

# Chapter 2

## Genetics, Genomics, and Proteomics of Sjögren’s Syndrome

Christopher J. Lessard, John A. Ice, Jacen Maier-Moore,  
Courtney G. Montgomery, Hal Scofield, and Kathy L. Moser

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### 2.1 Introduction

Dramatic advances in identifying the genetic basis of many human diseases are transforming our fundamental understanding of etiology and pathogenesis. Over the past decade, large global efforts to characterize sequence variation in the human genome have provided the foundation for this extraordinary progress. Success in mapping disease genes has also been fueled by revolutionary advances in our technical capacity for genotyping and analyzing complex genetic datasets. These advances include the technical capacity for genotyping millions of known variants and have ushered in a new era of powerful, large-scale, and highly successful genome screens for many diseases. Scanning the human genome for association of variants with disease is unbiased and not limited by prior selection of a putative candidate gene for testing. As a result, the genes that are associated with disease can be surprising, oftentimes linking previously unsuspected molecular pathways to

C.J. Lessard • J.A. Ice • J. Maier-Moore • C.G. Montgomery • H. Scofield • K.L. Moser (✉)  
Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation,  
Oklahoma City, OK, USA

numerous disease phenotypes. In many cases, an association may be located between genes and have no known or obvious functional effect. Thus, a dramatic shift in our knowledge of the genetic architecture of human disease is underway. Still, much remains to be learned.

Pinpointing specific genetic associations with disease and characterizing the effects on gene function offer fundamentally important opportunities for insight into disease etiology and pathogenesis. In Sjögren's syndrome (SS), the etiology remains poorly understood in part due to highly limited knowledge of the underlying genetic architecture. These limitations can largely be attributed to small sample sizes of patient cohorts currently available for study. Similar to most autoimmune diseases, epidemiologic and genetic studies in SS to date strongly support the hypothesis of a complex etiology involving variants in numerous genes with functional consequences across multiple biological pathways. In contrast, the genetics of related autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), are far more advanced and dozens of genetic associations have already been established. A multitude of genes are likely to be involved in SS as well. However, specific genes associated with SS are only beginning to be identified and characterized. In addition, the influences of epigenetic processes and environmental factors on the etiological complexity of SS remain largely undefined.

Gene expression profiling (GEP) and proteomic studies are complementary tools for revealing important disease-associated pathways. Application of these powerful approaches has begun to offer new global views of dysregulated pathways and networks that distinguish patients from controls. Integrating genetic, genomic, and proteomic data can be used for higher level "systems biology" approaches that may prove especially fruitful when combined with detailed clinical data.

In this chapter, we begin by summarizing the evidence for a genetic component to SS, emphasize the underlying concepts and approaches to new gene discovery, and discuss complementary high-throughput RNA and protein-based studies that are providing new and important insight into the underlying molecular pathways leading to this complex disorder. Overall, developing a much more comprehensive understanding of SS is expected in the future as these types of studies progress.

## 2.2 Genetic Epidemiology of SS

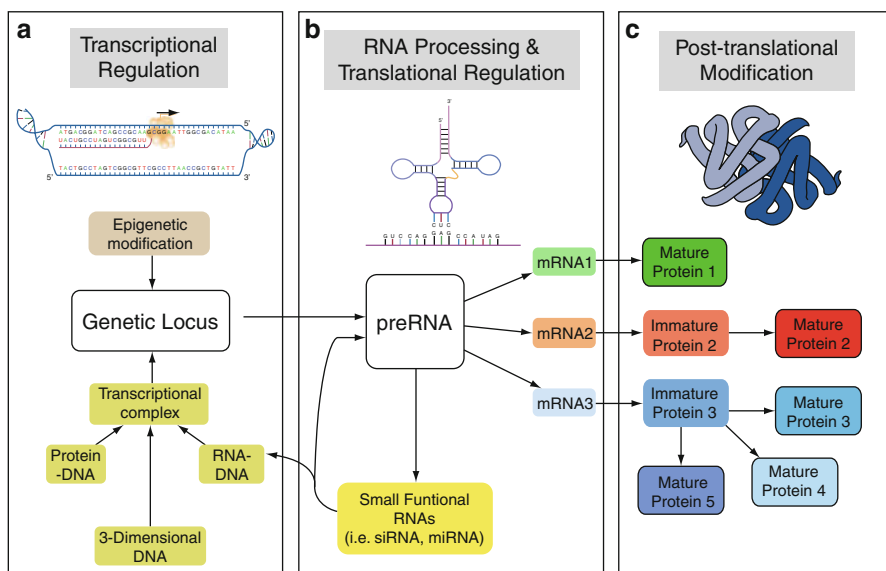
Evidence gathered to date for a genetic etiology in SS is consistent with an important and complex contribution of heritable factors; however, the genetics of SS is substantially understudied. Approximately 40 genetic studies have been reported thus far and are largely focused on candidate genes such as the HLA loci or genes demonstrating association in other autoimmune diseases. In the absence of large-scale genetic studies identifying robust associations, increased concordance rates of

disease among monozygotic twins and the frequency of familial aggregation are two measures commonly used as evidence for a genetic component.

A limited number of case reports describing twins with SS have been published, but studies to establish a reliable twin concordance rate are not available [1–4]. For example, Scofield et al. reported adult monozygotic twins with similar serological profiles with respect to high-titer anti-Ro/SSA, severe lymphocytic infiltration of the labial salivary glands, and mild clinical symptoms [4]. Another case of dizygotic twins with SS was reported by Houghton et al., in which one adolescent sister presented with pulmonary symptoms leading to the diagnosis of lymphocytic interstitial pneumonia, while the other sister demonstrated no respiratory symptoms [3]. Interestingly, these dizygotic twins feature both pulmonary involvement and familial aggregation, which are uncommon in juvenile SS. Familial aggregation in SS has also been observed. Several multiplex families with SS have been described, and increased prevalence of other autoimmune diseases in the families of SS patients is quite common (30–35%), including SS (12%), autoimmune thyroid disease (AITD, 14%), RA (14%), and SLE (5–10%) [5].

Clinical and serological features among related autoimmune diseases often demonstrate overlap in which subsets of patients may share similar symptoms (including, but not limited to, arthralgias, myalgias, fatigue, rashes, and visceral involvement from vasculitis) or serological biomarkers such as autoantibody profiles [6]. Many of the clinical features of SS are found in subsets of patients from a variety of other autoimmune disease groups. Direct evidence for associations of common genetic loci shared across related autoimmune disorders has been documented [7]. These studies suggest that sharing of underlying disease mechanisms across related phenotypes may account for overlapping clinical features. Based on phenotypic similarities, increased twin concordance rates in SS could be expected to be between those of RA (15%) and SLE (25%) [8, 9]. Likewise, female sibling or dizygotic twin rates of 2–4% and estimated odds of female sibling concordance ( $\lambda_s$ ) between 8 and 30 could be reasonable estimates for SS.

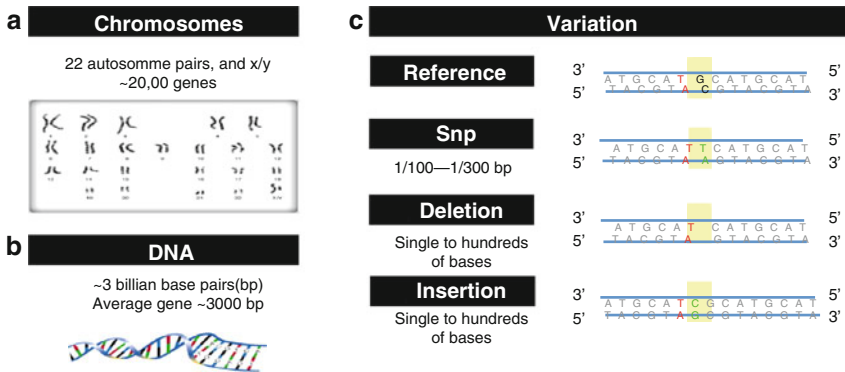
Various human leukocyte antigen (HLA) alleles demonstrate association with many autoimmune diseases, including SS, SLE, RA, and others [10]. A growing list of non-HLA genes have also shown association with multiple autoimmune diseases. For example, associations have been reported for *CTLA4* with AITD, Type 1 Diabetes (T1D), celiac disease, Wegener's granulomatosis, SLE, vitiligo, Addison's disease, and RA [5]. Other associations include *PD-1* with RA, T1D, and SLE, and *PTPN22* with SLE, RA, T1D, Graves's disease, and Hashimoto's thyroiditis [5]. Although most genes associated with SLE, RA, and other autoimmune diseases have not yet been fully evaluated in SS, *IRF5*, *STAT4*, and *BLK* are examples of genes strongly associated with SLE for which there is recent data suggesting association in SS [11]. Thus, although direct evidence overall is limited, the available studies are consistent with a heritable component similar to diseases such as RA and SLE, both of which have well-established genetic associations for dozens of genetic loci.



**Fig. 2.1** The central dogma of human diversity through RNA and protein regulation and modification. The figure illustrates the stepwise progression of genomic information from DNA to RNA to protein. With 20,000–25,000 genes in the human genome, increased complexity of the human protein repertoires comes from posttranscriptional and posttranslational modification of RNAs and proteins. **(a)** The process of transcribing DNA template into messenger RNA (mRNA) is a tightly controlled process governed by transcription factors, RNA pol II (typically), helicases, and topoisomerases. These molecules coalesce within the proximal promoter region and form the transcription machinery. In order for these molecules to bind the promoter region, the DNA must be open, or “melted.” Epigenetic modifications can silence regions of the genome by impeding the melting process. **(b)** Once transcribed, the preRNA can be modified through splicing, addition of the 5’ cap (modified guanine nucleotide), nucleotide editing, and polyadenylation on the 3’ end. Splicing of mRNA can dramatically increase diversity by removing intronic regions and alternatively splicing exons. Some intronic regions form small silencing RNAs (siRNAs) and/or microRNAs (miRNAs). These nonprotein coding RNAs also regulate the transcription and translation process. **(c)** Once mRNA are translated into protein, diversity is further increased by posttranslational modifications such as the addition of functional groups (acetate, phosphate, lipids, and carbohydrates) or structural changes (disulfide bonds)

## 2.3 Key Concepts in Genetics, Transcriptomics, and Proteomics

The central dogma of information flow from DNA to RNA to protein has grown in complexity over recent years as shown in Fig. 2.1 [12]. The entire human genome contains over 3 billion base pairs, approximately 20,000 genes, and millions of variant sites in the DNA sequence (Fig. 2.2). Genetic associations may occur in DNA coding sequences and alter the structure or function of a protein; however, most associations with complex diseases found to date reside in regions thought to regulate gene expression [12]. Genetic variation may also influence molecular processes

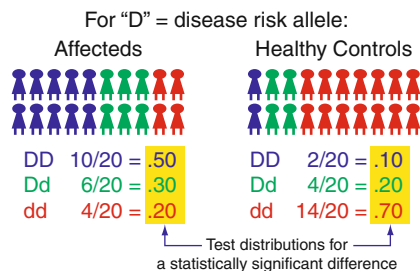


**Fig. 2.2** The human genome. The human genome is comprised of 22 autosomal chromosomes and 2 sex chromosomes (X and Y, panel **a**) consisting of ~3 billion base pairs (panel **b**). The average size of a gene is 3,000 base pairs. (c) Every human is ~99.9% identical with the 0.1% of variation leading to benign traits such as eye or hair color, or disease-causing traits such as Sjögren's syndrome. Variation is illustrated in panel **c** as compared to reference sequence at the top of the panel. The red and blue bases flank the site of variation. Single nucleotide polymorphisms (SNPs) are single base changes and are the most common polymorphisms in the genome (~15 million to date). Deletions and insertions can occur ranging in size from single base units to hundreds of bases

such as splicing and posttranslational modifications that have potential to generate over 100,000 protein isoforms from only 20,000 genetic loci. As the nature and extent of human variation has been revealed over the past decade, it is perhaps not surprising that many complex human diseases now have dozens of variants that are associated with increased risk.

In general, humans are between 99.5% and 99.9% identical, yet there are millions of polymorphic sites scattered throughout the genome that account for individual differences. Many different forms of DNA sequence variation have been discovered in the human genome including: single nucleotide polymorphisms (SNPs, pronounced “snips”), copy number variants (CNVs; variation in which the number of copies of a gene or DNA sequences differ), microsatellites (regions with short tandem repetitive sequences) and insertion/deletion events (indels or DIPs). Any type of variation can influence benign features, such as height, but it can also lead to an increase in the susceptibility of disease development.

SNPs are the most frequent type of polymorphism found in the human genome. Current estimates indicate that one out of every 100–300 base pairs could be a SNP [13]. Estimates of approximately 15,000,000 total SNPs in the human genome continue to increase as additional variants are discovered through ongoing sequencing projects. As such, these common variants are now frequently used to screen the genome when attempting to detect association of specific alleles with disease. Common genetic association study designs are focused on detecting statistically significant differences in allele frequencies for a given SNP between cohorts of cases and controls (Fig. 2.3). Large sample sizes (potentially thousands of subjects) for these types of studies and confirmation in independent cohorts are necessary



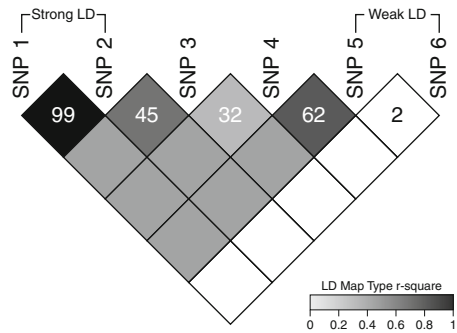
**Fig. 2.3** Calculation of association of variants with disease. Every human carries two copies of each chromosome and therefore two copies of every allele, one inherited from their mother and the other from their father. For disease allele D, alleles are counted for both affected individuals and healthy controls. The distribution of alleles between affected individuals and healthy controls is then tested for statistical significance using statistical tests such as Chi-squares ( $2 \times 2$  table) or logistic regression (covariates can be implemented to adjust for confounding)

when the allele frequencies between cases and controls are relatively small, as is the case for many of the associations now established in complex diseases.

When mapping disease loci, not all genetic variation must be genotyped to detect an initial association effect since some variants can serve as markers for causal alleles. Scientists have used the nonrandom, or linked, assortment of loci that occurs during meiotic recombination to map genetic locations on chromosomes. When two loci lie in close proximity on a chromosome, these loci tend to be inherited together more frequently than expected when random crossover events occur. This phenomenon, termed linkage disequilibrium (LD), is measurable by determining correlations between the recombination frequencies of specific alleles at two or more loci. Variable patterns of LD across the genome are defined by the length of the regions in which LD occurs, creating haplotype blocks, and the strength of LD is measured by these correlations [14]. A benefit of understanding these patterns for gene mapping studies lies in the ability to use this knowledge to reduce the genotyping burden while still detecting association of a variant with disease [14]. Essentially, any SNP present on a haplotype block may serve as a tag, or marker, for a causal variant if LD is sufficiently strong. Additional studies can then be used to precisely identify and characterize causal variants (Fig. 2.4).

The dramatic expansion of knowledge regarding human variation has been coupled with revolutionary advances in technologies in order to assay genetic variants. Rapid development of microarrays designed for genotyping have led to the current capacity of assaying up to approximately 2.5 million SNPs in a matter of days and typically capturing information for the majority of variation in Caucasian populations. Thus, it is now possible to efficiently search the entire genome for loci associated with the risk of developing disease in studies referred to as genome-wide association (GWA) scans. Additional genotyping and sequencing can be used to narrow the region of interest further and to identify the disease risk allele (Fig. 2.5).

Many studies of genetically complex diseases have used this approach to identify risk loci with approximately 500 GWA studies reported in the literature and with



**Fig. 2.4** Linkage disequilibrium (LD) between SNPs within the human genome. LD (or correlation) is present between variants within the genome where portions of sequence are inherited nonrandomly as units, and is typically expressed as  $r^2$  values. An example is shown with regions of strong LD and weak LD. Each diamond represents the  $r^2$  values between any 2 SNPs. In this example, the  $r^2$  value between SNP1 and SNP2 is  $r^2=0.99$ . The  $r^2$  between SNP5 and SNP6 is 0.20. The shading of each diamond is proportional to the  $r^2$  value. (e.g., black is  $r^2=1.0$ )



**Fig. 2.5** Mapping genetic effects in complex diseases. This figure illustrates the concept of mapping genetic associations within the genome. In the GWA scan phase, markers are selected to take advantage of haplotype block structure and reduce the genotyping burden within a given population. The GWA scan phase will identify SNPs that are in LD with the causal allele and not likely to type it directly. Further genotyping is required to narrow the effect to the region of interest surrounding the causal allele. While LD between SNPs allows researchers to test fewer polymorphisms to capture the majority of variation within an individual genome, it can also pose problems for fine mapping in regions with strong LD. Ultimately, functional assays that test biological effects such as altered transcript levels or changes in coding sequence that modify protein function are usually required to prove causality when multiple markers are in very strong LD with each other

more than 2,400 associations currently identified. Over 30 GWA scans have been reported for autoimmune phenotypes. Collectively, GWA scans have been highly successful in detecting novel associations with disease. For example, associations with over 35 loci have been established with SLE, with the continual confirmation of additional associations [7].

Although GWA studies have proven instrumental in identifying numerous genetic associations, complexities in the underlying architecture of disease can pose

significant challenges in the execution of gene mapping studies. The effect size, describing the strength of association between two variables, can be expressed as an odds ratio (OR). Furthermore, GWA scans are neither comprehensive nor very powerful for identifying rare variants (i.e., allele frequency < 1%). The potential for identifying false-positive associations becomes substantial when over a million statistical tests are performed. To compensate for multiple testing, corrections typically place the significance threshold at  $p < 5 \times 10^{-8}$ . GWA studies to identify low-level risk variants while minimizing false-positive discoveries require large patients cohorts to provide reliable data.

Cohort sizes for genetic association studies published to date in SS have included no greater than a few one-hundred cases (Table 2.1 and Fig. 2.6). In SS, the lack of large patient cohorts available for study has confounded the reliability and reproducibility of genetic associations reported thus far. With such small sample sizes, a seemingly significant association in one study may be nearly impossible to replicate in another given the imprecision in allele frequency estimates or heterogeneity of clinical subjects. This is illustrated in Fig. 2.7, where the power to detect genetic association is influenced not only by the study sample size, but also by the strength of the effect (as quantified by the odds ratio) and the allele frequency. In this context, efforts to build much larger patient cohort resources will be a critical step toward improving statistical power and developing a comprehensive understanding of the contribution of genetics to SS etiology.

## 2.4 Candidate Genes and SS Pathogenesis

Models of pathogenesis in SS have largely been based on traditional hypothesis-driven studies in which various components of the immune system have been dissected. Genetic studies completed to date have been informative from the perspective that most candidate genes have been chosen for association testing based on these discoveries. Other candidate genes have been evaluated in SS only after associations were identified in other related diseases, such as SLE or RA.

Clearly, the altered function of multiple cell types and molecular pathways is involved in SS, with each possibly having genetic variants that underlie these observations. Evidence to support genetic variants contributing to SS within both the innate and adaptive immune responses has been reported (Fig. 2.6 and Table 2.1). Numerous multifunctional proteins such as cytokines and chemokines have been implicated and are often produced by or act on more than one cell type, adding complexity to disease models. Overall, most of the associations reported to date should be interpreted with caution given that further studies in larger, more statistically powerful sample sets are needed. Additionally, any putative genetic association should also be replicated in independent cohorts. Nevertheless, we can begin to integrate genetic associations into models of disease pathogenesis for variants with significantly differing allele frequencies between cases and controls. We focus the discussion below to include primarily the genes (or loci) with the most



**Table 2.1** Summary of non-HLA association studies in Sjögren's syndrome

Locus	Phenotype	Cases	Controls	<i>p</i> -value	OR/RR	Reference
<i>ApoE</i>	Early onset SS	63	64	0.0407	–	Pertovaara et al. Rheumatology (Oxford) 2004;43(12):1484.
<i>BAFF</i>	Anti-Ro/anti-La in SS	123	136	<0.001	–	Nossent et al. Rheumatology (Oxford) 2008;47:1311.
<i>CCR5</i>	SS	39	76	0.043	0.35	Petrek et al. Clin Exp Rheumatol 2002;20(5):701.
<i>CD4A</i>	Anti-Ro or anti-La in SS	391	532	0.00092	0.45	Nordmark et al. Genes Immun 2010:1.
<i>CD40</i>	Anti-Ro or anti-La in SS	391	532	0.00098	0.73	Nordmark et al. Genes Immun 2010:1.
<i>CLTA4</i>	SS	111	156	0.032	1.78	Downie-Doyle et al. Arthritis Rheum 2006;54(8):2432.
<i>EBF1</i>	SS	540	532	0.000099	1.68	Nordmark et al. Genes Immun 2010:1.
	Anti-Ro or anti-La in SS	391	532	0.00051	1.65	Nordmark et al. Genes Immun 2010:1.
<i>FAM167A-BLK</i>	SS	540	532	0.00047	1.37	Nordmark et al. Genes Immun 2010:1.
	Anti-Ro or anti-La in SS	391	532	0.00082	1.40	Nordmark et al. Genes Immun 2010:1.
<i>Fas</i>	Anti-Ro/anti-La negative SS	101	108	0.04	–	Mullighan et al. Ann Rheum Dis 2004;63(1):98.
<i>FCGR3B</i>	SS with <2 copies of FCGR3B	774	409	0.074	2.01	Mamtani et al. Genes Immun 2010;1:155.
	SS with >2 copies of FCGR3B	774	409	0.048	2.26	Mamtani et al. Genes Immun 2010;1:155.
<i>GSTM1</i>	SS	106	143	0.035	1.72	Morinobu et al. Arthritis Rheum 1999;42(12):2612.
<i>HA-1</i>	SS	88	271	0.003	–	Harangi et al. Eur J Immunol 2005;35(1):305.

(continued)

**Table 2.1** (continued)

Locus	Phenotype	Cases	Controls	<i>p</i> -value	OR/RR	Reference
<i>IL-1RA</i>	SS	36	100	0.04	2.38	Perrier et al. Clin Immunol Immunopathol 1998;87(3):309.
<i>IL2-IL21</i>	SS	94	368	0.033	0.46	Maiti et al. Arthritis Rheum 2010;62(2):323.
<i>IL-4Rα</i>	SS	45	74	0.035	2.6	Youn et al. Immunogenetics 2000;51(8–9):743.
<i>IL-6</i>	SS	66	400	<0.0001	–	Hulkkonen et al. Rheumatology (Oxford) 2001;40(6):656.
<i>IL-10</i>	s-IgG concentration in SS	28 <sup>a</sup>	9 <sup>a</sup>	0.012	–	Origuchi et al. Ann Rheum Dis 2003;62:1117.
	SS	129	96	0.036	2.25	Gottenberg et al. Arthritis Rheum 2004;50(2):570.
	Early onset SS	63	150	0.001	–	Font et al. Rheumatology (Oxford) 2002;41(9):1025.
<i>ILT6</i>	SS	149	749	0.0093	2.65	Kabalak et al. Arthritis Rheum 2009;60(10):2923.
<i>Ig KM</i>	Anti-La in SS	6 <sup>b</sup>	56 <sup>b</sup>	0.016	–	Pertovaara et al. J Rheumatol 2004;31:2175.
	LSG histological severity in SS	35 <sup>b</sup>	27 <sup>b</sup>	0.004	–	Pertovaara et al. J Rheumatol 2004;31:2175.
	p-IgG3 in SS	35 <sup>b</sup>	27 <sup>b</sup>	0.002	–	Pertovaara et al. J Rheumatol 2004;31:2175.
	s-β2-m concentration in SS	35 <sup>b</sup>	27 <sup>b</sup>	0.024	–	Pertovaara et al. J Rheumatol 2004;31:2175.
<i>IRF5</i>	SS	210	154	0.01	1.93	Miceli-Richard et al. Arthritis Rheum 2007;56(12):3989.
	SS	368	711	0.000024	1.49	Nordmark et al. Genes Immun 2009;10:68.
	SS	368	711	0.00032	1.57	Nordmark et al. Genes Immun 2009;10:68.

**Table 2.1** (continued)

Locus	Phenotype	Cases	Controls	<i>p</i> -value	OR/RR	Reference
<i>IRF-5/TNPO3</i>	SS	540	532	0.0000055	1.70	Nordmark et al. Genes Immun 2010:1.
	Anti-Ro or anti-La in SS	391	532	0.0000017	1.81	Nordmark et al. Genes Immun 2010:1.
<i>IκBα</i>	SS	98	110	<0.001	16.2	Ou et al. J Clin Immunol 2008;28:440.
	SS	98	110	<0.007	34.14	Ou et al. J Clin Immunol 2008;28:440.
<i>MBL</i>	SS	104	143	0.011	–	Wang et al. Ann Rheum Dis 2001;60(5):483.
	SS	104	143	0.024	1.93	Wang et al. Ann Rheum Dis 2001;60(5):483.
	SS	14	129	0.0479	–	Tsutsumi et al. Genes Immun 2001;2(2):99.
<i>MECP2</i>	SS	460	1828	0.0016	1.33	Cobb et al. Ann Rheum Dis 2010;69:1731.
<i>PTPN22</i>	SS	70	308	0.01	2.42	Gomez et al. Genes Immun 2005;6(7):628.
<i>Ro52</i>	Anti-Ro52 SS vs. healthy controls	38	72	0.00003	–	Nakken et al. Arthritis Rheum 2001;44(3):638.
	Anti-Ro52 (+) vs. anti-Ro (–) in SS	39 <sup>c</sup>	23 <sup>c</sup>	0.011	2.67	Imanishi et al. Clin Exp Rheumatol 2005;23(4):521.
<i>STAT4</i>	SS	120	1,112	0.01	1.47	Korman et al. Genes Immun 2008;9(3):267.
	SS	368	711	0.0014	1.41	Nordmark et al. Genes Immun 2009;10:68.
	SS	540	532	0.00070	1.40	Nordmark et al. Genes Immun 2010:1.
	Anti-Ro or anti-La in SS	391	532	0.00069	1.44	Nordmark et al. Genes Immun 2010:1.
<i>TAP2</i>	Anti-Ro (+) vs. anti-Ro (–) in SS	51	57	0.001	–	Kumagai et al. Arthritis Rheum 1997;40(9):1685.

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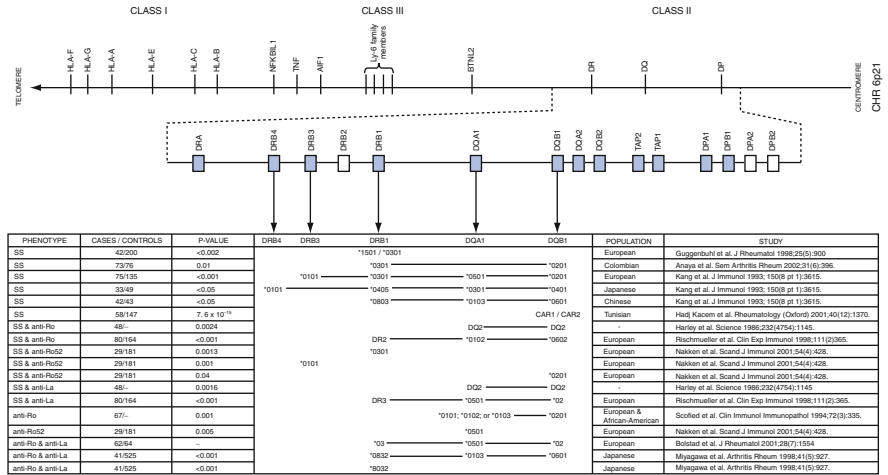
**Table 2.1** (continued)

Locus	Phenotype	Cases	Controls	<i>p</i> -value	OR/RR	Reference
<i>TCRBV</i>	SS	61	121	0.018	3.0	Lawson et al. Ann Rheum Dis 2005;64(3):468.
<i>TGFβ1</i>	SS with anti-La	129	96	0.0006 <sup>c</sup>	10.2	Gottenberg et al. Arthritis Rheum 2004;50(2):570.
<i>TNF2</i>	SS	129	96	0.00028 <sup>c</sup>	2.86	Gottenberg et al. Arthritis Rheum 2004;50(2):570.
<i>TNFSF4</i>	SS	540	532	0.00074	1.34	Nordmark et al. Genes Immun 2010:1.
	Anti-Ro or anti-La 391 in SS		532	0.000076	1.46	Nordmark et al. Genes Immun 2010:1.

<sup>a</sup>This study compares the allele frequencies of codon 54 wild type in subjects with SS by Fisher’s exact t-test, and has case and control groups comprised of these patients.

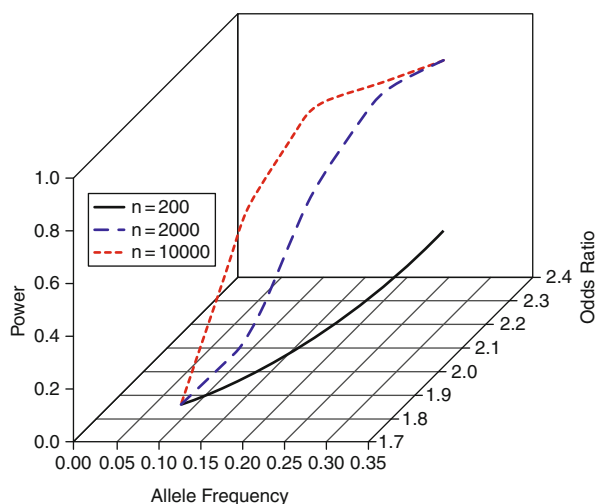
<sup>b</sup>There are more subclinical phenotypes referenced in the paper that exhibit statistical significance than are listed here.

<sup>c</sup>This study used Fisher’s exact t-test to compare subjects with primary Sjögren’s syndrome producing serum IgG >15g/L with those producing serum IgG <15g/L, and consists of case and control groups of SS subjects producing IgG.



**Fig. 2.6** HLA associations with Sjögren’s syndrome. This figure summarizes studies that have identified the association of certain alleles and/or haplotypes from the HLA region with Sjögren’s syndrome and/or the production of anti-Ro/anti-La autoantibodies. Note the small cohort sizes. Shaded blue boxes denote functional genes. Lines connecting alleles denote haplotype structure. DQ CAR1/CAR2 represents polymorphic CA repeat microsatellites located between the HLA-DQA1 and -DQB1 genes [42]. Note that DR2, DR3, DQ1, and DQ2 are older forms of HLA nomenclature based on serotype groups. These serotype groups are most commonly associated with: DRB1 for DR2 and DR3; DQA1 for DQ1; and DQB1 for DQ2

**Fig. 2.7** Power to detect association. This graph illustrates three different scenarios for studies with sample sizes of 100 cases/100 controls (majority of manuscripts published to date), 1,000 case/1,000 controls (current GWA scan), and 5,000 cases/5,000 controls (future GWA scan). Power to detect genetic association is a function of not only sample size, but also the allele frequency and the odds ratio of causal alleles



statistically significant evidence ( $p < 0.001$ ) and those which were evaluated in at least 200 cases, including *IRF5*, *STAT4*, *TNFSF4*, *HLA-DR/DQ*, *FAM167A-BLK*, and *EBF1*. We underscore that unbiased, genome-wide searches for novel genetic associations in SS will undoubtedly provide new details for existing disease paradigms and will most likely reveal new avenues for future study.

Innate immune processes in SS proceed with the activation of interferon (IFN) pathways, followed by cytokine and chemokine production by cells such as monocytes/macrophages and dendritic cells, and then with the presentation of antigen to lymphocytes to generate adaptive responses that ultimately lead to autoantibody production (Table 2.1). Evidence for association with genes involved in IFN pathways has been reported in SS and notably includes interferon regulatory factor 5 (*IRF5*) and signal transducer and activator of transcription 4 locus *STAT4* [11, 15–17]. *IRF5* is a transcription factor that acts downstream of the toll-like receptors and type I interferons to promote the expression of numerous antiviral and pro-inflammatory proteins [18, 19]. Associations with several independent genetic effects within the *IRF5* locus have been documented for SLE and are found in Asian, Caucasian, Hispanic, and African-American populations [20–26]. In SS patients, the GT or TT genotype at an *IRF5* SNP (rs2004640) was found to be more prevalent when compared to controls. The T allele results in the expression of the exon 1B isoform and significant overexpression of *IRF5* in cell lines, likely driving increased expression of Type I IFN [27]. Moreover, an association in the “G” risk allele of *TNPO3* at the SNP rs13246321, which is in linkage disequilibrium with rs10488631 within the *IRF5-TNPO3* locus previously demonstrated to have association with SS, was observed in a combined cohort of Norwegian and Swedish SS patients [17].

Genetic association has also been reported in SS with a SNP (rs7574865) found in (*STAT4*) involved in IFN signaling [11]. SNPs in *STAT4* have also been found to show strong association with SLE and RA [27]. *STAT4* encodes a lymphocyte signal transduction molecule that responds to Type 1 IFNs and other cytokines, including

interleukin-12 (IL12), IL-13, and interleukin-23 [11]. Upon activation by cytokines, STAT4 translocates to the nucleus and acts as a transcription factor to stimulate expression of IFN- $\gamma$ , a key inducer of T cell differentiation into type 1 T helper cells. The STAT4 protein is also required to regulate T helper cell responses [28, 29]. In addition to association analyses of SS with single variants, evaluation for more complex genetic models supports additive effects between the major risk alleles in *IRF5* and *STAT4* [16].

Plasmacytoid dendritic cells (pDCs) are a major producer of IFNs and have been observed in salivary gland tissues from SS patients [30]. Infiltrating macrophages secrete cytokines including IL-1, IL-6, macrophage chemotactic factor (MCP-1), and chemokines that can recruit T and B cells [31]. Lymphocytic salivary gland infiltrates consist of approximately 70% CD4<sup>+</sup> T cells and 20% B cells [32]. The CD4<sup>+</sup> T cells primarily express the  $\alpha/\beta$  T cell receptor and produce Th1 proinflammatory cytokines (TNF- $\alpha$ , interferon- $\gamma$ , TGF- $\beta$ , IL-6, and IL-10), several of which have been implicated, but not yet established, as robust genetic effects (Table 2.1). These cells become activated upon encountering antigens, secreting matrix metalloproteinases and additional chemokines and cytokines. These signaling cascades further exacerbate the immune response, promoting the continued production of Type I interferon and interferon-inducible genes, while driving loss of tolerance and ultimately creating a cycle of autoimmune reactivity [33–38].

Central to the interface between innate and adaptive immunity is antigen presentation. Additional genetic associations of SS with genes involved in this critical process have been identified and include several loci within the Major Histocompatibility Complex (MHC). Historically, studies evaluating alleles in the human leukocyte antigen (HLA) genes for association with SS dominated the literature prior to 1995 (Fig. 2.6). The extended MHC region, located at chromosome 6p21.31, covers approximately 7.6 megabases (Mb) of genomic DNA and encodes for approximately 252 genes and 139 pseudogenes [39]. HLA genes are the subset that encodes cell surface antigen-presenting proteins. Alleles of these genes are well-documented risk factors for the development of autoimmune disorders [40, 41]. The HLA genes found to be associated with SS vary in different ethnic groups and are summarized in Table 2.1 [42]. In general, studies have primarily focused on alleles at the Class II HLA-DR and -DQ loci. The most consistent associations to date have been with DR2 and DR3 alleles at the DRB1 locus in Caucasian populations (Fig. 2.6).

Interestingly, most HLA associations are stronger when evaluated in subsets of patients with anti-Ro/SSA and anti-La/SSB autoantibody responses. Particularly strong associations with these antibody responses were identified in patients heterozygous for DQw1 and DQw2 [43]. Other genes may also be involved in autoantibody production in SS, including the genes Transporter 2, ATP-binding cassette, sub-family B (*TAP2*), and Transforming Growth Factor- $\beta$ 1 (*TGF- $\beta$ 1*) (Table 2.1). The *TAP* genes, which are mapped to the MHC region, are important in peptide loading and cell surface expression of HLA Class I molecules. *TGF- $\beta$ 1*, mapped to chromosome19q13.1, is a pro-fibrotic, immunosuppressive cytokine

expressed by many cell types and is known to be underexpressed in the salivary glands of SS patients [44]. Gottenberg et al. identified an allele at codon 10 of *TGF- $\beta$ 1* with an elevated allele frequency in SS patients who had the HLA-DRB1\*3 haplotype and elevated levels of anti-La/SSB autoantibodies [44]. They hypothesized that both the *TGF- $\beta$ 1* polymorphism and the HLA-DRB1\*3 haplotype act in combination to promote the production of anti-La/SSB autoantibodies.

Several genes that function in adaptive immune responses, particularly in T and B cells, have been implicated in SS. A genetic association of SS has been recently identified by Nordmark et al. with tumor necrosis factor super family member 4 (*TNFSF4/OX40L*), a gene relevant to T cell functions [17]. *TNFSF4* is expressed on the surface of multiple cell types including pDCs, B cells, NK cells, and vascular endothelial cells. This ligand is involved in signal transduction leading to T cell proliferation and cytokine production. Significantly, binding of this ligand to its receptor, TNFSF4R (Ox40R), inhibits production of regulatory T cells that produce IL-10, plays a critical role in maintenance of peripheral tolerance, and can inhibit the development of autoimmune disease [45]. This interaction drives the Th1 T cell response and production of Type I interferon by pDCs. Candidate gene association studies demonstrated association with *TNFSF4* in Swedish and Norwegian SS patients (Table 2.1) [17].

Several genetic variants with evidence for association with SS could influence B cell function. B cells and plasma cells are important in the mechanisms leading up to glandular dysfunction, as is IgG deposition in the salivary glands and lacrimal ducts targeting receptors important in parasympathetic signaling. Furthermore, autoreactive lymphocytosis and an increase in circulating IgG antibodies have been implicated in the development of extraglandular effects prior to, during, or after glandular disease origination. There is significant autoreactive B cell expansion, hyperreactivity, and antibody formation in the exocrine glands [46]. Salivary infiltrates of some SS patients even demonstrate ectopic germinal centers (GC) with follicular dendritic cell networks lacking follicular zone expansion [47]. The number of anti-Ro/SSA- and anti-La/SSB-producing B cells present in the salivary infiltrate of patients with SS directly correlates with the number of anti-Ro60 producing B cells and the serum titer of circulating anti-Ro/SSA antibody [48].

An interesting example of a genetic association in SS with potential relevance to B cells has recently been identified in the region comprising two genes, *FAM167A* and the B lymphoid tyrosine kinase (*BLK*) locus [17]. *FAM167A* and *BLK* are transcribed in opposite directions, possibly from common promoter elements, and expression levels are inversely correlated. While the function of *FAM167A* remains unknown, *BLK* is expressed in B cells and is involved in cell signaling that results in activation of multiple nuclear transcription factors. Reduced expression of *BLK* is hypothesized to lead to a breakdown in tolerance by allowing autoreactive cells to escape deletion [17].

Early B cell factor 1 (*EBF1*) is a vital transcription factor involved in the enhancement of transcriptional activity during B cell development. EBF1 initiates

demethylation of DNA and activates PAX5, driving expression of important B cell activation markers. Immunoglobulin production also requires EBF1. Decreased or altered expression of EBF1 can lead to impaired B cell development. Association was observed with the presence of the “T” risk allele at a SNP (rs3843489) located in the tenth intron and in another SNP (rs869593) located in the sixth intron that appear to be independent genetic effects [17].

Significantly, increased levels of B-cell activating factor (BAFF, BLyS), a member of the tumor necrosis factor family, have been identified in the serum of SS patients compared to healthy controls and directly correlate to the degree of clinical activity and titer of circulating autoantibodies [49–51]. BAFF is essential for B cell maturation [52]. Three receptors have been identified almost exclusively on B cells that bind BAFF, including the BAFF-R (BR3), the cyclophilin ligand activator (TACI), and the B cell maturation antigen (BCMA) [53]. Increased expression of these receptors during B cell development in the presence of BAFF influences the differentiation of mature GC B cells into plasma cells [54]. BAFF levels are increased in the serum of SS patients and gene expression is upregulated in labial salivary gland tissue. Disease susceptibility for anti-Ro/SSA- and anti-La/SSB-positive SS has been associated with the CTAT haplotype of 4 SNPs located in the 5' regulatory region of the *BAFF* gene, while the TTTT haplotype has been associated with elevated BAFF levels in SS [55].

To facilitate understanding of how genetic variants identified to date can contribute to SS, it is helpful to view them in the context of their effect on relevant pathological pathways. As outlined above, many genetic associations have been observed within pathways important in the immune response, including interferon pathway signaling, antigen processing/presentation, and lymphocyte function. Other pathways with potential genetic associations include intracellular signaling, apoptosis, and inflammatory cytokine/chemokine regulation and cell recruitment. In related diseases such as SLE, multiple genes within a more limited number of common pathways have been observed. Other approaches, including genome-wide gene expression and proteomic studies, have also identified potential genes and pathways for further investigation and are highly complementary to the genetic studies outlined above.

## 2.5 Gene Expression Studies in SS

Genome-wide gene expression profiling (GEP) studies measure the levels of RNA transcripts for each active genetic locus in a given sample. These studies have identified multiple loci that are differentially expressed in either mRNA isolated from minor salivary glands or peripheral blood of SS patients when compared to healthy controls. This approach utilizes microarrays with oligonucleotides affixed to a glass slide, representing the various transcripts presently annotated in the human genome. When labeled test samples are hybridized to these arrays, the resultant binding



yields a florescent signal that can be used to quantitate the mRNA level for a given gene within a given sample. Current generation microarrays can interrogate the levels of ~48,000 transcripts in a single experiment. The analysis of this data often involves comparing transcript levels between groups such as patients and controls, and shows common expression patterns across subsets of presumably co-regulated genes, called signatures. The overexpression or underexpression of sets of genes in patients when compared to controls may point to dysregulation of recognized pathways and provide insight into disease mechanisms.

A review of four genome-wide GEP studies (three from mRNA isolated from labial salivary glands and one from peripheral blood) shows a significant and consistent dysregulation of IFN-inducible genes [30, 56–58]. These genes are overexpressed in SS patients relative to controls and form an “IFN signature” that has been observed in many related autoimmune diseases, such as SLE and RA [59, 60]. Genetic association studies in SS and other autoimmune diseases have found transcription factors, such as *IRF5* and *STAT4*, to be risk factors for disease that may contribute to the overexpression of the genes found within the IFN signature. Of interest, the overexpression of *IFITM1* (interferon-induced transmembrane protein 1) was observed in all four studies, and the genetic polymorphisms associated with SS in *IRF5* and *STAT4* correlate with expression levels of *IFITM1*. Furthermore, the peripheral blood GEP study found positive correlations between anti-Ro/SSA and anti-La/SSB titers and expression levels of the genes comprising the IFN signature in SS.

Other pathways that are dysregulated in SS patients have also been identified through GEP. For example, cytokine and chemokine signaling is important in the recruitment of inflammatory cells into tissues. Hjelmervik et al. and Pérez et al. found that the cytokine interleukin 6 (IL-6) was overexpressed within the labial salivary glands of SS patients when compared to healthy controls [56, 57]. Interestingly, while differences in IL-6 levels in peripheral blood have not been observed, downstream molecules in the IL-6 pathway do appear to be differentially expressed between patients and controls. Genetic association studies have found suggestive evidence that IL-6 is a risk factor for SS. However, additional studies will be required to establish a robust genetic effect and determine whether associated variants contribute to expression differences of IL-6 pathways. Other pathways identified in gene expression studies have been identified and relevance to disease pathogenesis is an active area of ongoing investigation.

## 2.6 Protein Expression Studies in SS

Although mRNA is an important intermediary molecule between DNA and proteins, not every mRNA molecule is translated into a functional protein. Therefore, the study of differential protein expression within tissues is critical to more clearly understand the functional impact on disease. Proteomics (the study of the structure,

function, and modification of proteins) is also rapidly developing in the field of autoimmune disease research. Multiple research tools are available to analyze the complex protein constituents in human tissues. Some of these include 2-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy, and multiple protein microarray chips having the capability to detect and quantify protein constituents in biological samples. Mapping of the SS proteome holds significant promise for revealing valuable biomarkers useful in the diagnosis and classification of the disease. It also stands to aid in drug discovery and monitoring of treatment efficacy with the goal of early treatment to prevent disease progression.

Recent studies aimed at cataloguing the normal human proteome have identified over 1,100 proteins in human saliva [55]. Multiple studies have evaluated the proteomes of whole saliva and minor salivary glands in SS [61–65]. Several hundred proteins have been described that differ in expression between SS patients and controls [61–63, 65]. At least two major trends have been observed in these studies. First, proteins involved in inflammation, including IFN pathways, are present at increased levels in salivary gland tissues and saliva from SS patients. Second, secretory proteins and other typical salivary proteins are decreased in samples from SS patients. The majority of proteins described thus far have not been evaluated as candidate genes in SS. Thus, one goal of future genetic studies will be to determine if specific variants contribute to the differences observed at the protein level, perhaps providing insight into the tissue specificity of autoimmune responses that are characteristic of SS.

## 2.7 Future Directions

Genetic, genomic, and proteomic studies are rapidly evolving and are expected to continue enhancing our understanding of SS etiology and pathogenesis. The next stage of SS genetics research will be to perform unbiased genome-wide association scans and large-scale replication studies in much larger patient cohorts. Initial efforts are underway. These studies require extensive collaboration and contribution from clinicians and researchers since no single group will be able to independently recruit the large number of well-characterized cases needed. Studies of this scale are expected to elicit many new associated loci and pathways not previously evaluated. Whole genome sequencing is now becoming feasible and the cost will likely be at a sufficiently low level to make this technique feasible on a broad scale in the next 5 or so years. This will be an important phase in SS genetic studies as many rare variants have yet to be explored and GWA scans are typically designed to evaluate more common variants. Transcriptome sequencing and expanded proteomics studies will also provide important insight. Integration of these rich datasets, coupled with detailed clinical information, will undoubtedly be informative in the coming years for dissecting the complex etiology and disease mechanisms in SS.

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Sjögren's Syndrome

Diagnosis and Therapeutics

Ramos-Casals, M.; Stone, J.H.; Moutsopoulos, H.M.

(Eds.)

2012, XX, 633 p., Hardcover

ISBN: 978-0-85729-946-8