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Epigenetic alterations in primary Sjögren's syndrome – an overview

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ABSTRACT

Primary Sjögren's syndrome (pSS) is a chronic autoimmune rheumatic disease characterized by inflammation of exocrine glands, mainly salivary and lacrimal glands. In addition, pSS may affect multiple other organs resulting in systemic manifestations. Although the precise etiology of pSS remains elusive, pSS is considered to be a multifactorial disease, where underlying genetic predisposition, environmental factors and epigenetic mechanisms contribute to disease development. Epigenetic mechanisms, such as DNA methylation, histone modifications and non-coding RNAs, may constitute a dynamic link between genome, environment and phenotypic manifestation by their modulating effects on gene expression. A growing body of studies reporting altered epigenetic landscapes in pSS suggests that epigenetic mechanisms play a role in the pathogenesis of pSS, and the reversible nature of epigenetic modifications suggests therapeutic strategies targeting epigenetic dysregulation in pSS. This article reviews our current understanding of epigenetic mechanisms in pSS and discusses implications for novel diagnostic and therapeutic approaches.

1. Introduction

Epigenetic modifications, such as DNA methylation, histone modifications and non-coding RNAs (ncRNAs) are mechanisms for controlling gene expression that have emerged as important contributing factors in the pathogenesis of autoimmune disorders, including primary Sjögren's syndrome (pSS) [1,2]. PSS is a female dominated autoimmune disease characterized by inflammation of salivary and lacrimal glands resulting in the clinical hallmarks dry eyes and dry mouth. Systemic manifestations include extraglandular organ involvement, debilitating fatigue and a 15-fold increased risk of lymphoma, most commonly non-Hodgkin lymphomas of the B cell type [3]. B cell activation is a prominent feature of pSS and is reflected by hypergammaglobulinemia and presence of anti-Sjögren's syndrome antigen A (SSA)/Ro and antigen B (SSB)/La antibodies in sera. Biopsies of the minor salivary glands reveal focal lymphocytic infiltrates, which in a subset of patients show germinal center-like formations with B cell maturation and local anti-SSA/SSB antibody synthesis [4]. The factors driving B cell activation in sera and locally in the salivary glands or the molecular mechanisms behind the transformation to B cell lymphomas are not well understood.

The etiology of pSS is multi-factorial where genetic, epigenetic, hormonal and environmental factors are thought to interact. Several genetic susceptibility loci have been associated with pSS, including human leukocyte antigen (HLA) genes involved in antigen presentation to T cells. Confirmed pSS risk loci also include interferon regulatory factor 5 (*IRF5*) and signal transducer and activator of transcription 4 (*STAT4*) in the type I interferon (IFN) system, and B lymphocyte kinase (*BLK*) involved in B cell activation [5,6]. PSS associated genetic variants in these loci are most often found in non-coding regions and may exert their effect on disease susceptibility by affecting the epigenetic machinery, which in turn modulates gene expression in target cells and tissues.

Different cell types involved in the pathogenesis of pSS have been subjected to epigenetic studies, namely naïve CD4+CD45RA+ T cells, CD4+ T cells, CD19+ B cells, CD14+ monocytes and salivary gland epithelial cells (SGEC). The characteristic focal infiltration in salivary glands consists mainly of activated CD4+ T cells, but also of macrophages, CD19+ B cells and plasma cells. Follicular dendritic cells are present in infiltrates with germinal center-like formations [7]. Factors initiating homing of immune cells to the salivary glands are not fully understood. However, epithelial cells express major histocompatibility (MHC) antigen class I and II, adhesion and costimulatory molecules, cytokines and chemokines, and can act as antigen presenting cells and participate in recruitment of lymphocytes [8]. Studies targeting naïve T cells can provide important information on epigenetic modifications preceding immune activation. Transcriptional upregulation of IFN-induced genes, a so-called IFN signature, has been demonstrated in different peripheral blood mononuclear cell (PBMC) subsets from patients with pSS [9–11]. Type I IFN (IFN α/β), which is responsible for pleiotropic activation of immune cells and eliciting the IFN signature, is mainly produced by plasmacytoid dendritic cells (pDC) and CD14+ monocytes [12]. While pDCs are infrequent in the circulation, CD14+

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monocytes have been subjected to epigenetic studies.

Epigenetic modifications are cell type- and cell state-specific; they constitute central mechanisms contributing to both chromosome architecture and spatial-temporal regulation of genome function. Epigenomic states are semi-stable, implicating that they are relatively stable over time and by that capable of maintaining cell identity, but still dynamic enough to be able to change in response to external influences. This reversible nature suggests epigenetic modifications as potential targets for therapeutic intervention. In this review human studies of epigenetic alterations in pSS will be summarized and their implications in disease pathogenesis and clinical presentation will be discussed.

2. DNA methylation studies

DNA methylation is the most commonly studied epigenetic mark in pSS. DNA methylation constitutes the transfer of a methyl group by DNA methyltransferases (DNMTs) from S-adenosyl-methionine (SAM) to the carbon-5 position of the cytosine residue in CpG dinucleotides, generating 5-methyl cytosine (5-mC). De novo DNA methylation is established by DNMT3 and maintained during cell division by DNMT1. Methylated CpG sites in gene promoter regions are generally considered as interfering with gene transcription by binding to methyl-CpG binding proteins which recruit chromatin inactivating complexes with histone modifications leading to inaccessible chromatin. Steric inhibition of transcription factor binding to methylated promoters is another mechanism for gene silencing [13]. DNA methylation during cell replication can be inhibited by pharmacological drugs affecting the DNMT3 enzyme, e.g. hydralazine, procainamide or isoniazid, and SS-like disease has been described after anti-hypertensive treatment with hydralazine [14]. 5-azacytidin is another DNMT inhibitor currently in use in hematological malignancies with the aim of maintaining unmethylated gene promoters, thus allowing transcription of tumor suppressor genes. Active demethylation independent of cell replication can be achieved by ten-eleven translocation enzymes (TET) which remove methyl groups through iterative oxidation, followed by base excision repair. In this multi-step process 5-hydroxymethylation (5-hmC) is passed as an intermediate state prior to reversion to unmethylated cytosine [15].

Early investigations of peripheral blood cells, minor salivary glands and SGECs in pSS have employed different techniques for assessing methylation at specific CpG sites in selected target genes or determined global methylation without single CpG site resolution. More recent studies have utilized epigenome-wide DNA methylation arrays for analysis of differential methylation in various cell types and tissues in pSS. A summary of DNA methylation studies in pSS is presented in Table 1.

2.1. Targeted and global DNA methylation studies in different tissues

2.1.1. Peripheral blood cells

There have been a number of studies investigating DNA methylation at promoters of candidate genes in isolated subsets of peripheral blood, mainly CD4+ T cells. Yin and coworkers used promoter bisulfite sequencing of *TNFSF7*, encoding the B cell costimulatory molecule CD70, to determine differential methylation in CD4+ T cells from 17 patients with pSS compared with 14 controls [16]. They reported hypomethylation at the *TNFSF7* promoter, which correlated with CD70 overexpression in pSS CD4+ T cells. Hypomethylation was more prominent in pSS patients with active disease. Yu et al. employed bisulfite sequencing to analyze differential promoter methylation of the tumor suppressor gene *FOXP3* in CD4+ T cells from 15 patients with pSS and 15 controls [17]. They observed hypermethylation at all eight promoter CpG sites in pSS T cells compared to controls, with a negative correlation between methylation levels and *FOXP3* mRNA and protein expression. FOXP3 is required for maintaining regulatory T cells (Tregs),

Table 1
DNA methylation studies in primary Sjögren's syndrome.

Method	Target	Cells/tissues	Cases/controls (n)	Main outcome	Reference
Bisulfite sequencing	*TNFSF7 (CD70)*	CD4+ T cells	17/14	hypomethylation and overexpression of *CD70*	Yin et al. [16]
Bisulfite sequencing	*FOXP3*	CD4+ T cells	15/15	hypermethylation and low expression of *FOXP3*	Yu et al. [17]
Bisulfite pyrosequencing	*IRF5*	T cells, B cells, monocytes, SGECs	19/24	no difference	Gestermann et al. [21]
ELISA, anti-5mC Ab	global methylation	SGECs	8/8	global hypomethylation	Thabet et al. [25]
ELISA, anti-5mC Ab	global methylation	minor salivary glands	22/10	hypomethylation associated with lymphocyte infiltrates	Konsta et al. [26]
Bisulfite pyrosequencing	*LINE-1*	minor salivary glands	31/12, 44/8	hypomethylation and overexpression of *LINE-1*	Mavragani et al. [29,30]
Methylation-sensitive PCR	*BP230/DST*	minor salivary glands	18/14	hypermethylation and low expression of *BP230*	González et al. [32]
HM450k bead chip array	epigenome-wide	naïve CD4+ T cells	11/11	hypomethylation of *LTA*, IFN-induced genes	Altorok et al. [35]
HM450k bead chip array	epigenome-wide	CD4+ T cells, CD19+ B cells	26/22	larger methylation differences in B-cells than in T-cells	Miceli-Richard et al. [24]
HM450k bead chip array	epigenome-wide	whole blood, CD19+ B cells, minor salivary glands	100/400, 24/47, 15/13	hypomethylation of IFN-induced genes	Imgenberg-Kreuz et al. [23]
HM450k bead chip array	epigenome-wide	whole blood	24/24 case/case	hypomethylation of ncRNA in high fatigue	Norheim et al. [49]
HM450k bead chip array	epigenome-wide	minor salivary glands	13/13	enrichment for differential methylation in promoters	Cole et al. [43]
HM 450k bead chip array	epigenome-wide	SGECs	8/4	hypomethylation of IFN-induced genes	Charras et al. [44]

BP230/DST, Bullous Pemphigoid Antigen 1, 230/240 kDa alias dystonin; FOXP3, forkhead box P3; HM450k, Illumina HumanMethylation array 450,000 CpG sites; IFN, interferon; IRF5, interferon regulatory factor 5; LTA, lymphotoxin alpha; LINE-1, long interspersed nuclear element 1; SGECs, salivary gland epithelial cells; TNFSF7, tumor necrosis factor ligand superfamily member 7.

and decreased Tregs populations have been described in pSS salivary glands and peripheral blood, contributing to the autoimmune process [18]. Although these two targeted studies have been performed in relatively a small number of individuals they point to an important role of CD4+ T cells in pSS pathogenesis and support the concept of promoter methylation as a mechanism of regulating gene expression.

The strongest genetic susceptibility locus for pSS outside the HLA-region is found at the interferon regulatory factor 5 (*IRF5*) gene, which is a transcription factor involved in regulation of type I IFN-induced genes and production of IFN- α [5,19,20]. Following up on the finding of increased *IRF5* mRNA in patients with pSS, Gestermann et al. determined methylation profiles of the the CGGGG insertion/deletion region and the ATG start codon of the *IRF5* promoter in total PBMCs, CD4+ T cells, CD19+ B cells, CD14+ monocytes and cultured SGECs [21,22]. Both *IRF5* promoter regions displayed low levels of global methylation in all cell types from both patients with pSS and controls. Differential methylation was assessed in CD19+ B cells (n = 11 pSS, n = 15 controls) and CD4+ T cells (n = 14 pSS, n = 21 controls) where no difference in methylation levels between pSS and controls could be observed. Using a genome-wide methylation array, studies by Miceli-Richard et al. and by our own group identified differential methylation in patients with pSS in the *IRF5* promoter region at CpG sites that were located outside the previously investigated regions [23,24]. These findings underline the advantage of interrogating patterns of DNA methylation applying methods that cover a larger number of sites.

2.1.2. Salivary glands

Salivary and lacrimal glands are the main target organs in pSS with focal lymphocytic infiltrates and the clinical features dry mouth and dry eyes. What causes the homing of immune cells to the glands and the mechanisms behind the maintenance of the inflammation has not been fully elucidated. Two studies have investigated global DNA methylation levels in minor salivary glands and SGECs from patients with pSS compared to controls [25,26]. Global methylated DNA content was analyzed by an enzyme-linked immunosorbent assay (ELISA) utilizing an anti-5mC antibody. Thabet et al. investigated SGECs extracted from eight patients with pSS and eight patients with sicca symptoms undergoing investigations for pSS where no diagnosis was made [25]. They found global DNA methylation to be reduced in pSS SGECs and minor salivary glands compared to controls with concomitant decrease in *DNMT1* gene expression and an increase in expression of the demethylating stress protein Gadd45-alpha. Global DNA methylation was particularly low in salivary gland biopsies with abundant B cells. However, no difference in peripheral T cell or B cell global methylation was found between patients and controls. After treatment of two patients with Rituximab, an anti-CD20 B cell depleting antibody, the decrease in infiltrating B cells was accompanied by an increase in global methylation levels in the investigated minor salivary glands.

Konsta et al. found decreased global DNA methylation in minor salivary glands from 22 patients with pSS compared with glands from ten controls, with more pronounced hypomethylation in glands with extensive lymphocytic infiltrates [26]. Reduced global DNA methylation in minor salivary glands was also correlated with anti-SSB positivity in sera, but not with disease activity measured by the European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index (ESSDAI) [27]. In pSS patients positive for anti-SSB in their sera, methylation status of the *SSB* gene promoter was determined by restriction enzymes and found to be demethylated in minor salivary glands with concomitant overexpression of SSB mRNA and protein. Both authors conclude that infiltrating B cells may control DNA methylation in pSS minor salivary glands, and that treatment with B cell depleting agents can restore DNA methylation, thereby controlling the expression of genes that are involved in promoting inflammation and autoimmunity [25,26]. These studies have analyzed global methylation where methylation levels at single CpG sites are not investigated and information regarding genes and pathways involved in pSS pathogenesis is limited. Minor salivary glands consist of both epithelial and inflammatory cells, the latter practically absent in control glands. Depletion of B cells will also affect other immune cells and possibly epithelial cell activation, and it is therefore difficult to conclude which cell types contribute to the observed global differential methylation.

A few studies have investigated differential methylation in specific genes in SGEC or minor salivary glands. In the study by Konsta et al., reduced global DNA methylation in minor salivary glands was associated with overexpression in glandular acini of the epithelial protein cytokeratin-19 (gene *KRT19*), while methylated acini expressed low amounts of cytokeratin-19 as determined by immunohistochemistry [26]. In a follow-up study by the same authors a human salivary gland cell line was treated with the DNA demethylating agent 5-azacytidin, which resulted in *KRT19* mRNA and cytokeratin-19 protein overexpression [28]. The authors conclude that DNA methylation levels in SGEC may contribute to pSS pathophysiology in part by controlling *KRT19*/cytokeratin-19 expression. Mavragani et al. investigated promoter methylation and gene expression of long interspersed nuclear element-1 (LINE-1) in minor salivary gland biopsies from patients with pSS and controls [29,30]. *LINE-1* is a retroviral like, endogenous DNA sequence, with the ability to transpose within the genome. The authors found hypomethylation of the *LINE-1* promoter with concomitant *LINE-1* mRNA overexpression in pSS salivary glands, which correlated with the presence of IFN-α assessed by immunohistochemistry. *LINE-1* hypomethylation was also found to be associated with a methylene tetrahydrofolate reductase (*MTHFR*) risk genotype for pSS non-mucosal associated lymphoid tissue (non-MALT) lymphomas [31]. This suggests aberrant methylation of *LINE-1* as a contributing factor for type I IFN activity, lymphocyte infiltration and possibly lymphoma development in pSS. An additional targeted DNA methylation study of pSS minor salivary glands revealed hypermethylation accompanied by low expression of the *BP230*/dystonin gene (*DST*), possibly leading to defective anchoring of acinar cells to the basal lamina and thereby contributing to destruction of pSS glands [32]. Taken together, these studies implicate DNA methylation as an important way of controlling expression of several genes in the salivary glands. However, a more comprehensive analysis of epigenetic modifications in fractionated cell populations derived from salivary glands will facilitate a better understanding of the pathogenetic mechanisms underlying salivary gland autoimmunity.

2.2. Genome-wide DNA methylation studies

Facilitated by technological advances, studies of DNA methylation in pSS have moved from assessing candidate genes or global methylation levels to the analysis of methylation patterns on a genome-wide scale. The Infinium Human Methylation450 (HM450k) BeadChip array, which interrogates quantitative DNA methylation levels at more than 480,000 CpG sites across the genome with single-base resolution, has been widely applied for epigenome-wide association studies (EWAS) in various complex traits and diseases [33,34]. In pSS the HM450k array has been used in several studies analyzing differential methylation in whole blood, fractionated blood cells and salivary gland tissue from patients and controls.

2.2.1. Peripheral blood cells

In 2014, the first EWAS in pSS was published by Altorok et al. [35]. The authors investigated DNA methylation in naïve CD4+CD45RA+ T cells originating from eleven female patients with pSS and eleven matched controls. With a cut-off of 0.2 differential methylation between patients and controls, 753 differentially methylated CpG sites (DMCs) were detected; 553 hypomethylated CpG sites in 311 genes and 200 hypermethylated sites in 115 genes. The main findings were hypomethylation of several sites of lymphotoxin-α (LTA) and at type I IFN-induced genes, including *STAT1*, *IFI44L* and *IFITM1*. LTA forms heterotrimers with lymphotoxin-β (LTB) and signals via LTβ-receptor,

which is present on lymphoid cells. LTβ-receptor pathway signaling is essential for activation of follicular dendritic cells and maintenance of tertiary lymphoid tissue as well as it is involved in induction of IFN-α [36]. Interestingly, genetic polymorphisms in *LTA* have been associated with pSS susceptibility, and LTA protein expression is detected in salivary glands and sera from patients with pSS [37,38].

Miceli-Richard et al. analyzed genome-wide methylation patterns in fractionated peripheral CD19+ B cells and CD4+ T cells in 26 female patients with pSS and 22 age-matched controls, and observed larger differences in DNA methylation between patients and controls in B cells compared to T cells [24]. Despite a lower cut off of 0.07 differential methylation between cases and controls compared with the study by Altorok et al., only 119 DMCs were detected in CD4+ T cells. On the contrary, as many as 6707 DMCs in 3619 genes were identified in CD19+ B cells, out of which 44% of the differentially methylated sites were hypomethylated in patients. Several IFN-induced genes were among the most hypomethylated CpG sites in CD19+ B cells.

A larger comprehensive study of genome-wide DNA methylation in whole blood, peripheral CD19+ B cells and minor salivary glands was performed by our group [23]. Analyzing methylation in whole blood from 100 patients with pSS and 400 control individuals, disease associated alterations were observed at a total of 11,785 CpGs sites, of which 52% were hypomethylated in pSS. Differentially methylated sites were found at 5623 unique genes with most significantly hypomethylated sites annotated to genes involved in immune response mechanisms, in particular type I IFN-induced genes such as *MX1*, *IFI44L*, *PARP9* and *IFITM1*, replicating previous observations and establishing an IFN signature at the methylation level in pSS. Investigating fractionated peripheral B cells from 24 patients and 47 controls identified even more distinct differences in methylation levels between patients and controls at IFN-regulated genes compared to the whole blood analysis. Promoter hypomethylation in these genes was associated with elevated mRNA expression levels in pSS B cells.

To summarize the findings from the three EWAS above, hypomethylation of IFN-induced genes was observed in all analyzed cells and tissues from patients with pSS, and in B cells increased gene expression was demonstrated for the most differentially methylated genes. IFN production can be induced by endogenous stimuli such as immune complexes (IC) composed of nucleic acid-binding proteins and SSA/SSB antibodies. These IC can bind to endosomal toll-like receptor (TLR)7 and TLR9, followed by signaling cascades via several molecules including IRF5, resulting in IFN production [39]. Type I IFNs bind to the type I IFN receptor (IFNAR), and signaling via the JAK-STAT pathway elicits the expression of IFN-induced genes, i.e. the IFN signature (Fig. 1). IFN has pleiotropic activating effects on the immune system and an IFN signature has been observed in whole blood, different PBMCs and salivary gland tissue from patients with pSS [9,10,40–42]. From these pSS EWAS studies we can conclude that the IFN signature is also detectable at the DNA methylation level, and is strong enough to be observed also when studying modest numbers of samples and different cell types.

2.2.2. Salivary glands

Genome-wide methylation analyses have also been performed in pSS salivary glands. Cole and coworkers performed a genome-wide methylation study on minor salivary glands obtained from 13 patients with pSS and 13 controls and detected 7820 differentially methylates sites, of which 5699 were hypomethylated and 2121 hypermethylated in pSS [43]. They identified 57 genes enriched for differential methylation in their promoters, among these hypomethylation in *LTA*, as seen in CD4+ T cells in the study by Altorok et al., and *CXCR5*, a gene associated with pSS and found to be hypomethylated in whole blood in the study by our group [5,23,35]. Analyzing minor salivary gland biopsies from 15 patients and 13 controls, we reported 45 differentially methylated sites, with the most significantly hypomethylated site located at the IFN-induced gene *OAS2* [23]. Both studies are limited by the analysis of minor salivary gland tissue, consisting of multiple cell types, where glands from patients with pSS include inflammatory cells which are not present in glands from controls. Considering the potential confounding by differential cell type distribution the results must be interpreted with caution, but notably the same IFN-induced genes are hypomethylated in pSS minor salivary glands as in peripheral blood cells.

To overcome the effects of cellular heterogeneity, Charras et al. studied long-term cultured SGECs derived from minor salivary glands from eight patients with pSS and four controls [44]. Using a cut-off of 0.07 differential methylation, 4662 differentially methylated sites in 2650 genes were characterized, out of which 21% were hypomethylated in pSS SGECs. IFN-induced genes were among differentially methylated genes, corroborating the results obtained in whole minor salivary glands. An IFN signature is well established in different PBMCs in pSS, and hypomethylation of IFN-induced genes in minor salivary glands has been attributed to infiltrating lymphocytes. This study suggests similar signaling pathways in immunologically active epithelial cells and supports the concept of pSS as autoimmune epithelitis [45].

2.3. DNA methylation patterns of clinical sub-phenotypes

Epigenetic changes can provide insights into disease sub-phenotypes, such as specific disease manifestations, disease activity, flares and progression, or population and sex differences in disease prevalence and severity. So far, distinct DNA methylation signatures have been analyzed in a limited number of clinical subsets of patients with pSS. In the studies by Miceli-Richard et al. and our group, hypomethylation of IFN-induced genes was more prominent when only patients positive for anti-SSA and/or SSB-antibodies were included in the analyses [23,24]. This is in concordance with the IFN signature predominantly seen in cells from antibody-positive patients with pSS [46]. DNA methylation reflects the epigenetic status at a certain time-point, which might influence disease activity by altering gene expression and cellular downstream mechanisms. One EWAS investigated the correlation of DNA methylation with ESSDAI and found considerably more abundant differentially methylated sites in patients with high ESSDAI compared to low [24].

The biological basis of chronic fatigue, which is the main cause for disability in pSS, is poorly understood. The clinical observation that fatigue varies largely between individuals and that fatigue levels are not associated with disease activity or other common laboratory tests, could point to an implication of underlying genetic variation or other mechanisms affecting genetic regulation [47,48]. Norheim et al. investigated a possible role for DNA methylation in fatigue in patients with pSS [49]. In a case-case association study differential DNA methylation was analyzed in whole blood samples from patients with either high (n = 24) or low (n = 24) fatigue, assessed by fatigue visual analogue scale (fVAS) at the time of blood sampling. A total of 251 differentially methylated CpG sites were identified with hypomethylation of a ncRNA in high fatigue as the main finding. Limitations of this study are the relatively small sample size and a study design with fatigue level as a categorical factor. Although correcting for major blood cell types in the regression analysis, the results may still be affected by a differential distribution of cellular sub-types.

2.4. Genetic regulation of DNA methylation

It is suggested by numerous studies that DNA methylation levels at a fraction of CpG sites can be under genetic control [50]. Since genetic risk variants in pSS as well as in other complex diseases are predominantly found at intergenic regions, interpretation of their functional role is a major challenge. Genetic risk variants may potentially confer functional impact by influencing DNA methylation patterns of target genes, and thereby modulating transcriptional accessibility. Miceli-Richard et al. reported that differentially methylated CpG sites in

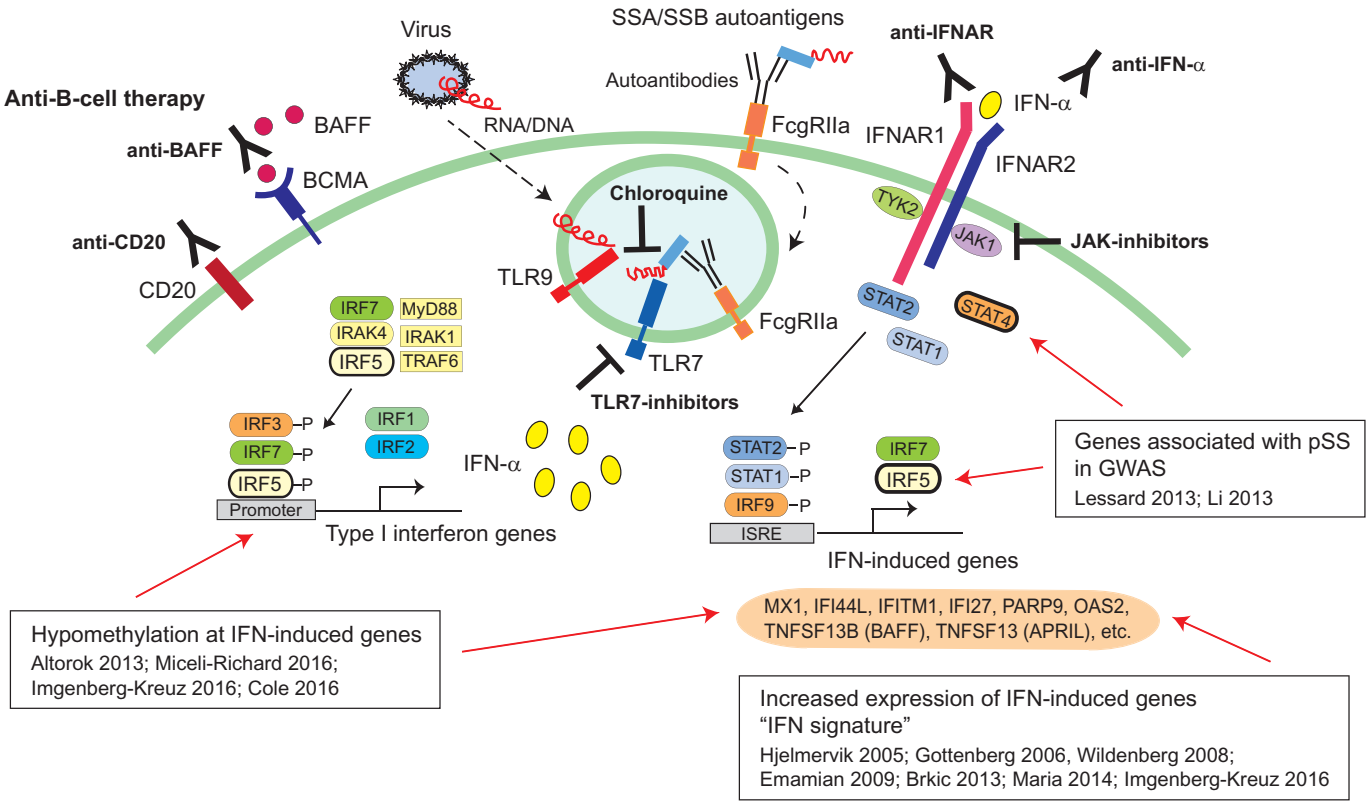

Fig. 1. The type I interferon system in B cells Production of interferon (IFN)-α can be induced by either exogenous stimuli, such as viral DNA/RNA, or endogenous stimuli, such as immune complexes (IC) composed of nucleic acid-binding proteins and SSA/SSB antibodies. Upon binding of ICs bind to Fcγ-receptor IIa on the cell surface, the complex is internalized into endosomes for ligation with toll like receptors (TLR) 7 and 9. This, in turn, activates signaling through a series of transcription factors, including IRF5, leading to gene and protein expression of IFN-α, the main type I IFN. IFN-α binds to the IFN-α-receptor (IFNAR) and by signaling via the JAK-STAT pathway, transcription of IFN-induced genes, the so-called “IFN signature”, is induced. DNA hypomethylation at CpG sites located in promoter regions of IFN-induced genes is observed in B cells from patients with pSS and is thought to contribute to the elevated expression of these genes. Genes associated with pSS susceptibility in GWAS are marked with bold circles. Therapeutic targets in off-label use in pSS are the B cells themselves by blocking with anti-CD20 or anti-BAFF antibodies. Chloroquine interferes with endosomal TLR-signaling. Potential therapeutics are antibodies against the IFNAR1 subunit (Anifrolumab) or IFN-α (Sifalimumab) and molecules targeting JAK1, TLR7 or IRAK4.

pSS B cells overlapped with loci, which had been associated with pSS susceptibility in a GWAS [24]. These results may be indicative of a close interaction between genetic and epigenetic variation in the pathogenesis of pSS.

For a systematic investigation of genetic regulation of DNA methylation, methylation levels can be tested for genotype association in methylation quantitative trait loci (meQTL) analyses. In our study analysis of genetic variants at seven pSS GWAS loci for association with methylation levels in whole blood from control individuals revealed evidence for genetic regulation of DNA methylation at pSS risk loci [23]. The results indicate that all tested pSS GWAS alleles have the potential to affect DNA methylation levels at nearby genes. The most significant meQTLs were observed within the HLA region and at the *IRF5-TNPO3* locus, which are also the regions where the strongest genetic associations for pSS have been identified [5]. Taken together, these studies point to a connection between pSS susceptibility loci and epigenetic regulation, suggesting a regulatory role for pSS-associated genetic variants. However, studies where both genetic variation and epigenetic marks, such as DNA methylation, are investigated in tissues from the same patients are still lacking.

3. Histone modifications

Modification of histone proteins constitutes another epigenetic mechanism. Histone proteins are essential for nuclear architecture and genome stability, but they are also involved in regulatory processes of gene transcription by modifying the accessibility of chromatin for the transcription machinery [51]. Histone tails protrude out of the nucleosome and are subject to a variety of covalent post-translational modifications, with methylation, acetylation, phosphorylation and ubiquitination being the most abundant modifications. These so-called histone marks can modulate chromatin structure and hence transcriptional accessibility directly, and they typically constitute binding sites for non-histone proteins to chromatin. Transcriptionally active or inactive chromatin are distinguished by specific post-translational modifications of histone proteins.

Integration of multiple sources of genetic and epigenetic data can infer additional functional information about identified variants. In an in silico approach study by Konsta et al., pSS susceptibility variants were found to be specifically associated with histone marks for promoters and enhancers in reference B cells, and with enhancers in reference monocytes [52], underlying once again the importance of analyzing epigenetic marks in relevant cell types. Our group found an enrichment of differentially methylated sites in pSS for overlap with specific histone modifications in reference B and T cells [23]. More specifically, CpG sites hypomethylated in whole blood from patients compared to controls were overrepresented in enhancer regions (H3K4me1 and H3K27ac), whereas hypermethylated sites were underrepresented in these regions, and instead enriched for the histone mark for actively transcribed gene bodies, H3K36me3. Analyses approaches aiming at inclusion of different sources of data are hampered by limited availability of published data from different cell types and conditions. As of now, there are no public datasets available on histone modifications directly from primary cells from patients with pSS.

Table 2
Studies of microRNAs (miRNAs) in primary Sjögren's syndrome.

Method	Target	Cells/tissues	Cases/controls (n)	Main outcome	Reference
miRNA microarray	534 miRNAs	minor salivary glands	16/8	miR-768-3p and miR-574 inversely correlated with focus score	Alevizos et al. [70]
SOLiD sequencing	total/novel miRNAs	minor salivary glands	6/3	differential expression of miR-5100	Tandon et al. [71]
RT-qPCR	miRNAs predicted to target SSA/SSB	minor salivary glands, SGECs, PBMCs	14/13	downregulation of let-7b (SGECs) in AB-positive compared to AB-negative pSS	Kapsogeorgou et al. [72]
RT-qPCR	miR-132, miR-146, miR-155	PBMCs	25/10	overexpression of miR-146 and miR-155	Pauley et al. [57]
RT-qPCR	miR-146a/b	PBMCs	21/10	overexpression of miR-146a/b	Zilahi et al. [58]
RT-qPCR	miR-146a, miR-155	PBMCs	27/22	overexpression of miR-146a, downregulation of miR-155	Shi et al. [59]
RT-qPCR	miR-155	PBMCs	23/10	overexpression of miR-155 and *SOCS1*	Chen et al. [60]
miRNA microarray, RT-qPCR	2042 miRNAs	PBMCs	4/3 (33/10)	overexpression of miR-181a	Peng et al. [62]
RT-qPCR	miRNAs predicted to target SSA/SSB	PBMCs, minor salivary glands, SGECs	29/24	dysregulation of miR-16, miR-200b-3p, miR-223 and miR-483-5p	Gourzi et al. [63]
miRNA microarray	2063 miRNAs	peripheral CD14+ monocytes	18/10 (10 SLE/10 RA)	dysregulation of miRNAs in TGF-β pathway	Williams et al. [64]
miRNA microarray	miRNome-wide	peripheral CD4+ T cells and CD19+ B cells	17/15, 16/12	miR-30b-5p inversely correlated with *BAFF* expression in B cells	Wang-Renault et al. [66]
NGS small RNA-sequencing	miRNome-wide	PBMCs	8/7 (8 SLE)	downregulation of miR-105-5p	Chen et al. [68]

AB, autoantibody; BAFF, B cell activating factor alias TNFSF13B; NGS, next generation sequencing; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; RT-qPCR, reverse transcriptase quantitative PCR; SSA/SSB, anti-Sjögren's syndrome antigen A and B; SGECs, salivary gland epithelial cells; SLE, systemic lupus erythematosus; SOCS1, suppressor of cytokine signaling 1; SOLiD sequencing, small oligonucleotide ligation and detection system sequencing; TGF-β, transforming growth factor beta.

4. Non-coding RNAs

While protein coding genes account for less than 2% of the sequence of the human genome, transcription of ncRNAs from intronic or intergenic genomic regions can constitute an important regulatory mechanisms with implications in tissue differentiation, development, proliferation and cell metabolism [53]. Aberrant expression of microRNAs (miRNAs) has been linked to essentially all complex autoimmune diseases, including pSS [54]. MiRNAs are a class of evolutionary conserved small non-coding, single-stranded RNA molecules with a length of around 19–25 nucleotides, that can interfere with gene expression at the post-transcriptional level. Long non-coding RNAs (lncRNAs) are defined as ncRNAs with a length above 200 nucleotides. An overview of studies investigating the role of ncRNAs in pSS is provided in Table 2.

4.1. microRNAs (miRNAs)

4.1.1. Peripheral blood cells, serum and saliva

Aberrant expression of miRNAs in different blood cells, sera and saliva in pSS has been reported by a number of studies, while only a few of the target genes have been identified so far. Evidence suggests a role for miR-146 and miR-155 in regulation of inflammatory immune responses [55,56], consequently, several studies have been undertaken with the aim to evaluate a possible contribution of miR-146 and miR-155 in pSS pathogenesis. These studies have in common that miRNA expression was analyzed in PBMCs using reverse transcriptase quantitative PCR (RT-qPCR) and that sample sizes were rather limited (Table 2) [57–60]. Zilahi et al. investigated the expression of miR-146a and miR-146b and their putative target genes *IRAK1*, *IRAK4* and *TRAF6* in PBMCs from 21 patients with pSS and ten controls [58]. They found overexpression of mirR-146a/b and *TRAF6* in pSS, whereas *IRAK1* expression was downregulated. Expression of *IRAK4* was not significantly different in this relatively small study. An implication of miR-146 in negative feedback mechanisms regulating TLR signaling has been proposed, and decreased expression of miR-146 may promote excess inflammation leading to generation of autoimmune responses [61]. Expression of miR-146a/b was found to be elevated in pSS PBMCs in all investigated cohorts, while the results for miRNA-155 are more conflicting: Overexpression of miRNA-155 in PBMCs from patients with pSS has been described by Pauley and Chen [57,60], downregulation by Shi et al. [59]. Small sample sizes, variation in cell type distribution and differences in clinical features and medical treatment between the investigated cohorts may explain these contradictory results.

The development of expression microarrays has facilitated simultaneous analyses of expression patterns of a larger number of miRNAs. Using microarray technology Peng et al. identified overexpression of miR-181a in PBMCs in a Chinese pSS cohort compared to controls [62]. They speculated that aberrant miR-181a expression may compromise B cell and T cell maturation leading to onset of autoimmune processes. Gourzi et al. investigated differential expression of a subset of miRNAs that were predicted to target mRNA of SSA/Ro and SSB/La, in salivary gland tissue, long-term cultured non-neoplastic SGECs and PBMCs from 29 patients with pSS and 24 non-pSS sicca controls [63]. They observed tissue-specific patterns of aberrant miRNA expression in pSS with miR-16 upregulation in salivary glands, miR-200b-3p upregulation in SGECs, and miR-223 and miR-483-5p upregulation in PBMCs. In addition, the authors reported lower expression of miR-200b-5p in minor salivary gland tissue from pSS patients with mucosal associated lymphoid tissue (MALT) lymphoma. The results suggest a role of aberrant miRNA expression in generation of autoantibodies against SSA and SSB as well as and in lymphomagenesis in patients with pSS. Williams and coworkers focused on CD14+ monocytes using expression microarrays and reported upregulated expression of several miRNAs involved in TGF-β signaling [64]. Gallo et al. demonstrated the presence of miRNAs in exosomes in saliva and serum, and suggested these as suitable sources for biomarker detection [65].

Recently, Wang-Renault et al. studied miRNA expression patterns in 17 patients with pSS and 15 controls in fractionated T cells and B cells using a miRNA expression panel [66]. They observed differential expression of 21 miRNAs in T cells and 24 in B cells, out of which seven and four, respectively, were replicated in an independent cohort. Restriction of analysis on anti-SSA positive patients resulted in increased statistical significance for the majority of differentially expressed miRNAs in B cells, while the effect in T cells was negligible, underlining the important role of B cells in pathogenic mechanisms in antibody-positive pSS. In addition, expression of miR-30b-5p was inversely correlated with B cell activating factor (*BAFF*) mRNA expression in B lymphocytes from patients with pSS, implicating a role for miRNAs in regulating *BAFF* expression. Liang et al. suggest a direct functional involvement of the SSB antigen in global miRNA expression via binding to miRNA precursors through mechanisms of stem-loop recognition [67].

Using next-generation RNA-sequencing Chen et al. interrogated differential miRNA expression in PBMCs from eight patients with pSS, seven healthy controls as well as eight patients with systemic lupus erythematosus (SLE) [68]. Compared to SLE, differential miRNA expression in pSS was in general more moderate. In pSS, a total of 25 miRNAs, including miR-146a, were elevated, all of which were also found to be overexpressed in SLE. On the contrary, only miR-105-5p was decreased in pSS PBMCs compared to controls. Downregulation of miR-105-5p was uniquely observed in pSS. miRNA-105-5p has been characterized as a key regulator of immune cell differentiation and immune response mechanisms, in particular with regard to B cell activation and differentiation [69]. Decreased expression of this miRNA may contribute to B cell activation triggering autoimmune processes in pSS. However, further studies comprising larger study populations and investigating fractionated cell types are needed to clarify if aberrant miR-105-5p expression is specific for pSS, and to provide further insights into the functional pathogenic mechanisms.

4.1.2. Salivary glands

Dysregulation of miRNA expression has also been studied directly in the target organ of the disease, the salivary glands. Alevizos et al. investigated miRNA expression using microarray generated profiles from minor salivary gland tissue of 16 patients with either low- or high-grade salivary gland inflammation and eight control individuals [70]. Upregulation of miR-768-3p and downregulation of miR-574 in biopsies from patients compared to controls were demonstrated, and pathway analysis of the biological function of genes under control of differentially expressed miRNAs pointed to neurologic pathways involved in modulating salivation processes. In addition, the expression of these miRNAs was inversely correlated with patients' focus scores. Another study in minor salivary gland tissue from six patients with pSS and three controls applying deep sequencing on the SOLiD platform suggested increased miR-5100 expression in pSS [71]. However, target genes, affected cell types and potential functional consequences are still elusive. Kapsogeorgou et al. reported downregulated expression of miRNA let-7b in SGECs from antibody-positive compared to antibody-negative patients with pSS [72]. The authors conclude that dysregulation of let-7b may contribute to deficient transcriptional inhibition of the autoantigens SSA and SSB in pSS. Potential effects by underlying cell type heterogeneity and small sample sizes are major limiting factors of these studies, and findings are yet to be replicated independently.

4.2. lncRNAs

Although the exact functional roles and mechanisms of most lncRNAs remain largely elusive, emerging evidence has revealed that lncRNAs are involved in regulation of central cellular processes, such as genomic imprinting, RNA splicing, chromatin remodeling and mechanisms of protein transport, thus, suggesting a contribution of lncRNAs in the pathogenesis of complex diseases [73]. Recent studies reported a role for lncRNAs in chronic inflammatory immune diseases, such as SLE [74,75], rheumatoid arthritis [76] and psoriasis [77]. To this date, there is no study available investigating a possible role for lncRNAs in pSS.

5. Perspectives

As of today, a general finding in epigenetic studies of pSS is DNA hypomethylation of promoter regions at IFN-induced genes in minor salivary glands, the main target organ for the autoimmune process, and in peripheral blood cells. Increased expression of IFN-induced genes with detected promoter hypomethylation has been demonstrated in pSS B cells [23,49]. Focusing on B cells and the IFN system, several therapeutic targets can be identified, illustrated in Fig. 1. While the pattern of inverse correlation between DNA methylation in the promoter region and gene expression is striking for robustly hypomethylated type I IFN-regulated genes, this relationship is less evident for other genes. Further studies investigating large clinically well-characterized cohorts, and intersection of with genetic and additional epigenetic data, such as histone modifications or miRNA expression, are warranted.

For these attempts the choice of relevant cell and tissue types is crucial. Whole blood is an easily assessable tissue and is thought to represent the systemic component of pSS. However, as blood is composed of many different cell types, results may be affected by underlying heterogeneity in cell type composition between cases and controls. Therefore, epigenetic association signals from samples of mixed cell types may rather reflect differences in cell population frequencies than true epigenetic changes which are inherent to a specific cell type. In order to account for differential cellular composition in the analysis of whole blood or PBMC derived samples, reference-based methods for statistical deconvolution have been developed [78,79]. Since these methods are restricted to major blood cell types, analyses may still be confounded by cellular subtypes. In order to fully dissect the contribution of epigenetic changes in different immune cell types in pSS, analyses of fractionated cell populations are needed. Local epigenetic mechanism can be investigated in tissue samples derived from specific target organs of the disease, such as salivary glands. However, these approaches are complicated by the difficulty to obtain these tissues, which typically leads to restriction in sample sizes. In addition, biopsies from affected tissues are also composed of mixed cell types, and reference epigenetic data sets are not available for these tissues. The recent development of reference-free deconvolution methods may provide an alternative approach for analyzing these samples in the future, but they have not been applied in pSS yet [80,81].

To fully decipher the role of DNA methylation in pSS, the whole epigenome needs to be studied. As costs for DNA sequencing are coming down, whole-genome bisulfite sequencing studies and studies of histone modifications in multiple tissues from larger numbers of pSS patients will soon be feasible. The ultimate goal for studies of epigenetic mechanisms in pSS would be to facilitate understanding of disease pathogenesis, identify biomarkers for diagnosis, patient subsets and prognosis, as well as add novel perspectives for therapeutic approaches. For this, longitudinal studies in pSS are required, following patients over time to infer the role of epigenetic changes in disease progression, disease activity, response to treatment, and disease manifestations. The resulting associated epigenetic modifications could then be used as biomarkers for patient stratification in order to predict treatment response or risk for malignant transformation. For example, promoter hypomethylation of the gene *IFI44L* has already been suggested as a biomarker in SLE [82]. Further studies might aim at pinpointing a set of genes with DNA methylation patterns specific for pSS and for certain manifestations of the disease.

The development of lymphoma is a long-term risk in pSS, affecting about 5% of patients [3]. While clinical and serological risk factors have been identified [83], there is a need for improved detection of early immunological perturbations leading to lymphoma. Future prospective studies of epigenetic alterations in different cell (sub-)types and salivary

gland tissue in patients with pSS before and at the time of lymphoma development could possibly increase our understanding of gene regulation disturbances leading up to lymphomagenesis.

Once we have catalogued the epigenetic variations in pSS and associated clinical manifestations, their functional consequences will need to be investigated. Tools for site-specific epigenome editing are currently being developed, and will in the future allow us to study the function of individual epigenetic variations in *in vitro* cell systems [84]. Epigenome editing techniques will enable us to move from inferring function through correlation, such as by correlating methylation and expression levels, to instead gain insights into actual causal relationships. Once causal relationships between epigenetic modifications and pSS or its manifestations have been established, development of targeted therapeutic approaches can start. While epigenetic modifiers, such as DNMT inhibitors and miRNA (ant)-agonists are in clinical use or in clinical trials in cancer [85], no clinical studies affecting epigenetic modulation are currently undertaken in pSS. As for inhibition of DNA methylation with DNMT inhibitors, the opposite effect, i.e. maintaining methylation of e.g. IFN-induced gene promoters would be desired; however this approach is yet not feasible. In addition, systemic administration of epigenetically modifying compounds may raise major safety concerns attributed to the global impact on disease-unrelated genes. More targeted approaches will be needed before therapeutic strategies aiming at epigenetic modifications can find their way into clinical application. The continuous advances in experimental technologies and methods for bioinformatics analysis have a broad potential to reveal major insights into the molecular mechanisms underlying disease causation, pathogenicity and progression, and to provide guidelines on how to translate these discoveries into improved patient care and treatment options.

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