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Review

Proteomics: addressing the challenges of multiple myeloma

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Multiple myeloma (MM) is a malignancy of terminally differentiated B-lymphocytes that accounts for ∼13% of all hematologic cancers. Despite a wealth of knowledge describing the molecular biology of MM as well as significant advances in therapeutics, this disease remains incurable. Since proteins govern the cellular structure and biological function, a wide selection of proteomic approaches holds great promise for increasing our understanding of this disease, such as by investigating the dynamic nature of protein expression, cellular and subcellular distribution, post-translational modifications, and interactions at both the cellular and subcellular levels. The aims of this review are to introduce the available and emerging proteomic technologies that have potential applications in the study of MM and to highlight the current status of proteomic studies of MM. To date, although there have been a limited number of proteomic studies in MM, those performed have provided valuable information with regard to MM diagnosis and therapy. The potential future application of proteomic technologies is expected to provide new avenues in MM diagnostics, individualized therapy design and therapy response surveillance for the clinician.

Keywords proteomics; multiple myeloma; biomarkers; mass spectrometry; post-translational modifications

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Introduction

Multiple myeloma (MM) is a malignant plasma cell (PC) disorder that accounts for ∼13% of all hematologic cancers [1]. The incidence varies globally from 1 per 100,000 people in China to about 4 per 100,000 people in most developed countries [2]. Although major advances have enhanced the understanding of the multifactorial influence of genetic and environmental determinants on MM, the etiology of the disease remains elusive [2]. The array of proteins found within the cell and their interactions as well as modifications hold the key to understanding biologic systems [3]. 'Proteomics' describes the protein population of a cell, characterized in terms of localization, post-translational modification (PTM), interactions, and turnover, at any given time [4]. Therefore, the description of processes underlying MM cell development, pathogenesis, and the functional activity of MM cells will be enhanced by application of proteomic approaches for protein characterization, study of protein–protein interaction, and, in particular, relative quantification [5,6]. It is important to note here that, although the applications of proteomic approaches in MM are still in their infancy, those performed highlight the potential future impact of these technologies in the discovery of novel biomarkers, proteins associated with drug resistance and the identification of biomarkers, which may facilitate the development of a rapid diagnostic test applicable in the clinical setting [5,6]. The aim of this review is, in addition to discussing its current status in the study of MM, to introduce the currently available proteomic technologies to the MM research community.

MM: the disease

Despite advances in clinical care, MM remains an almost universally fatal disease with a median survival of 3–4 years following conventional treatment and 5–7 years with high dose therapy followed by autologous stem cell transplantation [1,2]. Significant advances in understanding the underlying genetic and epigenetic alterations that lead to tumor initiation and progression have been elucidated recently [7]. Current models assume that MM evolves through a multistep transformation process. The disease

springs from a PC and proceeds *via* monoclonal gammopathy of undetermined significance (MGUS) to clinically overt myeloma [8]. MM cells are characterized by genetic instability with several chromosomal abnormalities. Translocations involving the immunoglobulin heavy-chain (IgH) locus on chromosome 14 (14q32) are commonly observed in MM and MGUS are therefore thought to be early pathogenetic events [9,10]. The genetic alterations, as well as external stimulation from the microenvironment, lead to activation of several proliferative/antiapoptotic signaling cascades, including phosphatidylinositol-3 kinase/Akt, NF-κB, Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-related kinase (ERK), and Janus kinase (JAK) 2/signal transducers and activators of transcription (STAT) [11,12]. Interactions between these pathways lead to proliferation, survival, resistance to therapy as well as dynamic migration, and adhesion of MM cells to the bone marrow (BM) milieu [10].

The advent of new technologies, such as gene-expression profiling (GEP), has provided the necessary tools to study MM in unprecedented detail. Pioneering studies using microarray by Zhan *et al*. [13] have identified novel MM-associated genes suggesting a gene-based classification system for MM. Complementary DNA arrays have led De Vos *et al*. [14] to important findings on the role of intercellular signaling genes in the biology of malignant PCs. Several groups have used GEP to evaluate drug response in MM patients. Mulligan *et al.* [15] have identified a pretreatment expression pattern and predictive markers that could differentiate between bortezomib and dexamethasone (Dex) response. Gene expression arrays have provided important insights to clarify the anti-tumor activity of Dex in MM [16]. These studies have made significant contributions to our understanding of the molecular development as well as mechanisms of drug resistance of MM.

Proteomics technologies

Current proteomics techniques are facing the limitations in terms of their capacity to analyze the entire proteome of a tissue or biological fluid in a single reaction [17]. In bodily fluids like serum or plasma, protein concentrations vary over more than 10 orders of magnitude and the presence of high-abundant proteins invariably masks the detection of low-abundant proteins [18]. The strategy of many researchers in the field is thus oriented towards either combining two or more complementary technical approaches and/or analyzing the sub-proteome of interest [19]. Many techniques for de-complexion of the proteome, enrichment or depletion of particular sub-proteomes, and separation techniques for proteins/peptides have emerged in parallel with the development of mass spectrometry (MS) of high capacity, resolution, and accuracy [20]. Excellent in-depth reviews on MS are available elsewhere [21–23].

Gel-based proteomics

Over three decades, two-dimensional gel electrophoresis (2-DE) has been proven to be a reliable and efficient method for the separation of complex protein mixtures based on the mass and charge. Its advantages are powerful for protein separation, relative simultaneous quantification of proteins on gel images and identification of protein isoforms and posttranslational modifications. Unfortunately, 2-DE is a time-consuming and labor-intensive process. Besides, this technique has low dynamic range and gel-to-gel variability [24].

As an improvement, two-dimensional difference gel electrophoresis (DIGE) overcomes the problems associated with traditional 2-DE and allows more accurate and sensitive quantitative proteomics studies [25]. As a separation, detection, and quantification technique, DIGE is an important tool, especially for clinical laboratories involved in the determination of protein expression levels and disease biomarker discovery.

LC-MS/MS-based proteomics

In liquid chromatography (LC)-based proteomics, mixtures of proteins are specifically digested to peptides by proteases, separated by one or more dimensions of LC, and coupled with automated MS/MS [26]. Proteins are identified on the basis of one or more identified peptide sequences. The most common two-dimensional LC separation combines strong cation exchange chromatography with reverse phase (RP) chromatography coupled with automated MS/MS, which was first described as multidimensional protein identification technology [27]. The fractions separated by RP are injected into the mass spectrometer via online electrospray ionization or spotted to the MALDI target plates for analysis by MS/MS [28].

In label-free LC-MS-based proteomics, protein quantification is generally based on two categories of measurements: (i) measuring and comparing the mass spectrometric signal intensity of peptide precursor ions belonging to a particular protein [29–32] and (ii) counting and comparing the number of fragment spectra identifying peptides of a given protein [33–35]. Peptide peak intensity or spectral count is measured for individual LC-MS/MS or LC/LC-MS/MS runs and changes in protein abundance are calculated via a direct comparison between different analyses.

MS-based quantification methods are based on labeling proteins or peptides prior to the MS analysis. These include: stable-isotope labeling by amino acids in cell culture (SILAC), isotope-coded affinity tags and iTRAQ

and ^{18}O labeling. A detailed description of these techniques can be found elsewhere [36,37].

Protein arrays

Protein arrays are miniaturized, parallel assay systems that contain small amounts of purified proteins in a high-density format [38,39]. Protein arrays can be classified into two types, depending on their application: analytical and functional protein arrays. Analytical protein arrays use well-characterized molecules that have known specific activities as immobilized probes, such as antibodies, peptide–MHC complexes or lectins. Functional protein arrays are mainly used to screen various types of protein activities: including protein–protein, protein–lipid, protein–DNA, protein–drug and protein–peptide interactions; to identify enzyme substrates; and to profile immune responses [39,40]. These arrays, and in particular human proteome arrays, have been successfully applied to the screening of disease-related biomarkers [41,42].

PTMs: focus on phosphorylation

Protein PTM has an intrinsically important role in processes such as clotting, hematopoiesis, the immune response and so forth. Due to their crucial functions in cells, protein modifications have become one of the major focuses in proteomic technologies (collectively called 'modificomics') [43]. Protein phosphorylation is one of the most frequent PTMs in eukaryotic cells, where about one-third of all proteins in eukaryotic cells are phosphorylated at any given time [44]. Phosphoproteomics usually refers to a large-scale analysis of protein phosphorylation using MS-based strategies [45–47]. An efficient MS-based method that not only identifies the PTM proteins but also measures the degree of the modification would be very informative for understanding protein function and signaling cascades in various cells. With the recently developed relative quantification methods by MS, phosphoproteomic quantification has become possible [48–50].

Proteomic analysis of MM

To date, there have been very few published reports on the application of proteomic technologies in the study of MM. Concerning proteomic analysis of clinical samples, for example, one work describing the use of proteomic fingerprint technology combining magnetic beads with MALDI-TOF MS to analyze MM sera from MM patients has been recently reported [51]. Serum samples were tested in groups representing MM and non-MM. The results suggested that using proteomics approaches, such as magnetic beads and MALDI-TOF MS in combination with bioinformatics tools, could facilitate the discovery of new biomarkers for identifying individuals with MM. The panel of three selected biomarkers was suitable for preliminary assessment of MM and could potentially serve as a useful tool for MM diagnosis and differentiation diagnosis. However, these biomarkers were unable to differentiate between MM and other PC dyscrasias including MGUS, Waldenstrom's macroglobulinemia and solitary plasmacytoma. Therefore, it is necessary to increase their samples size in order to identify additional markers to identify MM patients unequivocally.

More recently, Xiao *et al.* [52] performed proteomic analysis of cellular protein extracts from MM cells and normal PCs. Plasma cells from nine patients with newly diagnosed MM and nine healthy donors were purified by using anti-CD138-based immunomagnetic bead-positive selection. The protein profiles of purified MM and normal PCs were compared using 2-DE. A total of 43 differentially expressed proteins were identified. Further functional studies showed that annexin A1 knockdown modestly induced lethality alone and potentiated the effects of Dex on both Dex-sensitive and Dex-resistant MM cells. Although more studies are needed, the proteins identified here could potentially be biomarkers and/or therapeutic targets for MM. Furthermore, Ge *et al.* [53] developed the first biological model to study MM reversion using the H-1 parvovirus as a tool. The SILAC method was employed to compare the protein profiles of parental MM cells and revertants. The quantitative proteomics analysis identified many proteins that potentially affect MM reversion and implicate previously unconsidered pathways in the process of tumor reversion. Through functional studies, they also demonstrated that myeloma reversion could be operated, at least in part, through inhibition of the STAT3 pathway. These findings thus provided some molecular explanations for tumor reversion in MM and could lead to the development of new anticancer drugs.

Proteomic tools have been used to investigate the effects of drug treatment on cell lines, which can contribute functional, system-level data sets, provide insight into suitable drug targets, more broadly profile compound activities, and identify key biomarkers to assess clinical outcomes. For instance, Rees-Unwin *et al.* [54] used global protein expression analysis to characterize the pathways of Dex-mediated apoptosis and resistance in MM. Analysis of MM.1S cells by 2-DE identified a series of proteins that were up- and down-regulated following Dex treatment. Most notably, they identified FK-binding protein 5, which was over-expressed and involved in protein folding and trafficking in the MM.1S but not in the MM.1R cell line following Dex treatment. Their results demonstrated that following steroid receptor signaling, the cells carried out a number of adaptive responses prior to cell death.

Interfering with these adaptive responses may enhance the myeloma killing effect of Dex. However, it is necessary to perform more functional studies to gain further insight into the signaling pathways in which these proteins are acting.

Bortezomib (also called PS-341 or Velcade) is the first proteasome inhibitor approved for newly diagnosed and relapsed MM and is currently being tested in many clinical trials against other types of cancers. To further elucidate the mechanism of apoptosis induced by bortezomib, Anderson group performed proteomic analysis of myeloma cells treated with bortezomib vs. control cells by using multiplex immunoblotting arrays [55]. The authors demonstrated that bortezomib down-regulated the expression of several proteins involved in DNA repair, confirming the observation that bortezomib sensitized MM cells to the action of DNA-damaging chemotherapeutic agents. Their study suggests that the combination of bortezomib with conventional chemotherapy will augment clinical effectiveness and overcome resistance in patients with relapsed refractory MM.

Arsenic trioxide (ATO) is a promising agent for progressive and refractory MM by inducing growth inhibition and apoptosis in MM cells [56]. In order to determine the mechanisms of ATO activity, Ge *et al.* [57] used proteomics to analyze the ATO-induced protein alterations in MM cell line and then investigated the molecular pathways responsible for the anticancer actions of ATO. Several clusters of proteins that altered the expression in U266 cells upon ATO treatment were identified, including down-regulated signal transduction proteins and ubiquitin/proteasome members, and up-regulated immunity and defense proteins. Significantly regulated 14-3-3ζ and heat shock proteins (HSPs) were selected for further functional studies. Over-expression of 14-3-3ζ in MM cells attenuated ATO-induced cell death, whereas RNAi-based 14-3-3ζ knockdown or the inhibition of HSP90 enhanced tumor cell sensitivity to the ATO induction. These results demonstrated the usefulness of proteomics in identifying additional therapeutic targets that may be exploited to overcome drug resistance. To further understand the molecular mechanism of 14-3-3ζ in MM pathogenesis, Ge *et al.* [58] performed a systematic proteomic analysis of 14-3-3ζ-associated proteins in MM cells. This method was termed as quantitative immunoprecipitation combined with knockdown (QUICK) [59]. By using this QUICK method, a total of 292 proteins were identified and 95 of them were novel 14-3-3ζ putative partners. These results provided new clues about the molecular mechanism of 14-3-3ζ in MM pathogenesis. This study also demonstrates that QUICK is a useful approach to detect specific protein–protein interactions with very high confidence.

With respect to GEP, an important advantage of proteomic technologies lies in the possibility of studying PTMs, which can ultimately give insights into protein function. One of the most common PTMs is phosphorylation. Given the extensive alterations of the ERK [60], jun kinase [61], STAT [62], and AKT kinase [63] signaling cascades in MM cells, phosphoproteomics holds promise in MM to investigate changes in phosphorylation by specific kinases of interest or on a global proteome-wide scale. The work by St-Germain and colleagues [64] can be considered as the first phosphoproteomic study on MM. To define the fibroblast growth factor receptor 3 (FGFR3) network in MM, phosphotyrosine (pY) proteomic method was used to identify and quantify pY sites modulated by FGFR3 activation and inhibition in MM cells. They identified and quantified several pY sites as a result of FGFR3 activation and drug inhibition. Their results substantially increased our understanding of FGFR3 function and provided a framework for studying appropriate signaling networks activated by this receptor in MM. Importantly, this approach coupled with label-free MS quantification may have particular utility to identify activated kinases and monitor their modulation in tumors and animal models.

By using a more elegant approach, the same group [65] demonstrated that the phosphorylation stoichiometries of two phosphorylation sites on Lyn kinase could be determined in human MM-derived cell lines and xenograft tumors. Lyn is the predominant Src family protein-tyrosine kinase in B cells and is implicated in B cell-related malignancies including MM [66]. Their method was based on the combination of high-resolution Fourier transform MS (FTMS) and selected reaction monitoring (SRM) MS. By analyzing extracted ion currents by FTMS, the phosphorylation stoichiometries of two tyrosine residues (tyrosine-194 and tyrosine-397) in the protein tyrosine kinase Lyn were determined in MM cells and MM xenograft tumors. This approach may have general utility for phospho-proteomics studies including the measurement of signaling pathways in clinical samples and preclinical models.

Recently, Ge *et al.* [67] performed the phosphoproteome analysis of MM cells using TiO_2 enrichment directly coupled with the LC-MS/MS approach. After database search, stringent filtering, and manual validation of neutral loss in the MS/MS spectra, a total of 530 phosphorylation sites on 325 phosphopeptides were identified in the primary MM cells. The newly identified phosphoproteins may contribute to the identification of novel regulatory mechanisms of MM development. By using SILAC in combination with phosphoprotein enrichment and LC-MS/MS analysis, Ge *et al.* [50] investigated the differential MM phosphoproteome upon proteasome inhibition. Many potential novel signaling proteins and associated signaling pathways were confidently identified. The functional results indicated that perturbations in stathmin phosphorylation could play a significant functional role in mediating

bortezomib-induced apoptosis in MM cells and the bortezomib-induced changes in the MT stabilization could also be attributed to the bortezomib-induced phosphorylation of stathmin. By correlating the phosphoproteomic data with further functional studies, these results provided novel insights into the mechanisms of bortezomib actions in MM cells.

Conclusions and future perspectives

In the past decade, proteomics technology has made great advances. And with the advent of powerful and sensitive mass spectrometers, sophisticated databases and bioinformatics software, it is now possible to investigate the protein changes that may underlie many diseases. However, the use of such technology to investigate MM remains a challenging problem. Initial proteomics studies aiming at the identification of biomarkers and molecular targets for MM are mostly small-scale gel-based approaches. In recent years, more large-scale approaches adopting MS/MS-based proteomics are reported. These studies generate large amounts of data that require extensive validation and follow-up analysis. Therefore, it is clear from a review of the literature that progress is being made in this area, but a great deal still remains to be done.

No proteomics technique is currently able to reveal the complete human proteome, therefore the choice of the technique should be guided by the specific research question and ideally a combination of complementary techniques should be applied. Especially, 'narrowing the field' and functional targeting of signaling complexes can offer improved chances of success. Sub-cellular fractionation is a relatively simple approach that can produce significant results. Affinity tagging of cell surface proteins with biotin and glycosylation techniques can also be used to identify the numbers of cell-surface or transmembrane proteins. Quantification of protein changes in MM PCs and comparison with normal PCs are also clearly important aims. Whilst techniques such as SILAC are perfectly applicable to cell line studies grown in heavy and light isotope-labeled amino acids, this technique is not readily appropriate for primary cells or tissues. However, SILAC is possible to be used in co-culture model systems that are designed to mimic the tumor microenvironment. Invariably, with primary cells we must rely on label-free MS quantification, ^{18}O labeling or iTRAQ approaches. In this respect, the increasingly sophisticated label-free quantification approaches that are coupled with sub-cellular fractionation and targeting of signaling complexes allow the possibility that critical protein changes will be identified in MM cells. The identification of such changes will provide important advances in understanding MM pathogenesis.

There are three main expectations for proteomic analysis of MM. The first is to decipher the molecular mechanisms and signaling events that lead to MM development. The second is to identify proteins that can be used for diagnosis or prognosis. The third is to identify potential targets for therapeutic intervention. It is clear from the above discussion that proteomics approaches have identified a number of proteins that can be potential targets for therapy in MM, but clearly there is still considerable scope for new discoveries. In conclusion, proteomics using advanced MS methods offers the opportunity to identify new therapeutic targets and biological mechanisms in MM. The challenge is to develop appropriate targeted, mechanistic and functional approaches that allow the identification of both novel and known protein species. However, successful proteomic studies on MM must be integrated and validated with biological and clinical studies. The challenge will be now to translate this fundamental knowledge into new prognostic, diagnostic, and therapeutic tools that can improve treatment and outcome of patients with MM.

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