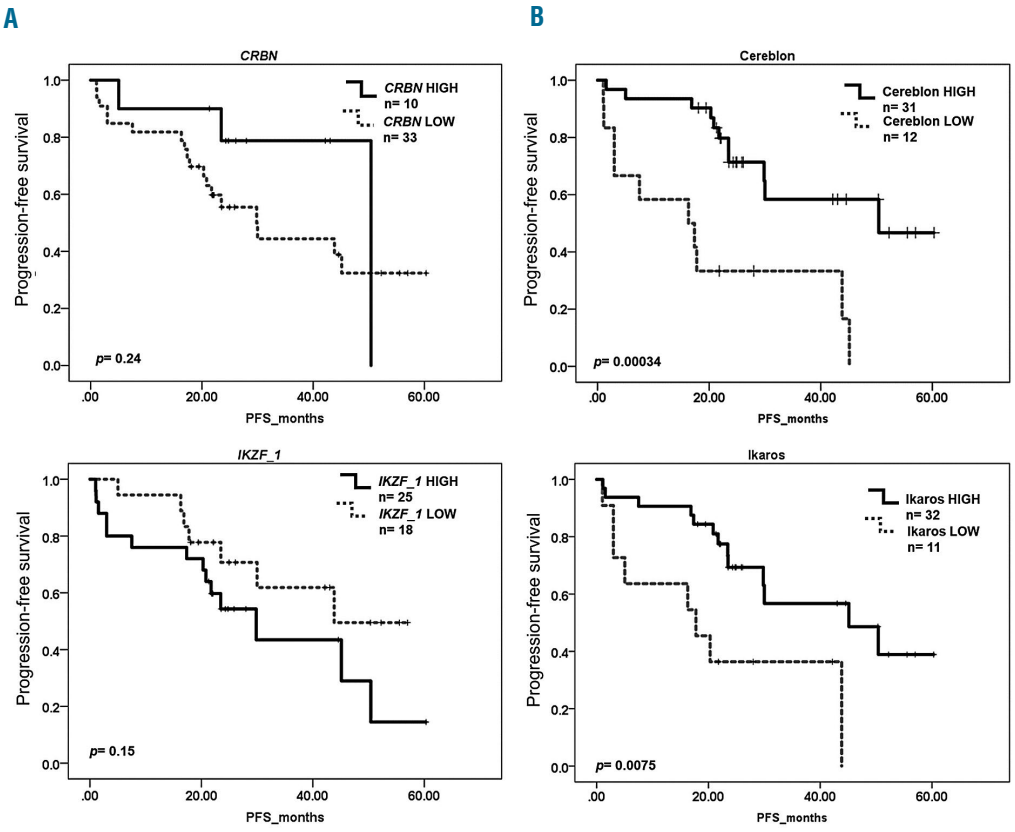

Figure 5. Progression-free survival according to levels of MRNA and protein expression. Progression-free survival in patients with low and high levels of mRNA (A) and protein (B) expression. The log-rank test was performed for each gene and protein and Kaplan-Meier curves represent the PFS of MM patients depending on mRNA and protein status. Cutoff Finder software (http://molpath.charite.de/cutoff) was used to obtain the optimal cutoff, which was defined as that producing the most significant split that discriminates between good and poor survival by examining all the possible cutoffs using the log-rank test.

tified the proteins extracted at the same time as the DNA and RNA from the RLT Plus buffer, we investigated the applicability of the method to the analysis of the expression of key proteins in MM biology, such as D Cyclins, c-myc, Cereblon, Ikaros, and Aiolos, among others. We also wanted to compare protein expression with the corresponding mRNA level, since many basic studies have revealed that only 30-40% of protein abundance can be explained by the mRNA level.[34] Our results showed a moderate or low correlation between mRNA and protein levels of expression, and are consistent with the general observation that ~60% of the variation in protein concentration cannot be explained by measuring mRNA alone.[34] We also observed that the mRNA level was less variable than protein expression among MM patients for all the mRNA and protein pairs analyzed. Indeed, protein abundance is regulated by a variety of complex mechanisms, such as post-transcriptional and post-translational modifications, and protein degradation regulation.[34,35] By measuring mRNA abundance, only the early steps in a long chain of regulatory events are considered.[36] However, the mRNA level is still often employed as a proxy for protein abundance, mostly because of the lack of appropriate technology to quantify proteins quickly and efficiently.

Our results reproduce the well-known pattern of *CCND1*/Cyclin D1 and *CCND2*/Cyclin D2 expression in MM with t(11;14) and t(4;14).[9] We also found a correlation between c-myc and Ikaros and Aiolos levels, analyzing either mRNA or protein expression, consistent with the previously demonstrated regulation of c-myc by Ikaros and Aiolos in MM.[19] Interestingly, the correlation between Ikaros and Aiolos levels was stronger for the protein than for the mRNA. To our knowledge, this is the first time that the protein levels of c-myc, Ikaros and Aiolos have been quantified and the relationship between their expressions analyzed in MM. In T-cell acute lymphoblastic leukemia, for example, the levels of mRNA encoding Ikaros and Aiolos were weakly, but significantly correlated.[37]

Among the proteins included in our study, we observed a significant association between protein level and PFS for Cereblon and Ikaros, while this association was not observed for the respective mRNA levels. Cereblon forms an E3 ubiquitin ligase complex together with the damaged DNA binding protein 1 (DDB1), Cullin4A (CUL4) and Roc1. Immunomodulatory drugs, such as lenalidomide or pomalidomide, bind to Cereblon in a region located at the C-terminus of this protein.[38,39] Our results did not demonstrate a correlation between Cereblon protein and mRNA level, and showed that only high levels of Cereblon protein were associated with a good prognosis in MM. These findings are concordant with those of previous studies and support the usage of protein expression to evaluate Cereblon levels.[40]

Several independent groups have identified Ikaros and Aiolos as the downstream targets of Cereblon after immunomodulatory drug activation.[41–43] The role of the level of Ikaros in MM survival is controversial. When Ikaros expression was investigated at the RNA level, a low level of mRNA *IKZF1* expression was associated with better prognosis in newly diagnosed patients treated with immunomodulatory drugs.[44] On the other hand, low *IKZF1* levels were found to predict a lack of responsiveness to immunomodulatory drugs and a shorter overall survival in refractory MM patients.[45] We observed that a high level of Ikaros protein was associated with longer PFS, while no significant impact on prognosis was observed when PFS was estimated from mRNA levels.

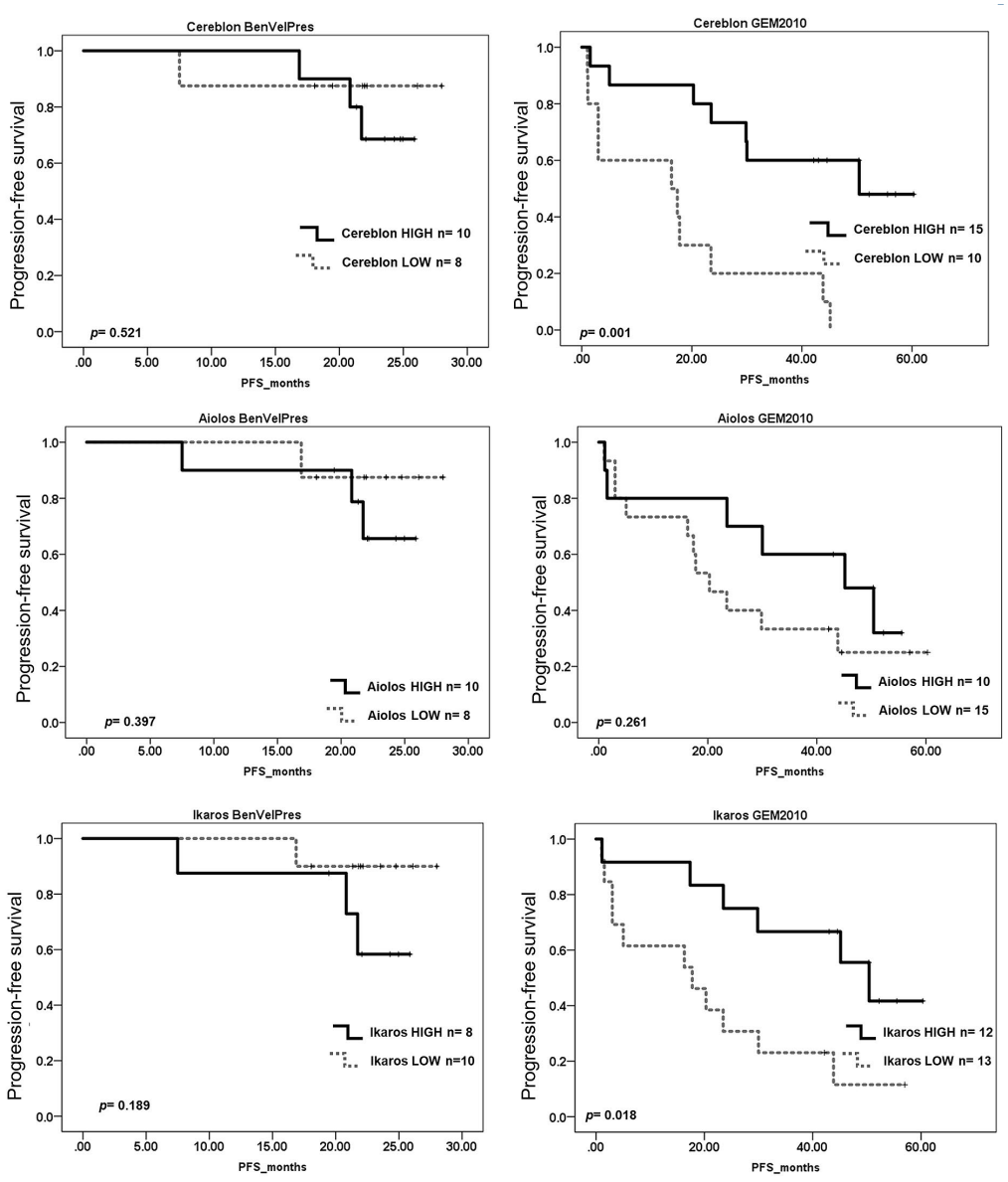

Figure 6. Progression-free survival in patients with low and high Cereblon, Aiolos and Ikaros protein levels, depending on the treatment scheme (only patients treated according to GEM2010 trial received lenalidomide). The log-rank test was performed for each protein and Kaplan-Meier curves represent progression-free survival of MM patients depending on protein status. Cutoff Finder software (http://molpath.charite.de/cutoff) was used to obtain the optimal cutoff, which was defined as that producing the most significant split that discriminates between good and poor survival by examining all the possible cutoffs using the log-rank test.

These results are consistent with the longer survival displayed by relapsed/refractory MM patients treated with lenalidomide who expressed high levels of IKZF1/3 protein, as evaluated by immunohistochemical staining.[46]

Although the number of patients analyzed in this study is relatively small, the survival analysis carried out dividing patients according to drug therapy showed that high levels of Cereblon and Ikaros proteins are associated with a longer PFS only in patients who receive immunomodulatory drugs and not in those who are treated with other drugs. Other studies reached the same conclusion that the level of Cereblon can predict the outcome of patients with MM mainly in those treated with immunomodulatory drugs.[47–50] By contrast, in the present series of MM patients, the level of Aiolos did not influence the outcome of the patients treated with immunomodulatory drugs.

In summary, we present the implementation of a novel technique based on capillary nano-immunoassay for quantifying protein expression in MM samples in the clinical setting. The requirement for only a relatively small amount of material means that, for the first time, more than 20 proteins can be analyzed using the same sample frozen for DNA and RNA analysis. This makes the CNIA platform a fast, effective and accurate tool for exploring the impact of different proteins on the survival of patients with MM and for investigating new protein biomarkers that could help to predict the response to new drugs that directly target specific proteins. These encouraging results require further validation in a larger cohort of patients with MM or other hematologic malignancies.

Acknowledgments

The authors thank Isabel Isidro, Teresa Prieto and Vanesa Gutierrez for their technical assistance.

Funding

This work was funded by a grant from the International Myeloma Foundation's Black Swan Research Initiative® and "Gerencia Regional de Salud, Junta de Castilla y León" (BIO/SA35/14). WES™ platform was acquired thanks to INNOCAMPUS Program (CEI10-1-0010).

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