Molecular Biology Approaches to Understanding Spondyloarthritis

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KEYWORDS

• Spondyloarthritis • Genetics • Epigenetics • microRNA • Microbiome
• Metabolomics • Immunophenotyping • Transcriptomics

KEY POINTS

- Recent advances in genetics, omics technologies, and immune profiling methods have contributed to significant advances in the clinical and scientific understanding of spondyloarthritis.
- Genetic studies have shifted from genome-wide association studies to newer technologies, such as whole-genome sequencing and identification of epigenetic modifications.
- Single-cell sequencing and time-of-flight cytometry allow identification of novel cell populations in tissues.
- Multiomic analyses will further integrate large data sets and inform pathophysiologic mechanisms.

INTRODUCTION

Numerous factors involved with the pathogenesis of spondyloarthritis (SpA) from genetic predisposition, transcriptional expression of genes, immune function, and environmental factors like the microbiome increasingly are identified as potential contributors to the pathophysiology. Recent advances in molecular techniques, including omics technologies and improved immune profiling methods, have allowed for further elucidation of important pathways and cell types, expanding understanding of diseases like ankylosing spondylitis (AS) and psoriatic arthritis (PsA). This article describes some of the molecular approaches utilized as well as some of the recent advances that are helping to progress the field and provide more targets for intervention.

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Rheum Dis Clin N Am 46 (2020) 203–211
https://doi.org/10.1016/j.rdc.2020.01.001
rheumatic.theclinics.com

GENETICS

The genetic landscape has evolved over the past 5 years away from traditional mendelian approaches toward genome-wide association studies (GWASs), next-generation sequencing (NGS), and epigenetics. These new molecular approaches have led to significant advances in science and have provided a powerful platform for studies in SpA, extending genetic understanding of SpA well beyond HLA-B27, which contributes only 20.1% of the heritability of AS.[1]

Genome-Wide Association Studies and Next-Generation Sequencing

GWASs are a complex genetic analysis tool that allows for the detailed profiling of genetic variants. GWASs allow for the analysis of hundreds of thousands of single-nucleotide polymorphisms (SNPs) to be assessed for association with a given disease.[2] A GWAS is performed most commonly as a case-control study design with a group of patients diagnosed with a disease of interest compared with healthy controls. For this type of study, DNA usually is isolated from peripheral blood and subsequently genotyped using commercial chip platforms. After data quality control, which can be assessed by several computational programs, data are analyzed for associations between SNPs and disease. Generally, an analysis is followed by replicating associations in an independent population sample.[3]

GWASs have been used extensively in SpA to identify genes linked to signaling pathways not previously identified.[4–6] This is further exemplified nicely in the study by Ellinghaus and colleagues,[7] in which 86,000 individuals of European ancestry with AS, inflammatory bowel diseases, primary sclerosing cholangitis, or psoriasis were compared with healthy controls. The analysis allowed identification of 27 new genetic susceptibility loci, 17 in AS alone, and demonstrated shared risk among these related diseases. Studies like this have identified novel pathways of disease based on susceptibility loci, such as interleukin (IL)-23 receptor (R), IL-12B, and chemokine receptor 6, which affect $CD4^+$ effector function.[8] Numerous other genes related to the IL-23/IL-17 pathway also have been identified through GWASs, including CARD9, PTGER4, TYK2, and STAT3[8] as well as ERAP1 and ERAP2, which relate to peptide trimming in the endoplasmic reticulum for peptide loading onto HLA class 1 molecules, such as HLA-B27.[9] Although these data have vastly expanded understanding of genetic susceptibility for SpA, GWASs are limited by their sample size and heterogeneity. They best identify common genetic variants but can miss rare polymorphisms and are unable to identify genetic interactions with other loci.

Better identification of rare genetic variants of clinical importance can be achieved through NGS methodologies like whole-exome and genome sequencing. In these methods, DNA is extracted from white blood cells, broken into short fragments and the DNA sequence determined through various sequencing technologies.[10] These fragments then are read as millions of short sequencing DNA reads, followed by alignment to the human genome reference sequence via a variety of available computational tools. In this manner, DNA composition can be determined in a specific order with individual nucleotides. Overall, NGS is a valuable tool for detecting single-nucleotide substitutions and/or insertions as well as differences in gene composition.[10] One study of exosome sequencing in AS confirmed previously identified susceptibility polymorphisms, such as ERAP1 and IL-23R; however, the study was underpowered to identify novel rare variants.[11]

Epigenetics: Methylation and Histone Acetylation/Deacetylation

DNA methylation is an epigenetic modification in which methyl groups are added to cysteine or adenine residues, thereby controlling transcription.[12]

Age, sex, smoking, medications, alcohol, and diet are known to affect DNA methylation.[13] Methylation patterns have been implicated in numerous biologic processes, such as aging, and several diseases, including SpA.[12] DNA methyltransferase 1 (DNMT1) is an enzyme that regulates patterns of methylated cytosine residues. Expression of DNMT1 is decreased in the setting of increased methylation of the DNMT1 promoter in AS patients compared with healthy controls, which is of unclear etiology in AS pathogenesis, because this did not correlate with clinical manifestations.[14] Furthermore, numerous genes in AS have been found to be differentially methylated, with hypermethylation of HLA-DQB1 having the most significant signal.[15] Another gene, B-cell chronic lymphocytic leukemia/lymphoma 11B (BCL11B), also was found to have increased methylation and decreased transcription in AS compared with healthy controls.[16] More recently, the first study to assess the role of HLA-B27 in methylation status of AS was performed. This study found hypomethylation of HCP5, tubulin folding cofactor A, and phospholipase D family member 6 in AS patients.[17] Thus, methylation studies have identified additional genes that may be important in disease development.

Histone modification allows activation (euchromatin) and deactivation (heterochromatin) of chromatin by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[18] These different acetylation patterns allow for chromatin stability, gene regulation, and transcription silencing. The concept of histone modification has been minimally studied in SpA. In peripheral blood mononuclear cells from patients with AS, HAT and HDAC activity were significantly reduced compared with healthy controls.[19] HDAC inhibitors, such as sirtinol, were able to decrease HDAC expression in healthy controls but not in AS. Sirtinol decreased production of tumor necrosis factor (TNF) in AS patients, suggesting an intriguing treatment strategy in AS requiring further study.[19] Targeting modifiable states, such as histone acetylation, versus unmodifiable states, such as genetic composition, is an attractive strategy that likely will be studied further in the future. For example, HDAC inhibitors have shown anti-inflammatory effects in vitro in rheumatoid arthritis (RA) models[20,21] but have not been studied thus far in SpA.

MicroRNA

MicroRNA (miRNA) is a type of small, noncoding RNA of approximately 22 nucleotides that can form complex networks that regulate cell differentiation, development, and homeostasis.[22] MiRNAs have been proposed to be involved in the pathogenesis of numerous rheumatic diseases, such as RA, systemic lupus erythematosus (SLE), and osteoarthritis.[23–25] The role of miRNA in the pathogenesis of AS has indicated a variety of expression variability of miRNAs. Common miRNA profiles in AS have identified individuals with increased miR-146a and miR-155.[26] Mechanistically, increased miR-146a expression has been studied in terms of gene regulation, in that miR-146a has been shown to inhibit dickkopf 1 (DKK1), which also has been implicated in AS pathogenesis, and miR-146a knockdown models can hinder AS progression.[27] Other miRNAs that have been implicated in AS include miR-125a-5p, miR-151a-3p, and miR-22-3p based on higher expression compared with healthy controls.[28]

Given that there is increased specific miRNA expression in patients with AS compared with healthy controls, miRNAs represent a potential biomarker for diagnosis, disease activity, or potential therapeutic targets. The previously discussed miRNAs, miR-146a and miR-155, have been found to correlate with disease activity using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).[26] Additional correlations with BASDAI and miR-625-3p and miR-29a also have been identified.[29,30] Other

miRNAs have been identified in AS patients with down-regulated miR-199a-5p correlating with increased TNF, IL-17, and IL-23.[31] This finding implies that miRNAs could distinguish SpA phenotypes responsive to inhibitors of TNF, IL-17, or IL-23.[32] This concept also has been assessed in PsA with specific miRNA signatures noted in patients with PsA compared with healthy controls, which have been proposed as biomarkers or potential therapeutic targets.[33]

PROTEOMICS AND METABOLOMICS

Proteomics and metabolomics are omics tools that focus on unbiased identification and measurement of the proteins or small molecule metabolites in a biologic system. These approaches rely on high-pressure liquid chromatography and mass spectrometry of a sample followed by alignment of results with a database of known proteins and metabolites for identification. Addition of known concentrations of specific proteins and metabolites allows further quantification within samples. These approaches have potential to identify biomarkers for disease diagnosis and activity as well as pathogenic mechanisms. For example, proteomics identified several candidate biomarkers for either the diagnosis of or conversion from psoriasis to PsA[34–36]; however, none of these candidates has been confirmed in independent cohorts. Similarly, in AS, 2 proteomic studies suggested biologic processes of cytotoxicity and vitamin D binding in the pathogenesis of AS,[37,38] although these have yet to be validated. With regard to metabolomics, analysis of multiple tissue, including plasma, urine, and hip ligament, found alterations in fat, glucose, and choline metabolism.[39,40] Given the known dysbiosis in the fecal microbiota that have been identified in SpA compared with controls,[41–43] metabolites also have been studied in fecal samples in a pediatric population with enthesitis-related arthritis. The investigaton of fecal metabolites in pediatric enthesis-related arthritis found decreased metabolic diversity and alterations in the tryptophan metabolism pathway relative to healthy controls.[44]

MICROBIOME

Alterations in intestinal bacterial communities (dysbiosis) have been identified in SpA, although the etiology and consequence of dysbiosis is not elucidated.[41–43] The microbiome represents an emerging area in SpA given the presumed gut-joint relationship in disease pathogenesis. There are numerous approaches toward studying the microbiome in patients with SpA: 16S sequencing identifies bacteria through limited sequencing of regions within the bacterial 16S ribosomal RNA gene and alignment with sequence databases; this approach limits identification of bacteria to the level of genera. Similar methods exist for sequencing fungi using the internal transcribed spacer. Shotgun metagenomics allows species-level identification through untargeted sequencing using random primer sets. This technique also provides the functional potential of a community after analysis of genetic pathways identified by sequencing. Metatranscriptomics and single-cell RNA sequencing (scRNA-seq) are still being optimized to microbiome analysis and will provide another level of analysis.

A limitation of microbiome studies to date in AS has been the variability of findings from one study cohort to another.[41,43,45] For example, Breban and colleagues[43] identified a significant expansion of *Ruminococcus gnavus* by 16S sequencing of stool in patients with AS compared with controls. Tito and colleagues,[46] however, identified a significant expansion of *Dialister* by 16S sequencing of intestinal biopsies from individuals with AS that correlated with disease activity scores. The variation of findings may be related to tissue sampling (eg, stool vs mucosa-associated bacteria), geographic differences, or other confounding factors that need to be considered when designing

and interpreting microbiome studies in AS. Furthermore, the specific microbiota may not be as important as the community function, which may be better assessed with newer technologies, such as metagenomics and metatranscriptomics.

IMMUNE PHENOTYPING

Numerous new techniques have been utilized in discovering novel immune phenotypes in SpA. Traditional immune cell profiling through flow cytometry has led to the identification of cells hypothesized to contribute to disease pathogenesis. These immune phenotypes include expanded helper T cell type 22, helper T cell type 17, γδ T cells, and mucosal-associated invariant T cells in the peripheral blood[47–50] as well as IL-17–producing natural killer cells in the intestine of AS patients.[51] As general knowledge of immune cells and function has expanded, traditional techniques have revealed additional cell types, such as innate lymphoid cells. Increased levels of type 3 innate lymphoid cells that produce IL-17 and IL-22 have been identified in the intestine, peripheral blood, synovial fluid, and bone marrow of AS patients[48] and in the peripheral blood of patients with PsA that correlate with clinical disease activity.[52] Newer molecular approaches, namely mass cytometry and scRNA-seq, have additive potential to reveal unique cell types and pathways.

Cytometry by Time-of-Flight and Imaging Mass Cytometry

Traditional flow cytometry is limited by use of fluorescent dyes that have overlapping spectra, thus limiting the resolution between dyes and number of antibody markers that can be combined (usually <20). Cytometry by time-of-flight (CyTOF) is a technology for single-cell analysis that relies on using heavy metal ions as antibody labels without the limitations of fluorescence,[53] allowing combinations of greater numbers of antibodies, upwards of approximately 40. Recently CyTOF was used to identify an expansion of unique $CD8^+$ T cells in the synovial fluid of patients with AS. These $CD8^+$ T cells expressed integrins β7, CD103, CD29, and CD49a.[54] Thus, CyTOF has the power to characterize immune cell populations more thoroughly. Coupling CyTOF technology with histology, imaging mass cytometry has the ability to add spatial information, which will be a powerful tool in understanding the function of immune cells in tissues relevant to SpA.

Single-Cell RNA Sequencing and Cellular Indexing of Transcriptomes and Epitopes by Sequencing

scRNA-seq allows more refined profiling of immune cells compared to flow cytometry and CyTOF. There are numerous different methods, each with specific strengths and weaknesses, for single-cell isolation followed by amplification and NGS of transcribed RNA, as reviewed by Papalexi and Satija.[55] Analysis of the data allows for characterization of distinct cell subsets, uncovering the heterogeneity within a population, and dissecting cell fate branch points.[55] The power of scRNA-seq is best highlighted by the efforts of the Accelerating Medicines Partnership in profiling peripheral blood and tissue cells in patients with RA and SLE,[56] which has revealed novel cellular functions for further study in the pathogenesis of these diseases. A significant limitation of scRNA-seq is that immune cells are characterized by cell surface proteins that are not highly expressed mRNAs and thus not easily detected through transcriptional sequencing. Cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq) adds on scRNA-seq through the use of nucleotide barcode-labeled antibodies targeting cell surface markers. These barcodes are sequenced along with the transcriptome of each cell, allowing the coupling of protein marker information in the

analysis of cellular populations. Although studies utilizing scRNA-seq in SpA have yet to be published, this represents an approach that could lead to significant advances in the field of SpA in terms of pathophysiology, diagnosis, and pharmacology.

INTEGRATED OMICS

With the rise of multiple omics technologies, integration of data from these approaches represents a powerful analysis for understanding aspects of SpA. This method can be used to study pathophysiology as well as identify candidate biomarkers for diagnostic purposes. For example, a recent study in inflammatory bowel disease utilized the techniques of multiomics to study microbial dysbiosis with regard to host factors toward dysregulation of microbial transcription, metabolite pools, and levels of antibodies in the serum.[57] A similar approach was used in the rat HLA-B27 transgenic model of AS in which microbiome analysis and host bulk RNA sequencing revealed correlations between specific bacteria and cytokine dysregulation, and bacterial metagenomics predicted pathways associated with inflammation.[58] Such approaches have great potential to expand understanding of SpA.

SUMMARY

With an ever-evolving scientific landscape, emerging molecular techniques continue to elucidate important pathways in complex diseases, such as SpA. Within SpA, many of the techniques, described in this article, are offering new approaches toward diagnosis, such as metabolites, miRNA, or immune cell profiling, as well as identifying new therapeutic approaches, such as epigenetic modifications. In addition to the development of new techniques is the expansion of analysis tools in handling data generated from the methodologies. Altogether, these approaches provide exciting pathways forward in the study of SpA.

ACKNOWLEDGMENTS

The authors are supported by grants through the National Institutes of Health (T32AR007534, K08DK107905, R01AR075033), the Rheumatology Research Foundation, and the Boettcher Foundation Webb-Waring Biomedical Research Award.

DISCLOSURE

The authors have nothing to disclose.

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