Differential expression of serum proteins in multiple myeloma

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Abstract. The exact cause instigating multiple myeloma (MM) has not been fully elucidated, and the disease has a median survival of 6 months without any treatment. To identify potential biomarkers of MM, serum proteins reflecting alteration in their proteomes were analyzed in 6 patients with MM compared with 6 healthy controls using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of flight mass spectrometry. The most notable differentially expressed proteins were validated by immunoblotting and changes in mRNA expression were evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A total of 11 differentially expressed protein spots were found. The expression levels of 7 proteins [Immunoglobulin heavy constant μ; proto-oncogene diffuse B-cell lymphoma (DBL2); 26S protease regulatory subunit 4 (P26s4); serum albumin; haptoglobin; and two unknown proteins with isoelectronic point (pI) of 6.41 and molecular weight of 35.4 kDa, and pI of 8.05 and molecular weight of 27.4 kDa, respectively] were downregulated in MM compared with healthy controls. Expression of gel actin-related protein 2/3 complex subunit 1A (ARPC1A); immunoglobulin heavy constant γ 1; fibrinogen α chain (FGA) fragment D; and zinc finger protein 70 were increased in serum of MM patients. Protein expressions of ARPC1A, FGA, P26s4 and DBL2 were measured by immunoblotting in an independent cohort of 12 MM patients and 10 healthy controls. RT-qPCR analysis demonstrated that *ARPC1A* expression only mimicked protein expression, whereas *FGA*, *PSMC1* (encoding P26s4) and *MCF2* (encoding DBL2) did not exhibit significant changes in mRNA expression between control and MM samples. These proteins represent putative serological biomarkers for patients with MM.

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Introduction

In clonal B cell malignancy, multiple myeloma (MM) lesions result from antibody-producing plasma cells that are terminally differentiated in the bone marrow (1). There are ~86,000 cases of MM each year, which accounts for 0.8% of new cancer cases (2). Each year ~63,000 MM patients succumb to the disease, which accounts for 0.9% of mortalities caused by cancers (2). Patients diagnosed with MM fulfill one of the following criteria: i) ≥10% of cells in bone marrow are monoclonal plasma cells or ii) plasmacytoma was confirmed by a biopsy (3). Prior to 2000, the main therapy drugs of MM were alkylators and corticosteroids (4). Recently, carfilzomib, pomalidomide, panobinostat, daratumumab, ixazomib and elotuzumab have used as to treat MM in the United States (3). Even though significant progress has been made in the diagnosis and treatment of MM in the past decade, the exact cause of MM has not been fully elucidated.

Common symptoms of patients with MM are fatigue and bone pain (5). A large fraction of MM patients have osteolytic bone lesions that can occur ubiquitously (6). However, such bone lesions are mostly observed in the pelvis, spine, ribs and skull (6). The composition of the oncogenic lesions remains to be elucidated, although it is well known that cells within the lesions have a high incidence of somatic mutations that are variants between clones; however, a post-switch isotype is present in almost every case (7-9). The underlying molecular mechanisms in pathogenesis of MM have not yet been elucidated.

Translation has been demonstrated to be a better indicator of gene expression compared with mRNA expression levels (10,11). Hence, proteomic approaches are perhaps more viable clinically in providing the correct estimate of differentially expressed proteins and their posttranslational modification, which might also impact its biological function (12). Therefore, it is of great interest to identify diagnostic and prognostic biomarkers for MM at the protein level rather than at the gene level.

Although certain studies have attempted to identify potential markers in MM, including integrin α-11 subunit, multimerin-1, complement C4 and paraoxonase/arylesterase (13,14), none of these markers have yet been clinically verified. There is compelling scientific evidence that serum proteome is an

astute indicator of changes in physiological function and disease processes (15). In the present study, differential serum proteome analysis was performed between MM patients and healthy controls using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS).

Materials and methods

Serum sample. Patients diagnosed with MM in the Department of Hematology, Chonbuk National University Hospital (Jeonju, Korea) and healthy controls involved in physical examinations in Chonbuk National University Hospital between January 2002 and December 2004 were enrolled in the current study. For patients with active (symptomatic) MM, the inclusion criteria were as follows: i) The proportion of monoclonal plasma cells in bone marrow was ≥10% or plasmacytoma was confirmed by a biopsy; ii) monoclonal M protein was identified in serum or urine; and iii) MM caused one or more impairments or target organ injuries, including hypercalcaemia, lytic bone lesions, renal insufficiency, hyperviscosity, anemia, amyloidosis or recurrent infections. Patients were included if they satisfied all three conditions simultaneously. For patients with smoldering (asymptomatic) MM, the inclusion criteria were as follows: i) The content of serum monoclonal M protein was >30 g/l or the proportion of bone marrow monoclonal plasma cells was >10%; and ii) associated organ or tissue impairment, end organ damage, or symptoms were not identified. The exclusion criteria of patients with MM were as follows: i) Nonsecretory MM; ii) reactive plasmacytosis caused by chronic inflammation, metastatic tumors, cirrhosis, typhoid fever or systemic lupus erythematosus; iii) monoclonal gammopathy of undetermined significance; and iv) hyperthyroidism and senile osteoporosis.

All enrolled patients and healthy subjects provided written consent for participation in the present study, which was approved by the Ethics Committee of Chonbuk National University Medical School (Jeonju, Korea). Venous blood (5 ml) from 6 patients with MM (5 female and 1 male; age, 39-57 years) and 6 healthy controls (5 female and 1 male; age, 33-54) were collected for 2-DE. To validate the protein sets obtained from 2-DE, 5 ml venous blood from an independent cohort of 12 MM patients (8 female and 4 male; age range, 47-78 years) and 10 healthy controls (7 female and 3 male; age, 42-69 years) were also collected. To obtain serum samples, 5 ml blood samples with citric acid anticoagulant (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were centrifuged at 1,776 x g for 5 min at room temperature. Serum samples were put into 1.5 ml centrifuge tubes and stored at -80˚C. Post-thawing the serum samples were centrifuged to remove fibrinogen for 10 min at 6,000 x g at 4˚C and filtered using 0.45-μm filters (EMD Millipore, Billerica, MA, USA). Serum samples were denatured using 10% sodium dodecyl sulfate (SDS) and 2.3% dithiothreitol (DTT) for 5 min at 95˚C and then allowed to cool to room temperature.

Dialysis. Dialysis using 12 kDa molecular cut-off tubings (Sigma-Aldrich; Merck KGaA) were used to desalt the samples before 2-DE.

2-DE. The protein in the serum samples were extracted using lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the concentration of protein was quantified by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Serum protein samples (100 μg) resuspended in standard rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% immobilized pH gradients buffer, 1% DTT and 0.01% bromophenol blue) were applied onto an immobiline DryStrip (pH 3-10; Thermo Fisher Scientific, Inc.). First dimension isoelectric focusing was performed using the IPGPhor IEF system (Thermo Fisher Scientific, Inc.) at 20˚C for 66,000 Vhr. Following one-dimensional resolution, the gel was incubated in equilibration buffer I (50 mM Tris-Cl, pH 8.8; 6 M urea; 2% SDS; 30% glycerol; 1% DTT) for 30 min at room temperature and subsequently for 30 min in equilibration buffer II (50 mM Tris-Cl, pH 8.8; 6 M urea; 2% SDS; 30% glycerol; 2.5% IAA) at room temperature. Second dimension was run on a 12.5% SDS-PAGE slab gel using the Ettan DALT II system (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The gels were run for 1 h at 75 V, followed by 5 h at 95 V, and finally 9 h at 135 V.

Silver staining. The Silver Stain PlusOne kit (Thermo Fisher Scientific, Inc.) was used according to the manufacturer's guidelines with the following modifications: Glutaraldehyde was not used in the sensitization step and formaldehyde was removed from the silver staining step.

Image analysis. LabScan software version 6.0 on an ImageScanner was used to obtain digital images of the silver stained gels, which were then analyzed using ImageMaster 2D Platinum 7.0 software (all GE Healthcare, Chicago, IL, USA). Both spot standardization and spot intensity or volume were performed according to standard protocols (16).

Destaining and in-gel trypsin digestion. Silver stained gels were destained as described previously (17). In-gel trypsin digestion was performed as described previously (18,19).

Identification of proteins. MALDI-TOF MS (Voyager-DE PRO; Thermo Fisher Scientific, Inc.) was used for peptide mass fingerprinting as described previously (20). The analysis was performed in positive ion mode and using automatic acquisition data. The peptide mass fingerprints mass spectrum scanned range was 800-3,500 Da, and 10 peaks with a maximum strength were selected for the second phase of mass spectrometry. Data was analyzed using ProFound (version 4.10.5) or MSFit (version 4.0.8) algorithms (21,22), which are available at http://kr.expasy.org. There were three parameters provided in the results, including measured pI (isoelectronic point) and molecular weight, the pI and molecular weight in the ExPASy Bioinformatics Resource Portal proteomics database (https://www.expasy.org/proteomics/protein_sequences_and_identification) and the peptide coverage rate, which represents the number of amino acids detected in the peptide as a percentage of the full length of the protein.

Immunoblotting analysis. Serum proteins were resolved by SDS-PAGE on a 10% gel and processed for immunoblotting

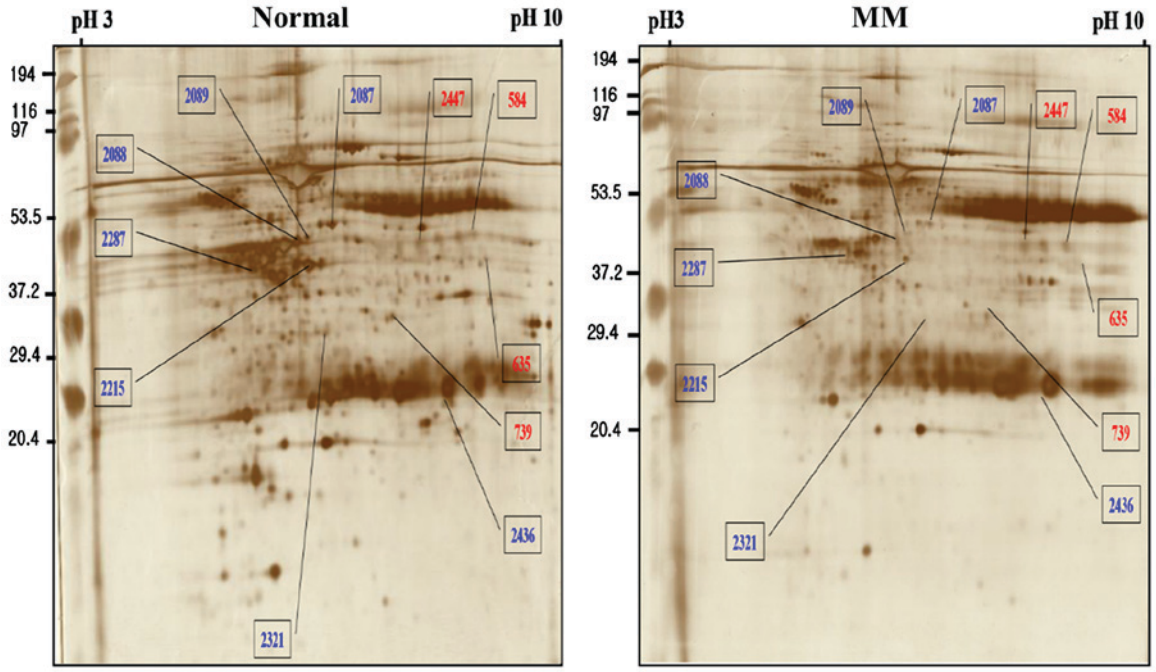

Figure 1. A pair of representative silver stained two-dimensional electrophoresis gels from human sera of 6 MM patients compared with those of 6 healthy controls. The 11 differentially expressed are indicated (red, high expression in MM; blue, low expression in MM). Numbers represent identification assigned to the spots during the experimental process. MM, multiple myeloma.

using standard protocols. Blots were blocked in TBS-Tween containing containing 5% of nonfat dry milk for 1.5 h at room temperature and then probed with antibodies against actin-related protein 2/3 complex subunit 1A (ARPC1A; cat. no. ab211124), proto-oncogene DBL2 (cat. no. ab33441), fibrinogen α chain (FGA; ab34269; all 1:500; Abcam, Cambridge, UK) 26S protease regulatory subunit 4 (P26s4; cat. no. HPA000872; 1:1,000; Sigma-Aldrich) and GAPDH (cat. no. ab9485; 1:1,000; Abcam) at 4˚C for 12 h. Then the blots were incubated with horseradish peroxidase-conjugated rabbit anti-human (cat. no. ab6759; 1:1,000; Abcam) for 1 h at room temperature. All blots were probed with GAPDH as a loading control. The bands were visualized using Pierce Electro-Chemiluminescence Western blotting substrate (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from serum samples using TRIzol LS (Thermo Fisher Scientific, Inc.). To obtain cDNA, the reverse transcription reaction was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR analysis was performed using Custom TaqMan Gene Expression Assay (Thermo Fisher Scientific, Inc.) to detect indicated genes according to the manufacturer's protocol. The primers used in PCR were as follows: *ARPC1A*: Forward, 5'-AACTCAAGGAGCACAACGG-3' and reverse, 5'-ATCAGCAACAGACACGGTG-3'; *FGA*: Forward, 5'-TCTGCCTGGTCCTAAGTGTG-3' and reverse, 5'-TGTTCAAGTTGCTTCTGCTG'; *PSMC1*: Forward, 5'-CAAGATGGTGAAACCTCGTC-3' and reverse, 5'-TGTGTCTCAGTTCTGTCGTCTG-3'; *MCF2*: Forward, 5'-ATCGGCAAATAAGTGGTGAC-3' and reverse, 5'-TCCTGCTGAGTTGTATCCG-3'. *GAPDH*: Forward, 5'-TAAGGCTTCCGCTCACTTG-3' and reverse, 5'-GAGACGAACAGTAAGGTCAACAAC-3'. Data was normalized to *GAPDH* expression and analyzed using the $2^{-\Delta\Delta Cq}$ method (23).

Statistical analysis. Data of relative protein expressions was presented as means ± standard deviation. Significant differences were identified using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Eleven differentially expressed proteins were found in MM patients compared to healthy controls. The 2-DE analysis was performed on serum samples obtained from 6 MM patients and 6 age-matched healthy controls (Fig. 1). Routinely, 600-800 spots from 100-μg protein samples were observed. There were 11 consistently different spots between the different MM samples compared with the healthy controls, 4 of which were upregulated and 7 were downregulated in the MM samples (Fig. 1).

MALDI-TOF MS was performed on these 11 spots. The 4 upregulated protein spots in the 2-DE gel were identified as ARPC1A (pI: 8.60; molecular weight: 47.3 kDa; 385±97%; $P<0.05$); Immunoglobulin heavy constant γ 1 (IGHG1; pI: 8.71; molecular weight: 42.3 kDa; 373±70%; $P<0.05$); FGA fragment D (pI: 7.32; molecular weight: 37.2; 306±44%; $P<0.05$); and zinc finger protein 70 (ZNF70; pI: 4.75; molecular weight: 47.9; 207±46%; $P<0.05$; Fig. 2; Table I). The downregulated proteins in the MM samples compared with the healthy controls were immunoglobulin heavy constant μ (pI: 6.33; molecular weight: 53.4; 35±16%; $P<0.05$); proto-oncogene DBL2 (pI: 6.02; molecular weight: 48.1; 37±7%; $P<0.05$);

Table I. Upregulated proteins in sera of MM patients.

Spot. no.	Protein name	Accession no. (GI)	Measured pI/M.W (kDa)	pI/mass	Peptide coverage rate (%)	Change in MM
584	Actin-related protein 2/3 complex subunit 1A	88984001	8.60/47.3	8.6/41.6	9.0	Increase
635	Immunoglobulin heavy constant γ 1	121039	8.71/42.3	8.5/36.1	26.0	Increase
739	Fibrinogen α chain, fragment D	1706799	7.32/37.2	7.1/36.3	23.0	Increase
2447	Zinc finger protein 70	20141062	4.75/47.9	8.7/50.8	14.0	Increase

pI/mass was obtained from the ExPASy Bioinformatics Resource Portal proteomics database. MM, multiple myeloma; pI, isoelectronic point.

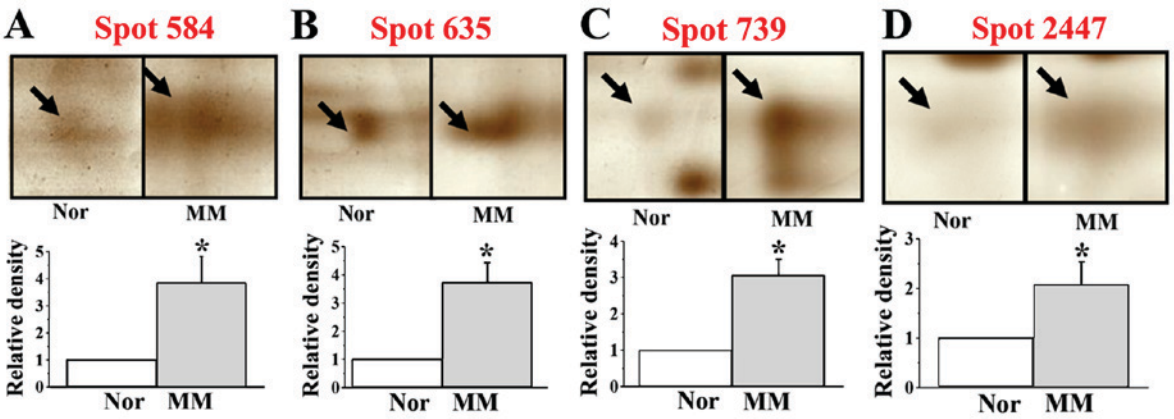

Figure 2. Upregulated spots among the 11 spots that were significantly and consistently changed in human sera of MM patients compared with healthy controls. A total of 4 spots (A-D) increased in the sera from MM patients. In each pair of two-dimensional electrophoresis from a healthy control and an MM patient, the density of a spot in MM gel was normalized to the density of the corresponding spot in the healthy control gel. The mean volumes of each spot from 6 pairs are displayed on the lower panel. Details of the identified proteins are provided in Table I. *P<0.05 vs. Nor. MM, multiple myeloma; Nor, normal subject.

P26s4 (pI: 6.13; molecular weight: 49.7; 32±2%; P<0.05); serum albumin (ALB; pI: 6.16; molecular weight: 43.7; 15±3%; P<0.05); haptoglobin (pI: 5.36; molecular weight: 42.5; 51±8%; P<0.05); and 2 unknown proteins with isoelectronic point (pI) of 6.41 and molecular weight of 35.4 kDa (spot number 2321; 20±16%; P<0.05), and pI of 8.05 and molecular weight of 27.4 kDa (spot number 2436; 26±15%; P<0.05), respectively (Fig. 3; Table II).

The mRNA and protein expression levels of ARPC1A, FGA, P26s4 and DBL2 were verified by immunoblotting and RT-PCR. Immunoblotting analysis of serum samples of MM patients and healthy controls on an independent set of age-matched cases confirmed that whereas ARPC1A and FGA were upregulated in MM, P26s4 and DBL2 were downregulated in the MM patients (Fig. 4A). RT-qPCR analysis demonstrated that among the 5 proteins whose expression were validated, only 1 (*ARPC1A*) was mimicked at the mRNA level (Fig. 4B). mRNA expression of *FGA*, *PSMC1* (encoding P26s4), and *MCF2* (encoding DBL2) did not change significantly between MM and control samples (P>0.05; Fig. 4B).

Discussion

MM is a malignant tumor characterized by alterations of various serum proteins, such as M proteins, β2-MG, C-reactive protein and serum free light chain, which are beneficial for the diagnosis and therapy of MM (24-26). In the present study, 11 serum proteins were differently expressed in MM patients compared with healthy controls. cDNA cloning and characterization of the 2 unidentified proteins that were significantly downregulated in the MM samples are required to characterize their identity and function.

Human actin-related protein 2/3 complex (Arp2/3) serves crucial roles in various cellular activities through regulation of actin polymerization and contributes to the pathogenesis of Wiskott-Aldrich syndrome, a genetic disease with recurrent infection and disordered immunity (27). Recent studies have demonstrated that Arp2/3 complex is critical for the formation of immune cell synapses and that a loss of Arp2/3 complex component predisposes to lymphocytic dysfunction and inflammatory disease (28,29). As a chronic antigenic stimulus, virus infection and gene mutation are closely associated with MM, and in the present study ARPC1A was upregulated in MM patients; thus it is speculated that ARPC1A may exert an effect on the development of MM.

Variants of immunoglobulin heavy chain and gene rearrangement have previously been observed in MM (30,31). It was also demonstrated that while IGM protein expression is downregulated, IGHG1 protein expression is upregulated in MM patients, consistent with previous reports of IgG gene rearrangements and variants in MM pathogenesis (32).

Defective fibrinogen to fibrin conversion has been previously reported in MM patients (33,34). It has been reported

Table II. The downregulated proteins in sera of MM patients.

Spot. no.	Protein name	Accession no. (GI)	Measured pI/M.W (kDa)	pI/mass	Peptide coverage rate (%)	Change in MM
2087	Immunoglobulin heavy constant μ	1160421986	6.33/53.4	6.06/8.5	25.3	Decrease
2088	Proto-oncogene DBL2	92087039	6.02/48.1	5.98/50.4	18.7	Decrease
2089	26S protease regulatory subunit 4	49065780	6.13/49.7	5.9/49.2	14.0	Decrease
2215	Albumin protein (homo sapiens)	113576	6.16/43.7	6.0/48.7	44.0	Decrease
2287	Haptoglobin	123508	5.36/42.5	5.21/40.8	19.1	Decrease
2321	Unknown		6.41/35.4			Decrease
2436	Unknown [protein for IMAGE 3934797 (homo sapiens)]		8.05/27.4	8.6/23.32		Decrease

pI/mass was obtained from the ExPASy Bioinformatics Resource Portal proteomics database. MM, multiple myeloma; pI, isoelectronic point.

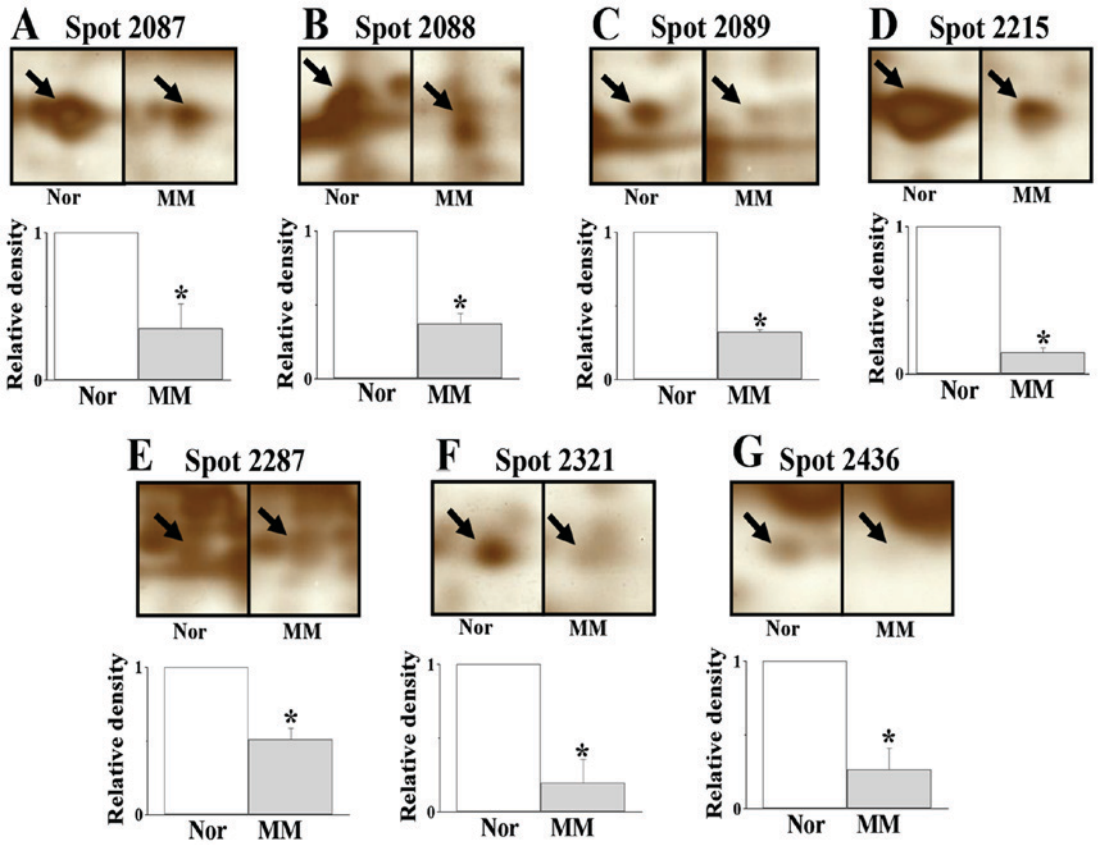

Figure 3. Downregulated spots among the 11 spots that were significantly and consistently changed in human sera of MM patients compared with healthy controls. A total of 7 spots (A-G) were decreased in the sera from MM patients. In each pair of two-dimensional electrophoresis gels from a healthy control and a MM patient, the density of a spot in MM gel was normalized to the density of the corresponding spot in the healthy control gel. The mean volumes of each spot from 6 pairs are displayed on the lower panel. Details of the identified proteins are provided in Table II. *P<0.05 vs. Nor. MM, multiple myeloma; Nor, normal subject.

that fibrinogen and its early degradation product, fragment D contribute to arterial constriction by binding endothelial intercellular adhesion molecule-1, leading to increased vascular resistance that compromises circulation (35), which may aggravate hemodynamic disorder in MM. That FGA fragment D was identified as one of the differentially upregulated proteins in MM cannot be attributed to fibrinogen for two reasons: i) Fibrinogen was removed during serum isolation, and ii) healthy control samples should also have high levels of the fragment D if it was a contamination issue stemming from incomplete fibrinogen removal.

ZFP is a specialized protein group that is characterized by a single zinc atom associated with DNA binding proteins (36). ZFP proteins function in DNA replication, repair and recombination, as well as in cell proliferation and apoptosis (37,38). ZNF70 is indicated to exert a role as a tumor suppressor in

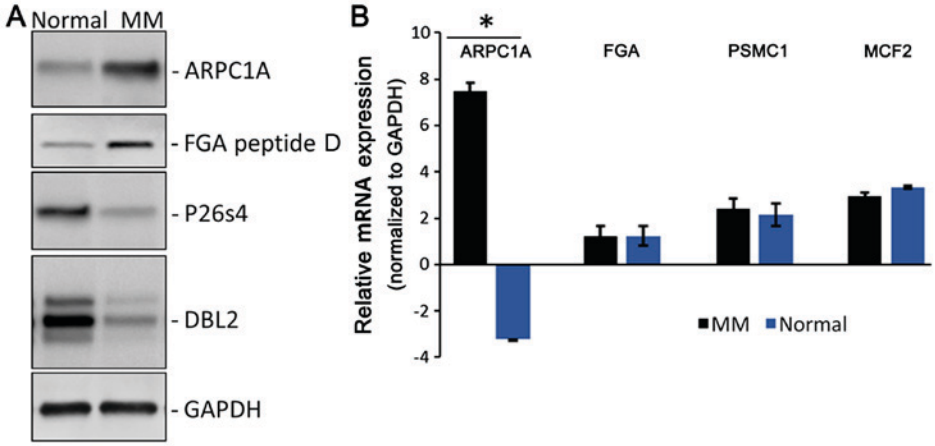

Figure 4. (A) Immunoblotting analysis-based validation of differentially expressed serum protein in an independent cohort of 12 MM patients and 10 healthy controls (n=3 each). Blots were stripped and probed with GAPDH to confirm equal loading across samples. Representative blots of three independent experiments are presented. (B) Relative mRNA expression of indicated genes in MM patients and healthy controls (n=6 each). Data were normalized to *GAPDH* expression and are represented as mean ± standard error of mean of three independent experiments. *P<0.05. MM, multiple myeloma; ARPC1A, actin-related protein 2/3 complex subunit 1A; FGA, fibrinogen α chain; P26s4, 26S protease regulatory subunit 4.

gastric cancer (39), however, ZNF70 was increased in the serum of MM patients in the present study. Therefore whether the role of ZNF70 in carcinogenesis is context dependent and its effects on the pathogenesis of MM remain to be determined.

DBL transforming protein has pro-oncogenic functions and regulates the Rho family of Ras-related GTPases (40,41). Despite harboring transforming activity, DBL protein was downregulated in MM patients in the present study. It remains to be elucidated whether there are other proteins that can mimic the function of DBL in the pathogenesis of MM.

Aberrant activity of the 26S proteasome, including P26s4, impacts the cell cycle and apoptosis. Moonlighting function of the 19S subunit was previously elucidated in regulation of gene transcription (42); whether a similar role is served by P26s4 in MM patients remains to be determined.

Serum ALB was previously demonstrated to be downregulated in MM (43), which was consistent with the present findings. Serum ALB is a critical prognostic factor for survival of Waldenstrom's macroglobulinemia and initial serum ALB prior to treatment is also an independent risk factor in MM (44,45). Therefore, serum ALB has prognostic significance in the diagnosis of MM and this efficacy will be evaluated in further studies.

Haptoglobin is a plasma α-2-glycoprotein and is highly expressed in neoplastic tissue and the inflammatory microenvironment (46-48). It has also been demonstrated to be downregulated in IgA MM (49), and it is speculated that the proinflammatory effects of haptoglobin may promote the development of MM.

Each one of the 9 identified proteins that were downregulated or upregulated in the present study require further analysis to elucidate their association with disease progression and survival of MM to define their actual clinical benefit. Additional studies will also be required to address what regulates these proteins and how the expression (or lack of expression) of these proteins impacts the pathogenesis of MM. The present study also highlights the importance of adopting a proteomic approach along with gene expression studies in order to correctly identify the proteins that are differentially expressed. Whereas gene expression studies continue to be the preferred platform to identify putative differentially expressed gene products, mere increases or decreases in mRNA expression does not count for much if the translation of the mRNAs is being differentially regulated in the context of a disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZM and YGK designed experiments and wrote the manuscript. ZP and SYJ performed the experiments and analyzed the data.

Ethics approval and consent to participate

All enrolled patients and healthy subjects provided written informed consent for participation in the present study, which was approved by the Ethics Committee of Chonbuk National University Medical School (Jeonju, Korea).

Patient consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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