

Detection of Abundant Proteins in Multiple Myeloma Cells by Proteomics

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Abstract

Abundant proteins of human multiple myeloma (MM) were globally analyzed and identified by using two-dimensional gel electrophoresis (2DE) and MALDI-TOF/TOF mass spectrometry (MS). Spots of 517 corresponding to 268 different proteins were detected on 2DE gels of protein lysate from plasma cells isolated from eight newly diagnosed MM patients. These identified proteins were classified into different categories based on their molecular functions and biological processes. The detailed experimental procedures and MS spectra of all the identified proteins have been deposited in the Proteomics Identifications Database (PRIDE) (<http://www.ebi.ac.uk/pride>) with Accession No. 8846 & 8847. This 2DE map of MM proteins will be an invaluable resource for further proteomics research that investigates proteomic changes associated with biomarker identification and carcinogenesis analysis of multiple myeloma.

Keywords: Multiple myeloma; 2-DE; Proteomic database

Introduction

Multiple myeloma (MM) is a clonal B-cell disorder in which malignant plasma cells (PC) expand and accumulate in the bone marrow (BM) leading to cytopenias, bone resorption, and the production (in most cases) of the characteristic monoclonal protein (Kyle et al., 2004). It is the second most common adult hematologic malignancy, and the most common cancer with skeleton as its primary site. It has an incidence of 19900 new cases per year in the USA, and accounts for 10% of hematologic malignancies and 1% of all cancer deaths (Jemal et al., 2006). MM remains incurable; but recent advances in cytogenetic and molecular profiling technologies may allow improving our understanding of disease pathogenesis, identifying novel therapeutic targets, rendering molecular classification, and thus providing scientific rationales for combining targeted therapies to increase tumor cell cytotoxicity and to abrogate drug resistance (Bergsagel et al., 2005; Tassone et al., 2006; Zhan et al., 2006).

To date, very few reports on the application of proteomic technologies to the study of MM have been published. In particular, there is no comprehensive 2DE protein database for the MM cells available to researchers. The current study sought to generate, for the first time, a proteome map of the human MM cells, composing a database with abundant proteins usually found in MM cells via 2DE display. The construction of this database

will aid as a reference for proteomic studies on the identification of pathological changes in the proteome caused by the disease.

Materials and Methods

Purification of plasma cells

BM aspirates were obtained from eight patients with newly diagnosed MM and without any treatment. Informed consent was received from all patients in accordance with the Declaration of Helsinki protocol and institutional policies. Mononuclear cells (including PCs) were isolated from BM biopsies by purification over a Ficoll-Paque (Amersham, Piscataway, NJ, USA) gradient centrifugation. Briefly, BM aspirates (approximately 15 ml) were diluted to 1:1 with prewarmed (37°C) PBS and overlaid onto 15 ml prewarmed Ficoll-Hypaque. After centrifugation at 2000 rpm for 20 min at room temperature, mononuclear cells were removed, washed again with PBS. PC isolation from mononuclear cell fraction was performed by immunomagnetic bead selection with monoclonal mouse anti-human CD138 antibodies in association with LS separation columns separation system (Miltenyi-Biotec, Auburn, CA, USA). PC purity was routinely above 95%, with analysis by 2-color flow cytometry using CD138+/CD45- and CD38+/CD45- criteria (Jennings et al., 1997). All purified MM cells were pool together for subsequent experiments.

Protein preparation

Purified plasma cells were washed twice with ice-cold washing buffer (10 μM Tris-HCl, 250 μM sucrose, pH 7.0) and transferred to a clean 2.0 ml Eppendorf tube, spun down at 500 g for 5 min. Whole cell lysate was prepared as previously described (Wang et al., 2006). Briefly, cell pellet was lysed by adding 80 μl lysis solution (7 M urea, 2 M thiourea, 4% CHAPS and 1% DTT, 2% v/v IPG buffer 3–10 NL, 0.2 mg/ml PMSF and protease-inhibitor (all from GE healthcare)). After centrifuged at 13200 rpm for 20 min at 4°C to clean the cell lysate, the

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lysis supernatant was used for 2-DE. Protein concentrations were determined using Bradford assay. All the samples were stored at -80°C prior to electrophoresis.

2DE and in gel digestion

2DE was performed with Amersham Biosciences IPGphor IEF System and Hoefer SE 600 (Amersham Biosciences, Uppsala, Sweden) electrophoresis units using the protocol suggested by the manufacturer. Briefly, total proteins (80 μg) were mixed up to 250 μl with rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT and 0.5% IPG buffer) and run in IEF using 13-cm immobilized pH 3–10 nonlinear or pH 4–7 linear gradient IPG strips (Amersham Biosciences). The rehydration step was carried out for more than 12 h at low voltage of 30 V. IEF was run by following a step-wise voltage increase procedure: 500 and 1000 V for 1 h each and 5000–8000 V for about 10 h with a total of 64 kWh. After IEF, the strips were subjected to a two-step equilibration in equilibration buffers (6 M urea, 30% glycerol, 2% SDS and 50 mM Tris-HCl pH 6.8) with 1% DTT w/v for the first step, and 2.5% iodoacetamide (w/v) for the second step. The equilibrated gel strips were placed on the top of 12.5% SDS-PAGE gels and sealed with 0.5% agarose containing a little bromophenol blue. SDS-PAGE was performed for 30 min at a constant current of 15 mA per gel and then 30 mA per gel until the bromophenol blue reached the bottom of the gels.

After 2DE, proteins in the gels were visualized using silver staining method, as developed by (Shevchenko et al., 1996). Each 2DE was repeated in triplicates. Analytical gels were scanned on an Image Scanner (GE healthcare, Uppsala, Sweden) at 300 dpi with 12-bit gray scale levels in tagged image file format (TIFF), and images were analyzed using the ImageMaster 2D Platinum (GE healthcare, Uppsala, Sweden) (Wang et al., 2006). All gels in the analyses were scanned with identical parameters. The individual spots of each gel were detected by their boundaries, and the spot volumes corresponding to the protein abundance were calculated automatically. Each spot intensity volume was processed by background subtraction and total spot volume normalization. The resulting spot volume percentages were used for comparison. Only those spots that were clearly and reproducibly visualized, as judged by software analysis of the silver-stained gels, were excised from gels for analysis by MS.

Protein spots were excised and transferred into siliconized 0.5 ml Eppendorf tubes. Each gel piece was rinsed twice with deionized water, destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and then equilibrated in 50 mM ammonium bicarbonate to pH 8.0. After hydrating with acetonitrile and drying in a Speed Vac (Thermo Fisher Scientific, Waltham, MA), the gel spots were rehydrated in a minimal volume of trypsin (Promega, USA) solution (20 $\mu\text{g}/\text{ml}$ in 25 mM NH_4HCO_3) and incubated at 37°C overnight. The supernatants were transferred into a 200 μl microcentrifuge tube and the gels were extracted once with extraction buffer (67% acetonitrile containing 2.5% trifluoroacetic acid). The peptide extract and the supernatant of the gel spot were combined and then completely dried in a SpeedVac centrifuge.

Protein identification and data analysis

Protein digestion extracts (tryptic peptides) were resuspended

with 5 μl of 0.1% trifluoroacetic acid and then the peptide samples were mixed in 1:1 ratio with matrix consisting of a saturated solution of α -cyano-4-hydroxy-trans-cinnamic acid and 0.1% trifluoroacetic acid in 50% acetonitrile. Aliquots of 0.8 μl were spotted onto stainless steel sample target plates.

Peptide mass spectra were obtained on an ABI-4800plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). PMFs and peptide sequence spectra were obtained using the settings presented in the Supporting Information Data 1 (http://life-health.jnu.edu.cn/data/Acta_Bioch/Supporting_Information_Data_1.pdf). Data were acquired in positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI-4700 Calibration Mixture). Mass spectra were obtained from each sample spot by accumulation of 600–800 laser shots in an 800–4000 mass range. For MS/MS spectra, the 5 most abundant precursor ions per sample were selected for subsequent fragmentation and 900–1200 laser shots were accumulated per precursor ion. The criterion for precursor selection was a minimum S/N of 50. Both the MS and MS/MS data were interpreted and processed by using the GPS Explorer software (V3.6, Applied Biosystems). The obtained MS and MS/MS spectra were then combined and submitted to MASCOT search engine (V2.1, Matrix Science, London, U.K.) by GPS Explorer software. The searching parameters were as follows: IPI Human database (V3.36), taxonomy of Homo sapiens (human), trypsin of the digestion enzyme, one missed cleavage site, partial modification of cysteine carboamido methylated and methionine oxidized, none fixed modifications, MS tolerance of 30–60 ppm, MS/MS tolerance of 0.2–0.3Da. Known contaminant ions (keratin) were excluded. Totally 69012 sequences and 29002682 residues in the database were actually searched. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 65 were considered statistically significant ($p \leq 0.05$). The individual MS/MS spectra with statistically significant ($p \leq 0.05$) best ion score (based on MS/MS spectra) were also accepted.

Identified proteins were classified based on the PANTHER (Protein ANalysis THrough Evolutionary Relationships) system (<http://www.pantherdb.org>), which is a unique resource that classifies genes and proteins by their functions (Mi et al., 2007). Some proteins were annotated manually based on literature searches and closely related homologues.

Results and Discussion

Figure 1 shows the representative 2DE images of MM cellular proteins separated in both pH 4–7 and pH 3–10 ranges. In total 517 gel spots were subjected to protein identification by MALDI-TOF MS/MS, and the identification resulted in 268 distinct proteins and their respective isoforms or subunits. These identified proteins were categorized into different functional and biological process groups as summarized in Figure 2 and Supporting Information Table (http://life-health.jnu.edu.cn/data/Acta_Bioch/Supporting_Information_Table.rar). The characterized proteins include cytoskeletal protein, chaperone, oxidoreductase, protease, etc. Figure 2 provides an overview of the human MM proteome based on the known or postulated functions or biological processes of the identified proteins. The detailed experimental procedures and MS spectra of all the identified proteins have been deposited in the Proteomics Identifica-

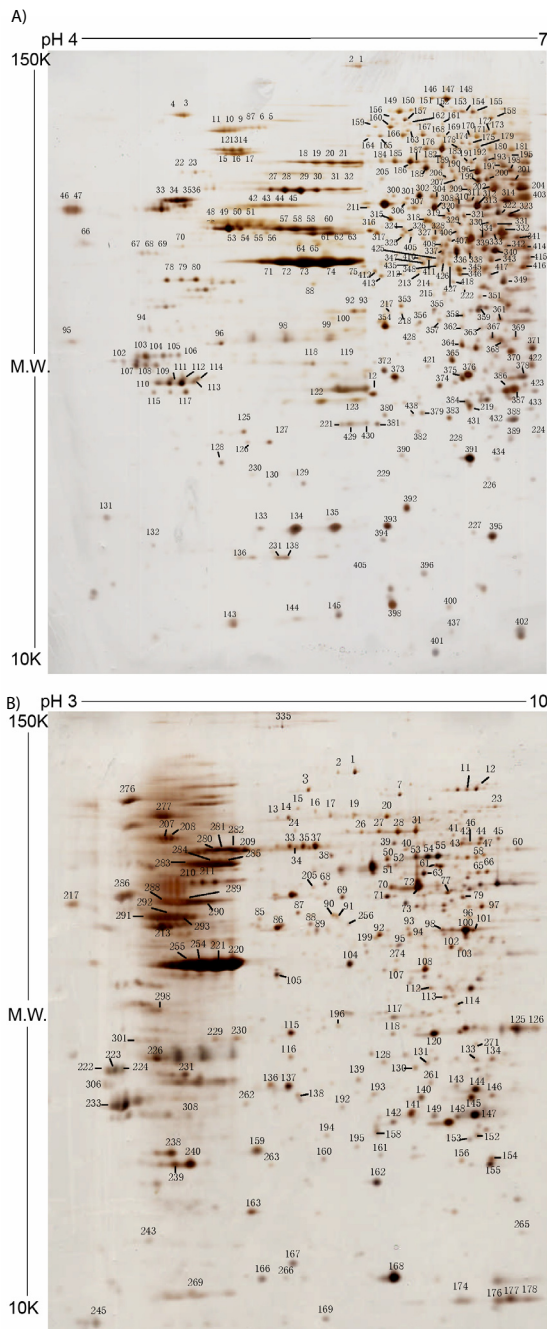


Figure 1: Representative 2DE gel images of MM proteome. Proteins from the purified MM cells were extracted and separated on (A) pH 4–7 Liner and (B) pH 3–10 NL IPG strips, and then on SDS-PAGE. After staining and image analysis, the protein spots were analyzed by MALDI-TOF/TOF MS. The identified proteins are labeled with spot numbers, which are also listed in Supporting Information Table (http://life-health.jnu.edu.cn/data/Acta_Bioch/Supporting_Information_Table.rar). Results were from one representative experiment out of three.

tions Database (PRIDE) (<http://www.ebi.ac.uk/pride>) with Accession No. 8846 & 8847.

The cytoskeletal proteins represented the largest group in all the identified proteins. The cytoskeleton is a highly complex and dynamic system comprising structural proteins forming polymers (actin, tubulin and intermediate filaments) and several associated proteins with regulatory functions. In addition to their well-known structural function, cytoskeletal proteins play important roles in cell mobility and migration, immunological synapse for-

mation and apoptosis (Richter-Landsberg, 2008). Tumor-associated changes in the cytoskeleton are well documented and even utilized in cancer diagnostics (Ditzel et al., 2002). The present study identified 89 protein spots representing 34 different cytoskeletal proteins, with 29 protein spots corresponding to three structural proteins, namely, actin, tubulin and vimentin. Twenty different actin-binding protein members were also identified, including vinculin, cofilin 1, villin 2, gelsolin, tropomyosin 3, coronin, *etc.* The actin-binding protein family represents a large number of cytoskeletal proteins with a crucial role in the regulation of microfilaments implicating in many pathologies (Khurana et al., 2008). For example, cofilins are implicated in several cellular processes including neuronal outgrowth, T-cell activation, phagocytosis, endocytosis, receptor recycling, regulation of ion channels, and maybe, via the formation of actin-cofilin rods, in cellular ATP-energy management (Ono, 2007). Cofilins could be important in the progression of Alzheimer’s disease and ischemic kidney disease. Furthermore, altered expression of cofilins may lead to inflammation, infertility, immune deficiencies and other pathophysiological defects (Bamburg et al., 2002).

The chaperone class comprises twenty members, including 14-3-3 proteins, heat shock proteins (HSPs) and chaperonin containing TCP1 *etc.* 14-3-3 proteins are a family of multifunctional phosphoserine-binding molecules that can serve as effectors of survival signaling (Fu et al., 2000). They are involved in a variety of important cellular processes that include cell cycle progression, growth, differentiation and apoptosis (Aitken, 2006).

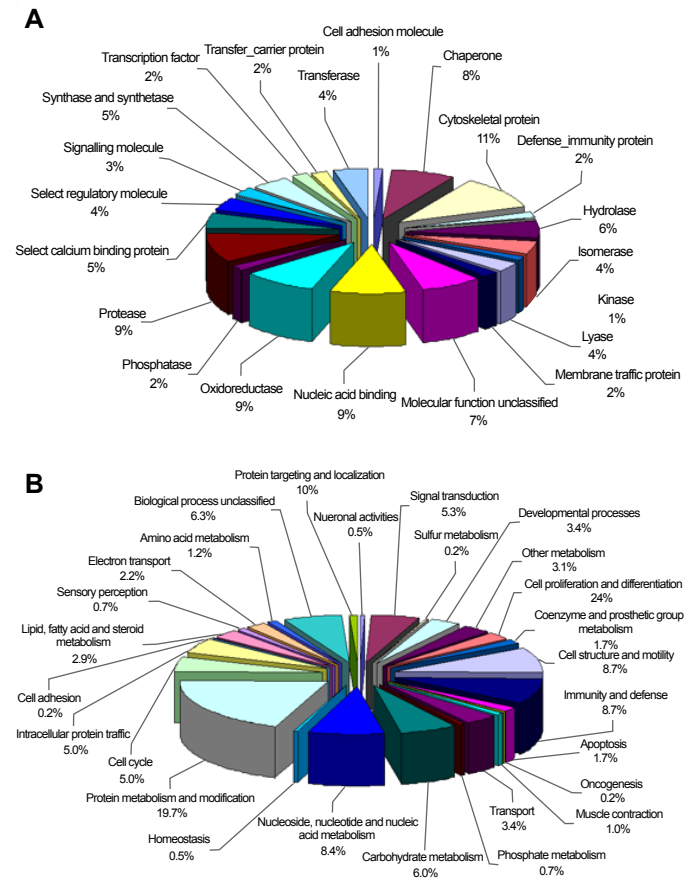


Figure 2: Pie chart representations of the distribution of identified MM proteins according to their (A) molecular functions and (B) biological processes. Categorizations were based on information provided by the online resource PANTHER classification system. Some proteins were annotated manually based on literature searches and closely related homologues.

In mammalian cells, seven different isoforms (β , ϵ , γ , η , σ , τ , ζ) have been identified, with each isoform having distinct tissue localization and function. Of particular interest is the role of 14-3-3 ζ , a protein that has multiplex functions in addition to its role as chaperone. In lung and oral cancers, 14-3-3 ζ was found up-regulated (Fan et al., 2007; Matta et al., 2007); the oncogenic function of 14-3-3 ζ was further proposed (Niemantsverdriet et al., 2008). The current up-regulation of 14-3-3 ζ may be part of the oncogene addiction machinery that MM cells rely on for survival.

HSPs are the products of several distinct gene families that are required for cell survival during stress. Different classes of HSPs play diversified roles in governing proper protein assembly, folding, and translocation. Regulation of HSP synthesis creates a unique defense system to maintain cellular protein homeostasis and to ensure cell survival (Beere, 2004; Calderwood et al., 2006). HSP90 is an emerging therapeutic target that may be interest for the treatment of MM. Its role in protein homeostasis and the selective chaperoning of key signaling proteins in cancer survival and proliferation pathways has made HSP90 an attractive target of small molecule therapeutic intervention (Francis et al., 2006; Mitsiades et al., 2006). Two cytosolic forms of HSP90, HSP90 α and HSP90 β , have been identified. The HSP70 and HSP60 families were present with three and one isoforms respectively. The small HSP family was represented by one protein, HSPB1.

In the identification, the oxidoreductase class is the third after the cytoskeletal proteins and the chaperones in terms of expression intensity: a value that testifies the relevance of this class of proteins in the cell economy. This class includes glutathione S-transferase P, Cu-Zn superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), glutathione peroxidase 1, thioredoxin, eight kinds of dehydrogenases and four members of the peroxiredoxin family. It has been reported that oxidative stress mediates various cellular responses, and that the control of reduction/oxidation (redox) is fundamental in maintaining the homeostasis of the whole organism (Nishinaka et al., 2001). Among these enzymes, the thioredoxin and glutathione systems are considered to be two major redox systems, serving as putative targets in animal cells for cancer therapy (Biaglow et al., 2005). The peroxiredoxin system has received much attention for its high antioxidant efficiency in recent years. Some of the peroxiredoxin members have tumor preventive functions and could be used as the potential drug targets (Neumann et al., 2007).

Twenty five different proteases were identified in this study. Among them, 15 proteins are related to the proteasome system, including 26S protease regulatory subunit 7, proteasome activator subunit 2, proteasome subunit alpha type, proteasome subunit beta type-4, etc. The proteasome plays a pivotal role in the degradation of short-lived regulatory proteins which are components of cell cycle regulation, cell surface receptors, ion channels modulation, and antigen presentation. It is believed that once the disposal system fails to work, the substances, such as regulatory molecules p53, NF κ B, and Bax that promote apoptosis, may accumulate to a high level harmful to the cell (Hernandez et al., 2004; Schwartz et al., 1999). Proteasome inhibitors constitute a class of antitumor agents with preclinical evidence of activity against several malignancies, including multiple myeloma and a

variety of solid tumors (Adams, 2004; Hideshima et al., 2002; Park et al., 2004).

The identified proteins categorized into other functional groups include select calcium binding proteins (5%), cell adhesion molecules (1%), defense/immunity proteins (2%), hydrolases (6%), isomerases (4%), kinases (1%), lyases (4%), membrane traffic proteins (2%), nucleic-acid binding proteins (9%), phosphatases (2%), select regulatory molecules (4%), signaling molecules (3%), synthases and synthetases (5%), transcription factors (2%), transfer/carrier proteins (2%) and transferases (4%); all are essential for maintaining the structure and function of the MM cells. Other identified proteins with their subfamilies not being classified were categorized as "molecular function unclassified" (7%).

In the 2DE protein dataset, some proteins are represented by multiple spots. Examples are HSP90 β 1, represented by at least six spots; and 14-3-3 ζ , represented by at least four spots. The multiple spots may result from phosphorylation, glycosylation, or other PTM. Each of these isoforms could play a specific cellular role, which awaits for further functional investigations.

In summary, the first 2DE dataset of human MM proteome was described here as a step towards our long-term goal to clarify the molecular mechanisms of MM formation. Totally 517 selected gel spots, corresponding to 268 proteins, were characterized by 2DE, mass spectrometry and database analysis. These characterized proteins correspond to different functional categories, and represent a preliminary functional profile of MM proteome. This database can serve as a reference map for the proteomic comparison between MM and normal plasma cells or among different stages in the process of MM formation. In addition, the availability of this reference map of MM cells could be very useful for possible biomarker identification and for the study of proteomic modulation associated with cancer progression.

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The authors have declared no conflict of interest.

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