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REVIEW ARTICLE

Proteomics in Rheumatoid Arthritis Research

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Although rheumatoid arthritis (RA) is the most common chronic inflammatory autoimmune disease, diagnosis of RA is currently based on clinical manifestations, and there is no simple, practical assessment tool in the clinical field to assess disease activity and severity. Recently, there has been increasing interest in the discovery of new diagnostic RA biomarkers that can assist in evaluating disease activity, severity, and treatment response. Proteomics, the large-scale study of the proteome, has emerged as a powerful technique for protein identification and characterization. For the past 10 years, proteomic techniques have been applied to different biological samples (synovial tissue/fluid, blood, and urine) from RA patients and experimental animal models. In this review, we summarize the current state of the application of proteomics in RA and its importance in identifying biomarkers and treatment targets.
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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint destruction, functional impairment, disability, and premature mortality (1-3). The bone and cartilage destruction rarely heals, the damage accumulating over time (4-7). With regard to inflammation, interfering with the inflammatory cascade before it is fully established is most effective. Therefore, it is evident that therapeutic intervention will have greater effect on the outcome if started early, and ideally, if commenced even before damage has occurred. Presently, RA is defined by the presence of four of the seven criteria developed by the American College of Rheumatology (ACR) in 1987 (8), or a total score of six or greater (of a possible 10) from the individual scores in the four domains in the 2010 Rheumatoid Arthritis Classification Criteria of the American College of Rheumatology/European League Against Rheumatism (EULAR) collaborative initiative (9,10).

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Abbreviations: RA, rheumatoid arthritis; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; DMARDs, disease-modifying antirheumatic drugs; DAS28, disease activity score 28; MS, mass spectrometry; LC, liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; IEF, isoelectric focusing; CID, collision-induced dissociation; PMF, peptide mass fingerprinting; AMT, accurate mass and time tag; iTRAQ, isobaric tags for relative and absolute quantitation; SILAC, stable isoptope labeling with amino acid in cell culture; ICPL, isotope-coded protein label; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; TOF, time of flight; FT−MS, Fourier transform ion cyclotron; LC−ESI-MS/MS, liquid chromatography−ESI-tandem mass spectrometry; HPP, human Proteome Project; HPPP, human Plasma Proteome Project; TAP, tandem affinity purification; IP, immunoprecipitation; CBP, calmodulin binding peptide; TEV, tobacco etch virus; FLS, fibroblast-like synovial; DEPs, differentially expressed proteins; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; OA, osteoarthritis; sCD14, soluble CD14; MRM, multiple reaction monitoring; IL-6R, interleukin-6 receptor; iTRAQ, isobaric tag for relative and absolute quantitation; ICAT, isotope-coded affinity tag; cICAT, cleavable ICAT

However, the current classification criteria do not allow early diagnosis (11,12).

Treatment and prevention of the joint destructive process are possible, mainly with the use of steroids, disease-modifying antirheumatic drugs (DMARDs), biologics, or combinations thereof (13-16). Unfortunately, the use of drug combinations may rely on recommendations and expert opinions rather than on algorithms or criteria derived from clinical studies (17,18). Moreover, no precise universal and/or easy-to-use assessment methods exist that allow for the evaluation of disease activity and the prediction of disease severity. The disease activity score 28 (DAS28) (19) and the Sharp/van der Heijde scoring systems (20) are used to guide treatment decisions, but these assessment tools cannot be easily applied in daily practice. Thus, there is an unmet need for novel biomarkers that can complement conventional measures and that allow precise monitoring of the disease activity and severity of RA.

The proteome, the entire set of proteins produced by a cell or organism (21), varies with time and the distinct requirements, or stresses, that the particular cell or organism undergoes. Proteomics is the large-scale study of proteomes (22,23). It is an emerging area that includes such technical disciplines as light and electron microscopy, array and chip experiments, yeast two-hybrid assay, and mass spectrometry (MS). Because proteomics investigates the overall picture of intracellular protein composition, structure, and activity, it is capable of identifying biomarkers and improving the understanding of pathogenesis. Therefore, this useful tool meets the needs of RA research. During the last 10 years, proteomic techniques have led to numerous advances in the analysis of different types of biological samples collected from RA patients, including synovial tissue/fluid, blood, and urine (Table I). In this review, we summarize the status of the applications of proteomics for RA and their importance in identifying potential biomarkers and treatment targets.

PROTEOMICS

Methods of studying proteins

Proteomics is the large-scale study of the expression, structure, function, modifications, and interactions of proteins as well as how these aspects of the proteins change in different environments and conditions. Transformational new technologies of MS and liquid chromatography (LC) have enabled rapid advances in proteomics. A typical MS-based proteomic experiment consists of six steps: protein extraction, protein fractionation, peptide fractionation, LC-MS/MS analysis, peptide/protein identification, and protein quantification (24). In step 1, a body fluid or biopsy specimen is obtained for the extraction of proteins. In step 2, the proteins to be analyzed are isolated from the cell lysate or tissue by biochemical fractionation tools, such as one- or two-dimensional gel electrophoresis, capillary electrophoresis, or affinity selection including affinity depletion and immunoprecipitation. In step 3, the proteins from the sample are digested enzymatically, usually with trypsin, into peptides. Step 4 requires that the peptides be separated based on their hydrophobicity using techniques including reversed-phase high-performance liquid chromatography (RP-HPLC) and isoelectric focusing (IEF). The fractionated peptides are ionized and analyzed by the mass spectrometer, which measures mass-to-charge (m/z) ratios of the peptides and their intensities (abundances). After the preliminary scans, those peptides with relatively high intensities are isolated in a data-dependent manner and fragmented by collision-induced dissociation (CID) (25), followed by tandem mass spectrometry (MS/MS) experiments (26). In step 5, peptide/protein identification is performed by various methods including database searching, de novo sequencing, peptide mass fingerprinting (PMF), and accurate mass and time tag (AMT). Finally, in step 6, protein quantification is performed using various labeling methods including isobaric tags for relative and absolute quantitation (iTRAQ), stable isoptope labeling with amino acid in cell culture (SILAC), ^{15}N or chemical protein labeling isotope-coded protein label (ICPL), as well as label-free methods involving the identification of peptides and alignments of the peptides (27).

MS is at the heart of all proteomic studies because it plays a key role in the analysis of proteins. A mass spectrometer consists of three parts: an ion source for the ionization of the peptides, a mass analyzer to measure the m/z of the ionized peptides, and a detector to detect the number of ions at each m/z value. For the ionization of the peptides, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two most frequently used techniques. As for the second part of the mass spectrometer, mass analyzers fall into four basic types: ion trap, time of flight (TOF), quadrupole, and Fourier transform ion cyclotron (FT－MS). The combina-

Table I. Various proteomic approaches in rheumatoid arthritis (RA) research

Sample used	Methods	Identified DEPs	Patients	Clinical implications	Ref.
Serum	2-DE LC-MS	CRP, S100A8 (calgranulin A), S100A9 (calgranulin B), S100A12 (calgranulin C)	Erosive RA vs non-erosive RA	CRP, S100A8, S100A9, S100A12 have been identified for prognosis of the erosive form of RA. These proteins are elevated in the serum of patients with erosive RA.	(43)
Plasma	LC-MS	Calgranulin A, B, C, CRP, thymosin β4, actin, tubulin, vimentin	RA vs Controls	This study observed elevated levels of cytoskeletal proteins and calgranulins in plasma samples from patients. RA is associated with inflammation, dysregulation of protease inhibitors and cytoskeletal fraction.	(49)
	2-DE MALDI-TOF-MS	COTL1	RA vs Controls	COTL1 were highly expressed in RA. The COTL1 polymorphism in RA patients has significant association with the levels of ACPA. Such findings demonstrated polymorphisms of the COTL1 gene could be associated with the genetic susceptibility of RA.	(50)
	SELDI-TOF MS	Apolipoprotein A-1, platelet factor 4	RA responder vs non-responder to infliximab	Apolipoprotein A-1 was predictive of a good response to infliximab, whereas platelet factor 4 was associated with non-responders.	(51)
Cells				The studies identified a number of proteins that have been implicated in the normal or pathological FLS function or that have been characterized as potential autoantigens in rheumatoid arthritis.	
Synovial fibroblasts	2DE	Uridine diphosphoglucose dehydrogenase, galectin 1 and galectin 3, BiP, colligin, HC gp-39	RA vs Control	The cytosolic enzyme uridine diphosphoglucose dehydrogenase is involved in the synthesis of hyaluronic acid; it has been suggested that the protein HC gp-39 may be involved in tissue repair and remodeling or possibly in innate host responses to pathogens containing chitinous elements; BiP can function as an autoantigen for both antibodies and T-cell responses.	(38)
	2-DE MALDI-TOF-MS	S100A4, S100A10, cathepsin D, annexin, SOD, PRX	RA vs OA	S100A4, S100A10, and cathepsin D are increased in RA and have been implicated as involved in the healthy or pathological synovial fibroblasts s function or alpha-enolase, and TPI has potential diagnostic and prognostic value for RA or annexin; SOD, PRX may be the new therapeutic targets.	(52)
	2-DE MS	PIMT, PIR, TRX1	RA vs OA	PIMT, pirin and Trx-1 affect the functions of FLS in some style and can be the drug targets of RA. They have found that the protein PIMT and PIR has a lower expression in RA. Trx-1 is only expressed in RA and may be associated with the functions of FLS. Western Blot confirmed that the expression of PIMT and pirin is lower in RA, and Trx-1 is expressed only in RA.	(53)
Neutrophils/ synoviovytes	MALDI-TOF MS	NGAL	RA vs OA	The level of NGAL in synoviovytes was significantly higher in patients with RA.	(54)
PBMCs	2DE MALDI-TOF	ACTB, FIB, HSP-5,-60	RA vs control	ACTB, FIB, HSP-5,-60 are 2-fold or more highly expressed in patients than in controls, and these protein expression patterns may have diagnostic value for RA.	(55)
	2DE WB	16 altered protein forms (most of them protein fragments)	RA vs control	The majority of the proteins differentially expressed in RA patients can be detected as protein fragments in PBMCs obtained from RA patients. This set of deregulated proteins includes several factors that have been shown to be autoantigens in autoimmune diseases.	(56)

Table I. Continued

Sample used	Methods	Identified DEPs	Patients	Clinical implications	Ref.
Monocytes/ macrophage	MALDI-TOF -LC/LC	Citrullinated Grp78	RA	Grp78 protein levels are increased in RA. ACPAs enhance NF- κ B activity and TNF α production in monocyte/macrophages via binding to surface-expressed citrullinated Grp78. Proteomic analysis revealed that the Grp78 protein is one of the cognate antigens of ACPAs.	(57)
MSCs	2DE	Cell proteome profiling	RA vs control	This study revealed DEGs related to cell adhesion processes and cell cycle progression beyond the G1 phase. RA MSCs had impaired clonogenic and proliferative potential in association with premature telomere length loss.	(51)
Synovial tissue	2DE MALDI-TOF	Calgranulin A MRP-8	RA, SpA, OA	Calgranulin A MRP-8 was markedly up-regulated in RA and SpA patients in comparison to OA patients. The analysis of the cytosolic proteome of synovial tissue is a useful approach to identify disease-associated proteins in chronic inflammatory arthritis.	(58)
Synovial fluid	2-DE LC-MS/MS	CRP and 6 members of the S100 protein family	erosive RA vs non-erosive RA	Levels of CRP and 6 members of the S100 protein family are elevated and have been identified for the prognosis of the erosive form of RA.	(43)
	MALDI-TOF MS	NGAL	RA vs OA	NGAL in SF was significantly higher in patients with RA than in those with OA upregulation of NGAL in neutrophils.	(54)
	LC-MS/MS	Apolipoprotein J, fibrinogen, haptoglobin, serum amyloid A, and complement factors (B, C3, and C9)	RA vs OA	Proinflammatory HDL in patients with RA contains a significantly altered proteome, including increased amounts of acute-phase proteins and proteins involved in the complement cascade.	(59)
Saliva	2-DE MS	Calgranulin A, calgranulin B, apolipoprotein A-1, 6-phosphogluconate dehydrogenase, peroxiredoxin 5, epidermal fatty acid-binding protein, 78kDa GRP78/BiP, and 14-3-3 proteins	RA vs Controls	GRP78/BiP showed the greatest increase in RA patients. GRP78/BiP showed the greatest increase in RA patients.	(60)

DEPs, Differentially expressed proteins; Ref, references; 2DE, 2-dimensional gel electrophoresis; LC-MS, liquid chromatography－coupled tandem mass spectrometry; CRP, C-reactive protein; MALDI-TOF-MS, matrix-assisted laser desorption ionization mass spectrometry; COTL1, coactosin-like1; SELDI-TOF-MS, surface enhanced laser desorption/ionisation time-of-flight mass spectrometry; OA, osteoarthritis; PIMT, protein isoaspartyl methyltransferase; PIR, pirin iron-binding nuclear protein; Trx-1, thioredoxin 1; NGAL, neutrophil gelatinase-associated lipocalin; PBMCs, peripheral blood mononuclear cells; WB, western blot; GRP78/BiP, glucose-regulated protein precursor; ACPA, anti-citrullinated peptide/protein antibodies; TNF α, tumor necrosis factor- α; MSCs, mesenchymal stem cells; DEGs, differential expression genes; MRP-8, myeloid related protein-8; SpA, spondyloarthropathy.

tion of the ion source and mass analyzer determines the type of mass spectrometry, for example, ESI-ion trap and MALDI-TOF. Liquid chromatography－ESI-tandem mass spectrometry (LC－ESI-MS/MS) and MALDI－MS/MS (MALDI-TOF/TOF) are still commonly used methods because of their simplicity and excellent accuracy (26).

Application of proteomics to protein profiling and protein interactions

Thousands of proteins can be identified from the complex protein mixtures in each study using the methods described above. However, to achieve biologically useful data to guide a comprehensive understanding of cellular functions, it is necessary to link the quantitative proteomic data to genomic sequences, gene expression profiles, and phenotypic data as well. Such efforts generate comprehensive proteome maps in various types of samples including cells and tissues, as well as bio-fluids such as blood (plasma/serum), ascites, cerebrospinal fluid, urine, saliva, and tears. Currently, major efforts such as the Human Proteome Project (HPP) are under way to identify the products of human genes on a large scale (28). Moreover, to support the discovery of non-invasive diagnostic biomarkers, the Human Plasma Proteome Project (HPPP) was carried out, providing a comprehensive serum proteome that can be used to identify secreted biomarker candidates (29).

Most proteins do not exert their function in isolation, but do so rather in the form of protein－protein interactions. Thus, to understand functions of proteins, MS-based methods have been used to identify interaction partners of the proteins. These methods include tandem affinity purification (TAP)-tagging (30) and immunoprecipitation (IP)-MS methods (30). The official method involves the fusion of the TAP tag to the C-terminus of the protein of interest. The tag comprises calmodulin binding peptide (CBP), followed by the tobacco etch virus protease (TEV protease) cleavage site and Protein A, which binds tightly to IgG. Protein A is at the end of the fusion protein such that the entire complex can be isolated using an IgG matrix. The latter method involves immunoprecipitation of a protein of interest to isolate the interactors of the protein using LC-MS/MS analysis. Identifying the interactors of the protein with no interaction data available can incorporate it into the known cellular networks defined by protein-protein interactions. In addition to the global profiling and identification of interactors, MS-based methods have been also applied to measure cellular locations, post-translational modifications, structures, and enzymatic activities of the proteins, thereby providing the entire spectrum of information needed to understand the functions of the proteins (31-36).

EXPLORATION OF NOVEL BIOMARKERS USING A PROTEOMIC APPROACH

Biomarkers for diagnosis

The pathogenesis of RA is complex and multifactorial. ACR/EULAR developed a set of criteria for the diagnosis of RA (8-10). Although these criteria are designated as diagnostic criteria, more precisely they are less a diagnostic tool than a set of classification criteria intended to facilitate comparisons between RA and other diseases. The criteria were based on the experience of doctors, and it is thus evident that novel biomarkers are needed to facilitate the diagnosis of RA. A substantive effort is being made to identify biomarkers, including combinations of genetic and serologic information or protein profiling using proteomic approaches.

Proteomic studies in RA are largely focused around the identification of autoantigens and protein targets by the differential screening of serum/synovial fluid or synovial/cartilage tissue (37). Kumar and colleagues separated a number of proteins from fibroblast-like synovial (FLS) cells by two-dimensional polyacrylamide gel electrophoresis and analyzed the in-gel digested proteins (38). The identified proteins included uridine diphosphoglucose dehydrogenase, galectin 1, galectin 3, BiP, colligin, and HC gp-39, all of which have been implicated in FLS function or as potential autoantigens (38). Li and colleagues reported that differentially expressed proteins (DEPs) identified in RA-FLS could be candidates for promising diagnostic indicators of RA (39). These proteins included enzymatic and structural proteins (e.g., PKM1/M2, α-enolase, ERp60, and lamin-A/C), signal transduction proteins (e.g., annexin 11, peroxiredoxin 1, and TrpRS), and heat-shock/chaperone proteins (e.g. TCP-1, GRP75, HspB5, and Bip) (39). Using data derived from microarray studies, our group demonstrated that Bip is crucial for synoviocyte proliferation and angiogenesis (40). This approach to analyzing FLS proteins was based on the fact that the synovial membrane becomes the target of a persistent inflammatory process and immune cell accumulation, leading to funda-

mental changes in the phenotype and function of FLS cells. Thus, the investigation of DEPs in FLS is a promising method to identify novel diagnostic biomarkers for RA.

Biomarkers for monitoring disease activity and disease severity

Disease activity is a central component in the assessment of patients with RA. It comprises the signs and symptoms of the disease and is fundamentally responsible for joint destruction (disease severity). The most frequently used tool for assessing disease activity is the DAS28, based on tender joint counts, swollen joint counts, and the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) (8). However, this instrument has practical the limitations of preventing immediate assessment, requiring specialized expertise, and having poor transparency for patients. For the assessment of disease severity, radiography is widely used. Although the structural damage visible on radiographs is a reflection of the cumulative disease severity and a strong predictor of disability, there is often no visible manifestation for 1～3 years after disease initiation. Moreover, there is no early biomarker to predict a high risk of joint destruction and disability (41).

Previously, Kang et al. performed quantitative urinary proteome profiling of urine samples from RA and osteoarthritis (OA) patients using a label-free LC－MS/MS analysis (42). Using these urinary protein profiles, they identified 134 DEPs between RA and OA urine samples. Through the integration of the analysis of the 134 DEPs with the analysis of mRNA expression profiles in joints and mononuclear cells, they discovered that urinary soluble CD14 (sCD14) had a comparable diagnostic value to that of conventional serum measures (ESR or CRP). They further identified an even higher predictive power for disease activity when combined with serum CRP. Other groups have also searched for biomarkers through 2-dimensional liquid chromatography-coupled tandem mass spectrometry. Liao and colleagues (43) reported that levels of CRP, S100A8 (calgranulin A), S100A9 (calgranulin B), and S100A12 (calgranulin C) proteins identified through screening the synovial fluid proteome profile were also elevated in the serum of patients with erosive disease compared with those levels in patients with nonerosive RA and in healthy individuals. They used the 2-step proteomic approach in which biomarker discovery using semiquantitative protein profiling of diseased tissues was followed by candidate verification using quantitative multiple reaction monitoring (MRM) analysis in peripheral blood. In these processes, at least 33 biomarker candidates for RA were identified, and Liao et al. were able to certify a subset of promising biomarkers for disease severity. Although the sample size was very small (first step: n=5 and second step: n=15), this study demonstrated that proteomic techniques can be used to discover novel biomarkers in RA. As more efficient sample enrichment/separation techniques and more accurate mass spectrometers become available in the future, proteomic methods will have greater efficiency.

Biomarkers for assessing treatment response

The treatment of RA is primarily based on the use of DMARDs (44,45). The term "conventional DMARDs" will be used to include chemical agents such as methotrexate, hydroxychloroquine, sulfasalazine, and leflunomide, whereas tofacitinib, a new synthetic DMARD specifically designed to target janus kinases (JAKs), will be designated as a "targeted synthetic DMARD" (45). Biologics (or biological DMARDs) such as tumor necrosis factor (TNF) inhibitors, T cell costimulation inhibitor (abatacept), anti-B cell agents (rituximab), and the interleukin-6 receptor (IL-6R)-blocking monoclonal antibody (tocilizumab) have revolutionized the treatment of RA. Despite the availability of these therapeutic options, treatment decisions in clinical practice are based more on the physician's experience or expert opinion than on experimental evidence.

A variety of studies have attempted to identify biomarkers of therapeutic responses to various drugs (46-48). Inhibitors of TNF are the most widely used of the biological therapies in RA. Although anti-TNFα therapy has revolutionized the treatment of advanced RA, approximately one-third of patients have suboptimal responses or no response (46). Moreover, these agents are expensive compared with conventional DMARDs. Assessing the treatment responses to anti-TNFα agents based on biomarker profiling has the potential to improve the overall disease control and to reduce costs for healthcare providers.

Recently identified biomarkers of responses to biological treatments for RA are described below. Segigawa and colleagues (47), using 2D LC-MS/MS analysis, investigated serum or plasma proteins differentially expressed after anti-TNFα therapy. They identified FAM62A/MBC2 proteins related to the TNF-α-mediated pathway for nuclear factor kappa B (NF-κB) activation and/or CTGF

protein related to the metabolism (including regeneration) of articular cartilage. Sellam and colleagues (48), using whole-blood transcriptomic profiling, identified molecular signatures that could be predictive of clinical responses to rituximab in patients with RA. The protein signature for the EULAR responder group featured upregulation of the inflammatory pathway, NF-κB, IL33, and STAT5A, and downregulation of the interferon pathway (48). If these approaches are successful and a useful biomarker has been discovered, it could open new perspectives for clinical RA management.

CONCLUSION

Proteomics-based analysis of RA patients over the past 10 years has provided promising data. DEPs may be helpful for better understanding the pathobiology of RA, and those identified by several studies may be essential for the identification of new targets and to monitor current and new treatments. However, most studies are inadequate in allowing reliable conclusions. The analysis of RA may be more complicated than other inflammatory diseases because of its combination of inflammatory processes, including synovial inflammation and angiogenesis. Data obtained from proteomic analysis of studies including a larger number of patients must be considered a fundamental requirement for more targeted progress. In addition, strategies including specialized proteomic technologies such as an isobaric tag for relative and absolute quantitation (iTRAQ), isotope-coded affinity tag (ICAT), and cleavable ICAT (cICAT), which significantly reduce sample-to-sample variation and time-point variation, can drive basic scientific findings closer to clinical practice. Although RA research still has a long way to go, proteomics has helped shorten the distance.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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