REVIEW

Proteomic analysis of multiple myeloma: Current status and future perspectives

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Multiple myeloma (MM) is a malignant plasma cell neoplasm that accounts for slightly more than 10% of all hematologic cancers and remains incurable. The major challenge remains the identification of better diagnosis and prognostic biomarkers. The advent of proteomic technologies creates new opportunities and challenges for those seeking to gain greater understanding of MM. Although there is a limited number of proteomic studies to date in MM, those performed highlight the potential impact of these technologies in our understanding of MM pathogenesis and the identification of novel therapeutic targets. In this review, we introduce the proteomic technologies available for the study of MM, summarize results of the published proteomic studies on MM, and discuss the novel developments and applications for the analysis of protein PTM in MM. The application of proteomic technologies will be valuable to better understand the pathogenesis of MM and may in the future open novel avenues in the treatment of MM.

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1 Introduction

Multiple myeloma (MM) is a malignant neoplasm originating in plasma cells. MM causes about 1% of neoplastic diseases and 13% of hematological malignancies in the USA [1]. The incidence varies globally from 1 *per* 100 000 people in China, to about 4 *per* 100 000 people in most developed countries [1].

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Abbreviations: apoC-I, apolipoprotein C-I; **ATO,** arsenic trioxide; **BM,** bone marrow; **FGFR3,** fibroblast growth factor receptor 3; **MGUS,** monoclonal gammopathy of undetermined clinical significance; **MM,** multiple myeloma; **SRM,** selected reaction monitoring

MM is a currently incurable malignancy and the etiology of MM remains unknown [2]. A multistep development model suggests that monoclonal gammopathy of undetermined clinical significance (MGUS) might become smouldering MM, and ultimately symptomatic intramedullary and extramedullary MM, or plasma cell leukemia [3]. The bone marrow (BM) microenvironment plays a critical role in enhancing tumor cell growth, survival, migration, and drug resistance [4]. MM cells home to the BM and adhere to extracellular matrix proteins and to BM stromal cells, which not only localizes tumor cells in the BM milieu but also has important functional sequelae [5]. Although gene expression profiling has provided novel insights into the understanding the pathogenesis of MM, ultimately

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protein expression and mutual interaction between PCs and BM microenvironment determine MM formation. Global gene expression profiling shows that few differences exist between MGUS and MM [6], which underscores the essential role of protein expression and BM microenvironment in development, maintenance, and progression of MM. Thus, proteomic analysis of MM complements and extends the genomic findings [7–10]. In addition, proteomics can be utilized to study PTMs and protein–protein interactions, which may determine MM formation [11].

Various proteomic methodologies have been established for the analyses of protein expression and PTMs, quantitative characterization of protein mixtures, protein–protein interactions, and disease management [12–14]. A list of publications on proteomic studies of MM is summarized in Table 1. It should be noted that the application of proteomic approaches in MM is relatively new, but growing rapidly. This review will introduce the currently available proteomic technologies and discuss their contributions to understanding MM pathogenesis and therapy.

2 Proteomic approaches

Over the past decade, a number of proteomic platforms have been developed or perfected for the purposes ranging from studying protein–protein interactions and PTMs to protein detection and quantitation [13, 15, 16]. Proteomic analysis is achieved by a combination of techniques that are designed to profile, quantitate, and identify proteins or peptides. Figure 1 shows various proteomic methodologies that have the potential to be applied in MM research. Importantly, each of these technologies has distinct advantages, disadvantages, and limitations and the proteomic technologies described below should be considered as complementary tools.

2-DE is an approach in which mixtures of proteins are separated based on mass and charge. When combined MS and advanced image analysis software, 2-DE can be used for protein separation, relative quantification of protein expression, and identification of protein isoforms and PTMs [17, 18]. As an improvement, 2-D DIGE overcomes the problems associated with traditional 2-DE and allows more accurate and sensitive quantitative proteomic studies [19–21]. Though 2-DE or DIGE has its advantages and disadvantages, there is no doubt that it will remain as an essential technique for the characterization of proteomes for many years to come.

iTRAQ has been established as a comprehensive and efficient method for proteomic quantification [22, 23]. The method was first developed by Ross *et al.* [24] and was subsequently commercialized by Applied Biosystems. This potential benefit – to identify and quantify low-abundance proteins in complex samples – coupled with the ability to multiplex up to eight samples in parallel suggests that iTRAQ holds the most promise for biomarker discovery [25].

Stable-isotope labeling by amino acids in cell culture is a differential proteomic technique based on mass spectrum and has become a common technique in quantitative proteomics [26]. Stable-isotope labeling by amino acids in cell culture has the advantages of a predictable mass shift and the incorporation of the tags during cell growth, prior to sample preparation, with a consequent minimization of potential biases due to separate handling of the samples [27]. This versatile strategy has been applied successfully to study a large variety of biological systems and has resulted in many important discoveries [28–30].

Table 1. Summary table of publications on proteomic studies of MM

Materials	Technique	Comments	References
Serum	SELDI-TOF/MS	Analysis of SELDI-TOF/MS data of myeloma-associated lytic bone disease	Hong *et al.* [76]
Serum	MALDI-TOF/MS	Construction of a MM diagnostic model	Wang *et al.* [77]
BM	2-DE	Comparative proteomic analysis of primary MM cells	Xiao *et al.* [78]
MM cell lines	2-DE	Proteomic evaluation of dexamethasone-mediated apoptosis in MM cells	Rees-Unwin *et al.* [79]
MM cell lines	2-DE	Functional proteomic study of mechanism of ATO on MM cells	Ge *et al.* [80]
MM cell lines	Protein array	Proteomic analysis of the signaling state of bortezomib-treated MM cells	Mitsiades *et al.* [81]
MM cell lines	Label-free quantitation/ phosphoproteomics	Phosphotyrosine proteomic profile of MM cells	St-Germain *et al.* [83]
MM cell lines	SRM/phosphoproteomics	Measurement of protein phosphorylation stoichiometry in MM cells	Jin *et al.* [43]
BM	TiO_2 enrichment/ phosphoproteomics	Phosphoproteomic analysis of primary MM cells	Ge *et al.* [88]
Serum	LC/MS	Identification of potential bortezomib-response markers in MM patients	Hsieh *et al.* [82]

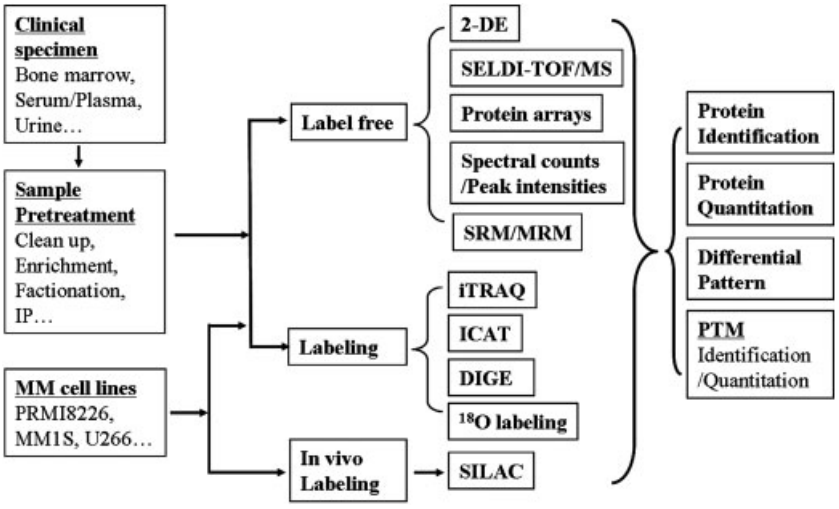

Figure 1. Schematic representation of various proteomic approaches available to study MM. SILAC, stable-isotope labeling by amino acids in cell culture; SRM, selected reaction monitoring; and MRM, multiple reaction monitoring.

Proteolytic ^{18}O labeling is a technique where proteolytic labeling and stable isotope incorporation occurs simultaneously during digestion [31, 32]. In addition to comparative quantitation [31–33], this method has also been used to provide labeled peptides for absolute quantitation [34], discover disease-specific biomarkers [35–37], quantify the *N*-glycosylation site occupancy [38], and identify changes in phosphorylation [39]. With continued advances in software and instrumentation, the $^{18}O_2$-labeling method promises increased the applications for large-scale proteomic applications.

Selected reaction monitoring (SRM, also known as multiple reaction monitoring) is a new promising approach that does not use labeling and stable isotopes to obtain quantitative information [40]. SRM is an MS technique for the targeted detection and quantification of selected proteotypic peptides with known fragmentation properties in a complex sample matrix by using a new generation mass spectrometers such as triple quadrupole MS [40, 41]. It has the unique feature to quantify multiple proteins in one analysis with high reproducibility [42]. Especially, this approach has proved particularly successful for protein modification profiling, such as phosphorylation and glycosylation [43]. In particular, the Human Proteome Detection and Quantitation Project has suggested that SRM with triple quadrupole LC-MS/MS should be the main platform to be used to detect and quantify the 21 500 human proteins in blood in the nanogram *per* milliliter range [44]. Therefore, this technique will have a crucial impact on the clinical data validation at the protein level in the future.

SELDI-TOF MS was introduced as a variation on the MALDI concept and holds the promise for biofluid clinical proteomics [45–47]. Lots of applications using SELDI-TOF MS were published for diagnostic of cancers [48–52]. Other recent articles concerning protein–protein interactions [53], phosphorylated, and glycosylated proteins [54] also demonstrated the great potential of the technique. In addition, improvements in MS instrumental performances and standardized procedures could be expected, contributing further to more reliable and faster biomarkers discovery.

Protein arrays, also known as protein chips, are miniaturized, parallel assay systems that contain small amounts of purified proteins in a high-density format [55, 56]. They allow the simultaneous determination of a variety of analytes from small amounts of samples in a single experiment. The major breakthrough came from a report by Zhu *et al.* in which a proteome wide protein array consisted of 5800 unique yeast proteins on a modified microscopic slide that bears all adjectives of a protein array was fabricated and applied to identify calmodulin- and phospholipid-binding proteins [56]. Protein microarray technology has been shown to be a useful tool for multiplexed detection and proteomic studies. Novel applications utilizing protein microarrays and new protein microarray technologies are continually emerging, especially for disease-related biomarker study.

PTMs of proteins control many biological processes, and examining their diversity is critical for understanding mechanisms of cell regulation. Due to their key functions in cellular processes, PTMs have aroused lots interest [57]. Altered levels of PTMs, sometimes in the absence of protein expression changes, are often linked to cellular responses and disease states. Therefore, the comprehensive analysis of the cellular proteome would not be complete without the identification and quantification of the extent of PTMs of the individual proteins [58]. Among more than 300 different types of PTMs, phosphorylation is one of the best-characterized modifications [59]. Protein phosphorylation–dephosphorylation events play a primordial role in cell functions. Characterization of phosphorylation status is critical to the elucidation of signal transduction pathways and to the understanding of the mechanisms of disease and drug actions [60, 61]. Phosphoproteomics usually refers to a large-scale analysis of protein phosphorylation using MS-based strategies [62–64]. Recent successes in this area owe much to the development of MS instrumentation such as

the linear ion trap and the Orbitrap, as well as novel phosphoprotein/peptide enrichment techniques [65–67]. Given the extensive alterations of the ERK [68], jun kinase [69], STAT [70], and AKT kinase [71] signaling cascades in MM cells, phosphoproteomics is critical to the elucidation of signal transduction pathways and the understanding of MM pathogenesis.

3 Proteomic applications to MM research

3.1 Proteomic analysis of clinical samples

Currently, MM is still an incurable plasma cell malignancy and frequently associated with poor prognosis. It usually evolves from an asymptomatic premalignant stage known as MGUS [72]. MGUS develops into MM or related malignancy at a rate of 1% *per* year [72]. There are no unequivocal biomarkers that distinguish MGUS from MM tumor cells, and hence it is not possible to predict if and when MGUS will progress to MM. Therefore, it is important to identify biomarkers that can distinguish MGUS from MM.

In addition to finding the biomarkers that can predict if and when MGUS will progress to MM, it is also important to identify biomarkers for diagnostic and/or prognostic purposes. A diagnosis of MM is established using blood and urine tests. Staging with serum calcium, creatinine, hemoglobin, and most importantly the concentration of the ''monoclonal serum protein'' was established in 1975 by Durie and Salmon [73]. The International Staging System determined in 2005 uses those markers as well as serum albumin and β2-microglobulin [74]. Recently, a quantitative MS assay for monoclonal proteins is developed and assessed for its value in monitoring MM progression and relapse [75]. The implementation of this single quantitative test could offer advantages over the qualitative tests currently used to follow MM patients.

Hong *et al.* [76] developed a method for assessing the quality of SELDI-TOF data based on a correlation matrix. The correlation matrix approach was applied to the MM-associated lytic bone disease study to efficiently identify low-quality spectra prior to postanalysis. The results demonstrated the potential application of this method in the identification of biomarkers for myeloma associated lytic bone disease. Recently, Wang *et al.* used proteomic fingerprint technology combining magnetic beads with MALDI-TOF MS to analyze MM sera to determine whether there are distinct and reproducible protein fingerprints potentially applicable for the diagnosis of MM [77]. Serum samples from 54 MM patients and 108 non-MM donors were tested and a panel of three biomarkers was identified. The results demonstrated that the biomarker classification model based on this technology was suitable for preliminary assessment of MM and could potentially serve as a useful tool for MM diagnosis. More recently, our group used 2-DE to compare the proteomes of purified MM and normal plasma cells [78]. Anti-CD138-based immunomagnetic bead-positive selection was performed to purify plasma cells from patients and healthy donors. A total of 43 differentially expressed proteins were identified. Furthermore, we showed that annexin A1 are involved in the effects of dexamethasone in MM cells and could represent a novel potential therapeutic target. These findings we reported may improve the understanding of the pathogenesis of MM and form the basis for the development of novel potential therapeutic targets.

3.2 Proteomic studies of mechanisms of drug action and drug resistance

The use of proteomic tools to investigate the effects of drug treatment on cell lines can provide relevant information regarding the mechanisms behind its efficacy; it can also give insights into the modifications that accompany drug resistance. For instance, one study used the classical 2-DE to identify the dysregulated proteins, following dexamethasone treatment in MM cells [79]. The authors identified a total of 18 dysregulated proteins in response to dexamethasone treatment. The data revealed significant upregulation of FK binding protein 5, following dexamethasone treatment in dexamethasone-sensitive cell line but did not change in intensity in dexamethasone resistant cell line, showing a possible involvement of this protein in the mechanism of resistance. This information is important since the spectrum of use of the drug might be widened by the knowledge of the mechanism of resistance.

The use of arsenic trioxide (ATO) in MM is a promising targeted therapy. Recently, our group [80] have investigated the effects of the ATO on the MM cell line U266 using a combination of 2-DE, RNAi, flow cytometry, and Western blotting. Several clusters of proteins altered in expression in U266 cells upon ATO treatment were identified, including downregulated signal transduction proteins and ubiquitin/proteasome members, and upregulated immunity and defense proteins. The in-depth functional studies suggested a pivotal role for the 14-3-3ζ in ATO-induced apoptosis. These findings implicate 14-3-3ζ as a potential molecular target for drug intervention of MM and thus improve our understanding on the mechanisms of antitumor activity of ATO.

The proteasome inhibitor bortezomib represents an important advance in the MM treatment. To elucidate the mechanism of apoptosis induced by bortezomib, Anderson group performed proteomic analysis of myeloma cells treated with bortezomib *versus* control cells by using multiplex immunoblotting arrays [81]. The authors demonstrated that in addition to downregulating the expression of anti-apoptotic proteins, bortezomib inhibits genotoxic stress-response pathways. These studies, therefore, provide the framework for clinical use of this agent in combination with conventional chemotherapy. To identify possible early

biomarkers of the bortezomib response in MM patients, Hsieh *et al.* demonstrated that the plasma levels of apolipoprotein C-I (apoC-I) and apoC-I′ increased in MM patients who responded to bortezomib therapy [82]. The results suggest that apoC-I and apoC-I′ may be two potential early biomarkers of the bortezomib response in MM patients. However, before apoC-I and apoC-I′ can be used for clinical applications as biomarkers of bortezomib response in MM patients, the functional relationship between these proteins and bortezomib response must be established.

3.3 Phosphoproteomic studies of MM

So far, three studies have used a phosphoproteomic approach to investigate MM. The research article by Moran and colleagues [83] can be considered the father of phosphoproteomic study on MM. In this study, label-free quantitative phosphoproteomic method was used to identify and quantify phosphotyrosine (pY) sites modulated by fibroblast growth factor receptor 3 (FGFR3) activation and inhibition in MM cells. FGFR3 is a receptor tyrosine kinase and interacts with various signaling pathways including MAPK and PI3K [84]. Activation of FGFR3 has been associated with the pathogenesis and chemoresistance of MM [85]. However, the FGFR3 signaling pathways in MM remain to be fully understood. The results presented in this study provide novel insight into the function of FGFR3 in MM cells and constitute an outline of the FGFR3 network in the myeloma model. Importantly, this study also demonstrated the potential utility of pY-directed phosphoproteomics to measure drug pharmacodynamics since it provided a measure of drug-target modulation and insight into drug mechanisms.

Lyn kinase is the predominant src family kinase in B cells and is reported to play a key role in the growth and apoptotic regulation of hematopoietic cells and their malignancies including MM [86]. It has been reported that phosphorylation of Lyn kinase is associated with mechanistically defined effects on Lyn activity [86, 87]. By using a more elegant approach, the same group [43] demonstrated that the phosphorylation stoichiometries of two phosphorylation sites on Lyn kinase could be determined in human MM-derived cell lines and xenograft tumors. Their method, based on the combination of high resolution FTMS and SRM with label-free quantification, allowed for the quantification of relative phosphorylation stoichiometries at less than 1% and over a range of at least two orders of magnitude. With their label-free approach, more accurate information about the signaling pathways in normal and diseased tissue may be retrieved and compared. Importantly, their method may have general utility for phosphoproteomic studies including the measurement of signaling pathways in clinical samples and preclinical models.

Recently, our group has attempted the phosphoproteomic analysis of primary human MM cells [88]. In this study, we used a separation strategy involving immunomagnetic bead-positive selection of MM cells, preparative SDS-PAGE for prefractionation, in-gel digestion with trypsin, and titanium dioxide (TiO_2) enrichment of phosphopeptides, followed by LC-MS/MS analysis employing a hybrid LTQ-Orbitrap mass spectrometer. This analysis led to the identification of 530 phosphorylation sites from 325 unique phosphopeptides corresponding to 260 proteins. This data set provides an important resource for future studies on phosphorylation and carcinogenesis analysis of MM.

4 Concluding remarks and outlook

This review aims to summarize the emerging impact of proteomics in MM research. There are two main expectations from proteomic analysis of MM. The first is to discover new molecular targets associated with different stages of MM development. The second is to decipher the molecular mechanisms and signaling events that lead to MM development. Currently, the application of proteomics to MM is at a very early stage, and without subsequent downstream analyses, proteomic experiments merely provide lists of protein data with little practical value. Proteomics is a rapidly developing science and anticipated to be very useful to improve our understanding of the MM pathogenesis, develop novel anti-MM therapeutic strategies, and discover novel MM biomarkers.

In the future, the use of SELDI-based techniques, for the identification of biomarkers, has the potential to forge a rapid diagnostic test for either the preclinical or the early stages of malignant change. In parallel, the further development of protein chip-based technologies highlights how a technology can be adapted for rapid large-scale analysis applicable in the clinical trial setting. This technology is still being developed to increase sensitivity, reproducibility, and depth of coverage, but where the investigator has prior knowledge of the proteins that need to be quantified from a sample, they provide a good alternative to more stochastic screening approaches such as 2-DE or MS. Newer generation mass spectrometers as well as emerging quantitative approaches such as iTRAQ and SRM will also provide enhanced sensitivity and accuracy for the detection and quantification of proteins in MM clinical samples. Especially, the promise of phosphoproteomics will revolutionize our understanding of the MM pathogenesis. It could be foreseen that proteomic technologies will eventually improve treatment and outcome of patients with MM.

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