SCIENCE REVIEWS JOURNALS

Volume 4, 2017

Proteomics Investigation of Rheumatoid Arthritis

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Abstract: The difficulty of diagnosing rheumatoid arthritis (RA) early and the heterogeneity of the disease makes treatments less effective. Current diagnostic tests lack accuracy and sensitivity in the early stages of the disease, meaning that symptoms can be difficult to alleviate once treatment begins. Therefore researchers have focused their interests on gaining knowledge about RA pathogenesis and searching for novel biomarkers that enable early diagnosis and stratification of RA. Many have turned to genomic, proteomic and metabolomic techniques to carry out their tasks. This review examines advances in proteomics for clarifying the RA process and identifying protein biomarkers useful for early diagnosis, prognosis and monitoring of treatment response. We believe that proteomics will soon provide novel therapeutic solutions to RA.

Keywords: rheumatoid arthritis; mass spectrometry; proteomics; biomarkers; protein arrays

Rheumatoid arthritis (RA) is a common systemic autoimmune disorder affecting approximately 0.5-1% of the adult population. RA is characterized by chronic inflammation of the synovial membrane and formation of pannus, leading to joint damage, loss of function and systemic organ damage [1]. Diagnosis of RA and assessment of disease during and following treatment are based mainly on clinical observations, serological analysis and histological criteria defined by the American College of Rheumatology and the disease activity score 28 scale[2-4].

Genetic and environmental factors together contribute to the pathogenesis of RA[1]. Genome-wide association studies (GWAS) and transcriptome analyses have sought to identify candidate genes involved in RA pathogenesis[5]. GWAS can capture a large number of common genetic variants, but these risk loci can not entirely explain the RA susceptibility, and it is difficult to identify the variants and explain the gene functions. For RA transcriptome, gene expression does not necessarily predict protein abundance because of transcriptional and translational alternatives, and because genetic studies cannot take into account post-translational modifications of proteins or their interactions. This suggests the usefulness of protein-based approaches for understanding RA.

Protein biomarkers currently used to diagnose RA, such as serum antibodies against rheumatoid factor (RF) and cyclic citrullinated peptides, have disadvantages[6]. Assaying for anti-RF antibodies lacks specificity because the antibodies have also been detected in several other autoimmune disorders and infectious diseases, as well as in healthy elderly individuals. As a result, RF assay results are usually interpreted subjectively based on clinicians' experience rather than on objective guidelines or recommendations. Thus, novel biomarkers are needed to complement conventional methods and monitor RA activity and severity precisely and reliably.

Proteomics may open the door to novel RA biomarkers. It is the principal technique for comprehensively analyzing the proteins expressed in cells or organisms (the so-called "proteome"). It allows comparison of proteomic profiles between normal and diseased tissues, or between two physiological states, such as in the presence or absence of a stress or other stimulus. This allows proteomics to identify proteins expressed specifically in response to a disease or stress. Proteomic studies have already identified many proteins differentially expressed between RA patients and healthy individuals. These proteins are found in peripheral blood mononuclear cells (PBMCs), blood, saliva, urine, and synovial tissue or fluid.

In this review, we summarize the advances of proteomics in identifying proteins that may be useful for RA diagnosis and treatment (Table 1). We discuss the advantages and disadvantages of the widespread clinical application of these proteins as biomarkers and treatment targets.

Proteomics methods

Proteomics allows large-scale study of proteins in different environments and conditions. Numerous aspects of proteins can be studied, including their expression levels, structure, activity, modifications,

and interactions. A typical proteomics experiment consists of six stages: sample extraction, protein fractionation, peptide fractionation, mass spectrometry, protein identification, and protein quantification. In Stage 1, proteins to be analyzed are isolated from biopsy, biofluid, or cell culture, often using gradient centrifugation. In Stage 2, proteins are isolated from the cell lysate or tissue using 1- or 2-dimensional gel electrophoresis or affinity chromatography; the latter serves either to obtain specific proteins in a pulldown (affinity selection), or to remove specific proteins and leave behind a target mixture (affinity depletion). In Stage 3, proteins are digested, usually by trypsin, generating peptides with protonated C-terminal residues. These peptides are fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC), cation exchange, or affinity chromatography (e.g. lectin affinity), and eluates are nebulized into small, highly charged droplets in an electrospray ion source. Multiply protonated peptides are then analyzed by mass spectrometry (MS). In Stage 4, the mass spectrometer measures the mass-to-charge (*m/z*) ratio of peptides in the gases phase. This is the most important stage in the proteomics process, because *m/z* data allow identification of proteins and measurement of their expression levels. Numerous variations and refinements on standard MS have been developed, including various mass analyzers like time-of-flight (TOF), ion trap, quadrupole, Orbitrap, and Fourier-transform ion cyclotron (FT-ICR), liquid chromatography-MS and tandem MS (MS/MS) methods, imaging MS and ion mobility MS, etc. In Stage 5, peptide sequences can be identified using various methods, including searching the protein sequence database with peptide mass fingerprints or MS/MS fragment ions, or by *de novo* sequencing. In Stage 6, expression levels of the same protein under different conditions can be quantified using non-labeling or labeling approaches. In non-labeling approaches, MS response (e.g., signal intensity, ion peaks) is compared directly between samples[7-10]. These approaches include spectral counting approaches(eg., emPAI or APEX), and quantification using XICs. In labeling approaches, parallel MS samples are prepared in the presence and absence of metabolic labeling (^{15}N) of the source tissue or organism (in a technique known as Stable isotope labeling with amino acids in cell culture [SILAC]), chemical labeling of intact proteins before trypsinization (chemical protein labeling [CPL]), chemical labeling of peptides after trypsinization (ICAT, cICAT, iTRAQ, TMT, methylation, esterification), or enzymatic labeling of peptides after trypsinization (^{18}O). Labeling approaches are based on the assumption that labeled and unlabeled molecules have identical physicochemical properties and so respond identically during sample preparation and analysis. As a result, any differences in measured levels are assumed to reflect differences in expression levels in the source tissue.

Proteomics advances in RA

Proteomic analysis of synovial fluid or tissue

Synovial fluid and tissue are the major sites of persistent inflammation and immune cell accumulation in RA, the composition of which can provide information about disease severity and treatment response. Therefore, studying the proteome of synovial fluid or tissue may be useful for detecting RA biomarkers. This approach is limited, however, by the fact that synovial fluid and tissue from normal individuals cannot be obtained for ethical reasons, meaning that parallel comparisons of proteome profiles from individuals with or without RA is rarely possible.

Kumar and colleagues [11] used 2-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization (MALDI) MS to identify proteins in fibroblast-like synoviocytes (FLS) in RA. Potential biomarkers included uridine diphosphoglucose dehydrogenase, galectin-1, galectin-3, BiP, collagen, and HC gp-39, all of which have been characterized as potential autoantigens in RA. Another proteomic study [12]used 2-dimensional gel electrophoresis followed by MALDI-TOF analysis to compare FLS proteins differentially expressed between individuals with or without RA. The 33 proteins identified include enzymatic and structural proteins (e.g. PKM1/M2, α-enolase, ERp60, lamin-A/C), signal transduction proteins (e.g. annexin 11, peroxiredoxin 1, TrpRS), and heat-shock/chaperone proteins (e.g. TCP-1, GRP75, HspB5, Bip).

Katano et al. [13]used MALDI-TOF MS to identify proteins up-regulated in neutrophils from synovial fluid upon stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF). The synovial fluid of individuals with RA contains abundant neutrophils and high levels of GM-CSF. This analysis identified neutrophil gelatinase-associated lipocalin (NGAL) and three enzymes (TERA, CATD and TG2) to be up-regulated in activated neutrophils.

More recently, Bhattacharjee and colleagues [14] used the combination of multiple affinity removal (MARS14) and LC-MS/MS to identify several proteins in synovial fluid from individuals with RA. These included vascular cell adhesion molecule-1, S100 proteins, AXL receptor protein tyrosine kinase, macrophage colony stimulating factor (M-CSF), programmed cell death ligand 2 (PDCD1LG2), TNF receptor 2, TNFRSF1B, as well as several novel

proteins including hyaluronan-binding protein 2, semaphorin 4A (SEMA4D) and osteoclast-stimulating factor 1. These proteins may be useful leads for studies aimed at elucidating RA pathogenesis and progression, and they may be candidates for diagnosing RA and monitoring treatment response.

Proteomic analysis of serum

Blood plasma and serum are more easily obtained than tissues or tissue-derived cells, making them attractive samples for identifying RA biomarkers. Li et al. [15]profiled proteins in sera from individuals with RA using the combination of magnetic bead-based separation and MALDI-TOF MS. They identified peaks with *m/z* values of 1,014.92 and 1,061.38 that were significantly greater in sera from individuals with early RA than in sera from individuals in later stages of RA or from healthy controls. Also using a combination of magnetic beads and MALDI-TOF MS, Zhang et al. [16]identified five peaks in sera from individuals with RA (*m/z* 15,715.5, 7,771.4, 8,959.4, 8,469.8 and 8,710.8) that may be useful for diagnosing RA. Using a different approach of surface-enhanced laser desorption/ionization (SELDI)-TOF MS, de Seny et al. [17]identified three peaks (*m/z* 11,632, 10,832, and 2,924) in sera from individuals with RA that may be useful as disease biomarkers.

The inconsistency among these three studies may reflect the inherent complexity of RA, technological differences between SELDI-TOF MS and magnetic bead-MALDI-TOF MS, as well as genetic differences between the European and Asian populations examined.

Proteomic analysis of PBMCs

PBMCs and several cytokines they produce play a pivotal role in RA pathogenesis. They can be easily isolated from the blood, which is easier to obtain from individuals than synovial fluid, which requires an invasive procedure. In addition, protein concentrations in PBMCs can be quantitated over a broader dynamic range than those in the serum. Comparison of PBMC proteomes from individuals with RA and the controls using 2-dimensional gel electrophoresis and peptide mass fingerprinting identified several peptides associated with RA[18]. Many of these had previously been shown to be auto-antigens in autoimmune diseases. Another such study using 2-dimensional gel electrophoresis and MALDI-TOF MS identified 18 proteins that were expressed at higher levels in individuals with RA than in controls[19]. One of these proteins (Hsp60) had already been implicated in RA pathogenesis. These proteins may be useful for diagnosing RA.

Proteomic studies of PBMCs have revealed several potential biomarkers, which should be verified with larger sample size. Proteomic studies of PBMCs in RA have also shed light on how this disease differs from systemic lupus erythematosus (SLE). LC-MS of iTRAQ-labeled proteins from PBMCs in healthy controls, individuals with RA and individuals with SLE revealed that 11 proteins were up-regulated and 8 proteins were down-regulated between stable SLE and RA. Meanwhile, 13 up-regulated proteins and20 down-regulated proteins were observed in active SLE compared to RA. Compared to stable SLE, RA, or healthy controls, some proteins (e.g. isoform 2 of zinc finger protein 549, histone H2A type 1, myeloblastin) were significantly high expressed in active SLE.[20].

Proteomic analysis of bone marrow cells

Bone marrow contains two types of stem cells: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs are the precursors of all cells, while MSCs are adherent fibroblast-like stromal cells. The proteomes of bone marrow-derived adherent cells (BMACs) from individuals with RA or osteoarthritis (OA) were compared using 2-dimensional difference gel electrophoresis (2D-DIGE) and MS[21]. Eleven proteins were found to differ significantly between OA and RA, including the alpha chain of collagen VI, heat shock protein 27, caldesmon, a membrane anchor for acetylcholine esterase, and cytoskeletal proteins such as beta actin and alpha tubulin. Kastrinaki et al. [22]compared expression of inflammatory cytokines between individuals with RA and controls using 2-dimensional electrophoresis. They failed to find any significant differences between the two types of samples using hierarchical clustering or Pearson correlation. These results suggest similar cytokine production between patients and controls.

MSCs have been proposed as therapeutic targets for rheumatic diseases because they help regulate immune responses and regenerate bone and cartilage. Further studies of proteins differentially expressed in MSCs in RA are needed, preferably with larger sample sizes.

Proteomic analysis of urine

Urine can be collected safely and routinely in large quantities, and analysis of urinary proteins can serve as a potentially rich source of biomarkers reflecting systemic inflammation. Kang et al. [23]used LC-MS/MS to identify 134 proteins differentially expressed between urine from individuals with RA or OA. One of these was urinary soluble CD14 (sCD14), which they found to have a diagnostic value comparable to conventional serum measures.

Proteomic analysis of saliva

Human saliva can be obtained non-invasively and collected repeatedly without discomfort to the patient. Comprehensive proteomic analysis of saliva from individuals with RA and healthy controls using 2-dimensional electrophoresis and MS identified 8 proteins differentially expressed in the disease[24]:

78-kDa glucose-regulated protein precursor (GRP78/BiP), 6-phosphogluconate dehydrogenase, peroxiredoxin 5, calgranulin A, calgranulin B, apolipoprotein A-1, epidermal fatty acid-binding protein, and 14-3-3 proteins. The chaperone GRP78/BiP showed the largest increase in RA, identifying it as a good candidate biomarker.

Table 1. Proteomics studies of RA

Sample	Methods	Patients	Proteins identified	Reference
Synovial fibroblasts	2D, MALDI MS	RA	uridine diphosphoglucose dehydrogenase, galectin-1, galectin-3, BiP, collagen, HC gp-39	Dasuri et al. [11]
Fibroblast-like synoviocytes	2D, MALDI MS	RA	PKM1/M2, α-enolase, ERp60, lamin-A/C, annexin 11,peroxiredoxin 1, TrpRS, TCP-1, GRP75, HspB5, Bip	Li et al. [12]
Neutrophils in synovial fluid	MALDI-TOF MS	RA	neutrophil gelatinase-associated lipocalin (NGAL), transitional endoplasmic reticulum ATPase (TERA), cathepsin D, transglutaminase 2 (TG2)	Katano et al. [13]
Synovial fluid	LC-MS/MS	RA	vascular cell adhesion molecule-1, S100 proteins, AXL receptor protein tyrosine kinase (incomplete list)	Bhattacharjee et al. [14]
Serum	MALDI-TOF MS	RA	peaks of *m/z* 1,014.92 and 1,061.38	Li et al. [25]
Serum	MALDI-TOF-MS	RA	peaks at *m/z* 15,715.5, 7,771.4, 8,959.4, 8,469.8 and 8,710.8	Zhang et al. [26]
Serum	SELDI-TOF MS	RA	peaks at *m/z* 3,899, 4,594 and 7,566	de Seny et al. [17]
PBMCs	2-D, PMF	RA	several autoantigens	Schulz et al. [18]
PBMCs	MALDI-TOF	RA	beta actin, chaperone HSPA5 precursor, ribonucleoprotein K isoform b, heat shock protein	Dotzlaw et al. [19]
PBMCs	iTRAQ, LC-MS	RA, SLE	proteins differing between stable SLE and RA, and between active SLE and RA	Wang et al. [20]
BMACs	2D-DIGE	RA, OA	collagen VI, heat shock protein 27, beta actin, alpha tubulin	Kamada et al. [21]
MSCs	2D, microarray	RA	cell proteome profiling	Kastrinaki et al. [22]
Urine	LC-MS	RA, OA	soluble CD14	Kang et al. [23]
Saliva	2-D, MS	RA	chaperone GRP78/BiP	Giusti et al. [24]

Abbreviations: 2D, 2-dimensional electrophoresis; BMAC, bone marrow-derived adherent cell; DIGE, differential image gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; MSC, mesenchymal stem cell; OA, osteoarthritis; PBMC, peripheral blood mononuclear cell; PMF, peptide mass fingerprinting; RA, rheumatoid arthritis; SELDI, surface-enhanced laser desorption/ionization; SLE, systemic lupus erythematosus; TOF, time of flight

Discussion and conclusions

Despite improvements in RA diagnosis since the introduction of an assay for antibodies against cyclic citrullinated peptides[3, 6, 25, 27-29], many patients with atypical early RA suffer delayed diagnosis and treatment. More sensitive and specific biomarkers are needed to allow earlier, and more reliable diagnosis of RA. Proteomics studies have aimed to provide such biomarkers and, in so doing, help elucidate the molecular mechanisms of disease onset and progression, clarify the mechanisms of action of existing therapeutics, and identify new therapeutic targets. This review has examined the latest advances in proteomic efforts to identify RA biomarkers in numerous tissues. Despite numerous studies identifying numerous candidate proteins differentially expressed in RA, no molecule has yet been validated as a robust biomarker of the disease.

One of the more promising candidates is the potentially theranostic protein S100A9, the levels of

which in PBMCs and serum have been verified to be associated with RA based on proteomics and follow-up ELISA[30, 31]. S100A9 is the only biomarker in these studies that correlated with treatment response. These results obtained with a relatively small number of patients should be verified and extended in further study.

While still in its early stages, proteomics-based searches for RA biomarkers seem destined to deliver clinically useful insights. Conventional proteomics methods of 2-dimensional gel electrophoresis and traditional MS are continuously being refined and extended through such innovations as DIGE, shotgun proteomics, iTRAQ, MALDI-TOF, electro spray ionization-time-of-flight mass spectrometry (ESIQ-TOF MS), ESI-Q-TRAP, and FT-ICR-MS[1, 9, 10, 14, 28, 32]. Protein arrays have also shown promise for differential diagnosis and molecular stratification of RA[33]. These array studies have illustrated the potential of multiplex biomarker panels for diagnosing RA with greater sensitivity and specificity than current clinical tests. As proteomics studies mature, we have no doubt that they will help elucidate our understanding of RA processes and open the door to better diagnosis and treatment.

Disclosure statement

The authors declare no conflicts of interest.

Acknowledgments

This research was supported by the China Scholarship Council and the International Training Program of High-Level Talents in Henan Province.

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