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EXTENDED REPORT

Genome-wide DNA methylation analysis in multiple tissues in primary Sjögren's syndrome reveals regulatory effects at interferon-induced genes

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ABSTRACT

Objectives Increasing evidence suggests an epigenetic contribution to the pathogenesis of autoimmune diseases, including primary Sjögren's Syndrome (pSS). The aim of this study was to investigate the role of DNA methylation in pSS by analysing multiple tissues from patients and controls.

Methods Genome-wide DNA methylation profiles were generated using HumanMethylation450K BeadChips for whole blood, CD19+ B cells and minor salivary gland biopsies. Gene expression was analysed in CD19+ B cells by RNA-sequencing. Analysis of genetic regulatory effects on DNA methylation at known pSS risk loci was performed.

Results We identified prominent hypomethylation of interferon (IFN)-regulated genes in whole blood and CD19+ B cells, including at the genes *MX1*, *IFI44L* and *PARP9*, replicating previous reports in pSS, as well as identifying a large number of novel associations. Enrichment for genomic overlap with histone marks for enhancer and promoter regions was observed. We showed for the first time that hypomethylation of IFN-regulated genes in pSS B cells was associated with their increased expression. In minor salivary gland biopsies we observed hypomethylation of the IFN-induced gene *OAS2*. Pathway and disease analysis resulted in enrichment of antigen presentation, IFN signalling and lymphoproliferative disorders. Evidence for genetic control of methylation levels at known pSS risk loci was observed.

Conclusions Our study highlights the role of epigenetic regulation of IFN-induced genes in pSS where replication is needed for novel findings. The association with altered gene expression suggests a functional mechanism for differentially methylated CpG sites in pSS aetiology.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterised by inflammation of salivary and lacrimal glands. Systemic manifestations such as arthritis, pulmonary or renal involvement may also occur.[1] The aetiology of pSS is multifactorial, where both genetic and environmental factors are thought to contribute to disease development. However, the molecular mechanisms underlying pSS remain largely elusive.[2] Activation of the type I interferon (IFN) system with elevated expression of type I IFN-regulated genes in blood cells and minor salivary glands, a so-called IFN signature, has been demonstrated in pSS.[3–7] B cell activation in pSS is reflected by autoantibody synthesis and an increased risk of non-Hodgkin's lymphoma, most commonly of the B cell type.[8]

For pSS, to date, about 10 genetic risk loci have been identified through genome-wide association studies (GWAS).[9 10] These variants only explain a limited proportion of the susceptibility to pSS, and the functional consequence of associated single nucleotide polymorphisms (SNPs) remains unclear for most loci.[11] Increasing evidence suggests an epigenetic contribution to the pathogenesis of autoimmune diseases, including pSS.[12] Epigenetic modifications constitute an additional layer of genomic regulation, and may serve as a dynamic link between genotype, environment and phenotype, for example by modulating gene expression. Methylation of the DNA base cytosine (5mC) can be studied in large sample sets with array-based methods such as the Illumina HumanMethylation450K array (HM450K), which allows for quantification of DNA methylation of 485 577 CpG sites across the human genome.[13]

In pSS, so far, two studies with relatively small sample sizes have applied the HM450K array.[14 15] Altorok *et al*[14] report a number of differentially methylated CpG sites (DMCs) in naïve CD4+ T cells from 11 patients and 11 controls, with implications in immune responses and lymphocyte activation, including hypomethylation of IFN-regulated genes. Miceli-Richard *et al*[15] found more prominent methylation changes in CD19+ B cells than in CD4+ T cells in 26 patients compared with 22 controls.

In order to evaluate the role of DNA methylation in pSS in a comprehensive manner we performed an epigenome-wide association study (EWAS) in whole blood, CD19+ B-cells and minor salivary gland biopsy samples from patients and controls using the HM450K array. To further explore the functional role of the DMCs we intersected the disease-associated CpG sites with publicly available chromatin state data[16] and performed gene expression analysis in CD19+ B cells. Finally, genetic regulation of methylation at pSS risk loci was investigated.

PATIENTS AND METHODS

For full details of methods see online supplementary text.

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Patients and controls

A total of 108 Caucasian patients with pSS from the rheumatology clinic at the Uppsala University Hospital, Sweden, were included in the study, all fulfilling the American European Consensus Group (AECG) criteria.[17] Whole blood was collected from 100 patients, CD19+ B cells from 24 patients and minor salivary gland biopsies from 15 patients. As control samples, DNA from whole blood from 400 healthy blood donors from the Uppsala Bioresource and CD19+ B cells from 47 donors were analysed.[18] Only controls falling within the main European cluster in our previous study were included.[19] Minor salivary gland biopsies obtained from 13 individuals that were examined for a possible pSS diagnosis, where the biopsies showed no inflammation and serology was negative for autoantibodies, served as control biopsies (table 1).

Genome-wide methylation analysis

Genomic DNA from whole blood, CD19+ B cells and minor salivary gland biopsies was isolated using standard procedures. DNA methylation levels of 485 577 CpG sites were determined on the HM450K BeadChip (Illumina, San Diego, California, USA). Signal intensities were parsed into the Minfi R package for Subset-quantile Within Array Normalization.[20–22] The post-quality control (QC) data set comprised 388 971 CpG sites. To determine differential methylation between patients with pSS and controls a linear regression model containing cell count estimates,[23 24] age and sex as covariates was fitted. DMCs with a Bonferroni-adjusted threshold of $p<1.3\times10^{-7}$ were considered significant.

Gene expression profiling of CD19+ B cells

Expression analysis on CD19+ B cells (n=16 patients, n=23 controls) was conducted using the TruSeq stranded mRNA sample preparation kit followed by sequencing on a HiSeq2500 instrument (Illumina). QC was conducted using RNA-seQC.[25] Reads were mapped with Tophat2 and analysis of differential gene expression was performed using the Cufflinks pipeline.[26 27]

Methylation quantitative trait loci analysis

Methylation levels were tested in PLINK for genotype association at loci that have previously shown an association with pSS with genome-wide significance.[9 28] Quality controlled genotype data for 135 503 probes generated on the Infinium ImmunoChip (Illumina) were available for 382 of the healthy control individuals in our study. All CpG sites within a gene locus plus 100 kb flanking regions were tested against all genotypes within the same region. A Bonferroni corrected $p<1.24\times10^{-7}$ was considered statistically significant.

RESULTS

Differential methylation in whole blood

First, we investigated the difference in methylation levels in whole blood between patients with pSS and controls. We used reference DNA methylation signatures of flow sorted blood cells types to estimate cell counts and found reduced CD4+ and CD8+ T cells in pSS, while CD19+ B cell proportions were estimated to be similar between patients and controls (see online supplementary figure S1). We identified 11 785 (6171 hypomethylated and 5614 hypermethylated) DMCs annotated to 5623 unique genes (see figure 1 and online supplementary table S1).

An average difference in β-values of >0.1 between cases and controls was identified at 12 of the 11 785 DMCs, of which 11 DMCs, annotated to seven different genes, were hypomethylated in pSS (table 2). The most pronounced difference in methylation was detected for a CpG site annotated to *MX dynamin-like GTPase 1 (MX1)* (also referred to as *MxA*). MX1 is a key mediator of human antiviral immune responses and is induced by type I and type II IFNs.[29] Two additional CpG sites in *MX1* were found to be distinctly hypomethylated in patients. Of interest, among the top hypomethylated DMCs in pSS we further note CpG sites in the IFN-induced genes *IFI44L*, *PARP9*, *PLSCR1*, *IFIT1*, *IFITM1* and *HLA-A*, meaning that all of the top hypomethylated sites in pSS are IFN-regulated (table 2). In addition, we detected a large number of DMCs in IFN-induced genes with a difference in methylation <0.1, for example, *STAT4*, *NFAT5*, *ELF1*, *OAS1-3* and *TREX1*. Multiple DMCs with a difference in methylation <0.1 were also observed in the human leucocyte antigen (HLA) region, both major histocompatibility complex (MHC) class I and class II, the majority being hypomethylated in the patients (see online supplementary table S1). We identified one hypermethylated CpG site with an average difference in methylation-β of >0.1 annotated to *EBF4*, which is a transcription factor belonging to the Olf-1/EBF family with central implications in neural development and B cell maturation (table 2).[30]

We then analysed the 12 top differentially methylated sites in patients with pSS stratified on the presence of anti-Sjögren's Syndrome antigen A (SSA) and/or anti-Sjögren's Syndrome antigen B (SSB)-antibodies. When analysing antibody-positive patients versus controls, a more prominent difference in mean methylation was seen, indicating that the difference in mean methylation for the IFN-induced genes is mainly driven by the antibody-positive patients (see online supplementary table S2). Analysing only patients with pSS (n=57) with early disease defined as ≤3 years from diagnosis to blood sampling, almost identical results of difference in mean methylation were seen as in the analysis of all 100 cases versus controls (data not shown).

Association analysis of sex-chromosomal CpG sites was conducted separately for female and male individuals. We identified 85 X chromosomal CpG sites (out of 11 232 X chromosomal sites included on the HM450K array), annotated to 56 unique genes to be differentially methylated in female patients compared with female controls, with DMCs in notable genes such as *VSIG4*, *TLR8*, *CD40L* as well as in several microRNAs (miRNAs) (see online supplementary table S3). There were no DMCs in male individuals on the X chromosome and the Y chromosome.

Pathway analysis of the 500 most significantly associated DMCs in whole blood identified antigen presentation, IFN signalling and graft-versus-host disease signalling as the top canonical pathways (see online supplementary table S4). The strongest gene-set enrichment in disease or function annotation of DMCs was observed for lymphohaematopoietic cancer ($p=6\times10^{-13}$) (see online supplementary table S5). Given the large number of DMCs between patients and controls, we analysed global DNA methylation levels in whole blood, CD19+ B cells and minor salivary gland biopsies and found no difference between patients and controls (see online supplementary figure S2).

Functional genomic distribution and overlap with chromatin marks

In general, DMCs were enriched in CpG island shelves and open sea regions and depleted in CpG islands and shores (figure 2A). Investigating the distribution of hypomethylated and hypermethylated DMCs separately, hypomethylated DMCs were over-represented in 5′-untranslated region (UTR), whereas

Table 1 Characteristics of patients with primary Sjögren's syndrome (pSS) and control individuals

	Whole blood		CD19+ B cells		Minor salivary gland biopsies	
	Patients with pSS	Controls	Patients with pSS	Controls	Patients with pSS	Controls
Individuals, n	100	400	24	47	15	13
Women, n (%)	89 (89)	351 (87.8)	24 (100)	35 (74.4)*	15 (100)	13 (100)
Age, years, (mean±SD)	56.1±13.6	47.1±13.2**	56.6±13.8	48.8±17.7	47.8±15.9	47.5±17.2
Autoantibody frequency, n (%)						
Antinuclear antibodies (ANAs)	80 (80)	n.a.	20 (83.3)	n.a.	14 (93.3)	0
Anti-SSA antibodies	75 (75)	n.a.	24 (100)	n.a.	15 (100)	0
Anti-SSB antibodies	41 (41)	n.a.	13 (54.2)	n.a.	8 (53.3)	0
Minor salivary gland biopsies†						
Focus score, median (range)	2 (1–12)	n.a.	2 (1–12)	n.a.	2 (1–10)	0
Medication, n (%)						
Prednisolone	7 (7)	n.a.	1 (4.2)	n.a.	0	n.a.
Hydroxychloroquine	14 (14)	n.a.	5 (20.8)	n.a.	1 (6.7)	n.a.
Immunosuppressants	4 (4)‡	n.a.	2 (8.4)§	n.a.	0	n.a.

Whole blood and CD19+ B cells from the same individual were available from 17 patients; whole blood, CD19+ B cells and biopsies from 10 patients.
*p<0.01, **p<0.0001 for the difference between patients with pSS and controls. Frequencies compared with Fisher's exact test and continuous variables with Mann-Whitney U test.
†Biopsies performed in 75/100 patients with whole blood samples and 21/24 patients with CD19+ B cells.
‡Azathioprine (n=2), mycophenolate mofetil (n=1), chlorambucil (n=1).
§Methotrexate (n=2).
n.a., not available.

hypermethylated DMCs were more than twofold enriched in 3′-UTR and moderately enriched in gene bodies (figure 2B). Analysing the intersection of pSS associated CpG sites with chromatin marks revealed that DMCs with hypomethylation in patients were enriched in enhancers (H3K4me1 and H3K27ac) and accessible chromatin (DNase I hypersensitive sites, DHS) compared with the distribution of all probes on the array. In contrast, hypermethylated DMCs were depleted for these modifications and also largely under-represented in the active promoter mark H3K4me3. On the other hand, DMCs with hypermethylation in patients were enriched for H3K36me3, which marks an actively transcribed gene body (figure 2C).

Differential methylation and mRNA expression in CD19+ B cells

Next, we analysed primary CD19+ B cells from 24 patients and 47 healthy controls and found 453 DMCs, (98 hypomethylated and 355 hypermethylated, annotated to 303 unique genes) (see online supplementary table S6). The top associated DMCs are shown in table 3. Similar to whole blood, several IFN-induced genes showed prominent hypomethylation at multiple CpG sites in CD19+ B cells from patients with pSS. In order to investigate whether differential methylation was associated with gene expression, gene expression analysis was performed in CD19+ B cells from a subset of patients and controls. Significantly upregulated expression was observed for all of the eight IFN-induced genes exhibiting DMCs with hypomethylation >0.2 (table 3). In contrast, for the two genes with hypermethylated DMCs >0.2, no significant association with gene expression was observed.

Differential methylation in minor salivary gland biopsies

Finally, we studied DNA methylation directly in the primary target organ of the disease, the salivary gland. Given the

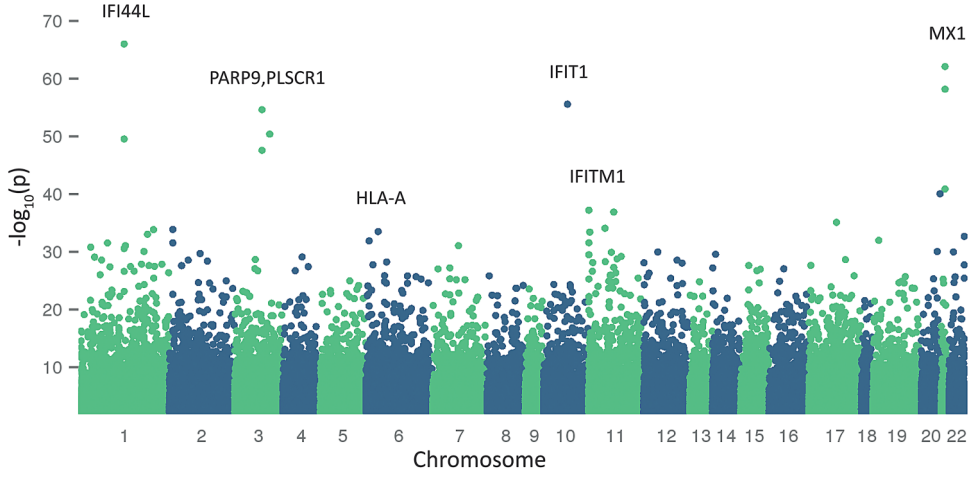

Figure 1 Manhattan plot of the genome-wide DNA methylation analysis in whole blood from patients with primary Sjögren's syndrome (pSS) and control individuals. The figure shows the $-\log_{10}$ p values of the association of CpG sites with pSS against the chromosomal position of the investigated sites. The hypomethylated CpG sites with a difference in mean methylation-β >0.1 are highlighted by their gene symbol annotation.

Table 2 Differentially methylated CpG sites (DMCs) with the largest difference in mean methylation values between patients with primary Sjögren's syndrome (pSS) and control individuals in whole blood (Δβ-value >0.1)

CpG site	Gene symbol	Gene name	Interferon-induced	CpG island information	Gene property	Mean methylation β in pSS	Mean methylation β in controls	Methylation Δβ*	p Value
cg21549285	*MX1*	MX dynamin-like GTPase 1	Yes	Shore	5′UTR	0.57	0.83	−0.26	6.9×10^{-59}
cg05696877	*IFI44L*	Interferon-induced protein 44-like	Yes	Open sea	5′UTR	0.47	0.67	−0.20	2.8×10^{-50}
cg22930808	*PARP9*	Poly (ADP-ribose) polymerase family, member 9	Yes	Shore	5′UTR	0.52	0.71	−0.19	2.4×10^{-55}
cg22862003	*MX1*	MX dynamin-like GTPase 1	Yes	Shore	TSS1500	0.51	0.70	−0.19	7.9×10^{-63}
cg03607951	*IFI44L*	Interferon-induced protein 44-like	Yes	Open sea	TSS1500	0.41	0.59	−0.18	9.9×10^{-67}
cg00959259	*PARP9*	Poly (ADP-ribose) polymerase family, member 9	Yes	Shore	5′UTR	0.41	0.58	−0.17	2.6×10^{-48}
cg06981309	*PLSCR1*	Phospholipid scramblase 1	Yes	Shore	5′UTR	0.39	0.54	−0.15	4.1×10^{-51}
cg05552874	*IFIT1*	Interferon-induced protein with tetratricopeptide repeats 1	Yes	Open sea	Gene body	0.57	0.71	−0.14	2.7×10^{-56}
cg26312951	*MX1*	MX dynamin-like GTPase 1	Yes	Shore	TSS200	0.29	0.44	−0.14	1.3×10^{-41}
cg23570810	*IFITM1*	Interferon-induced transmembrane protein 1	Yes	Shore	Gene body	0.55	0.69	−0.14	6.1×10^{-38}
cg17608381	*HLA-A*	Major histocompatibility complex, class I, A	Yes	Shore	Gene body	0.48	0.60	−0.12	3.4×10^{-15}
cg05825244	*EBF4*	Early B cell factor 4	No	CpG island	Gene body	0.57	0.47	0.11	2.4×10^{-11}

*Methylation Δβ refers to the difference in mean methylation β (β-values) between patients with pSS and healthy controls. A negative Δβ represents hypomethylation in the patients.
TSS, transcription start site.

described IFN signature in the minor salivary glands, we hypothesised there might be epigenetic changes in IFN-induced genes.[4 5] We found 45 DMCs annotated to 19 unique genes, where the most significant DMC showed hypomethylation in *OAS2*, which encodes a member of the IFN-inducible 2′-5′A synthetase family, involved in the innate immune response to viral infections (see online supplementary table S7).[31]

Genetic regulation of DNA methylation at pSS risk loci

To investigate whether the effects of established pSS risk alleles are mediated through changes in DNA methylation, we analysed genetic variants in 382 of our control individuals at the following pSS GWAS loci: *DDX6-CXCR5*, *FAM167A-BLK*, *IL12A*, *IRF5-TNPO3*, *STAT4*, *TNIP1*, and within the *HLA* region, for association with methylation levels in whole blood. Evidence for genetic regulation of DNA methylation, that is, significant methylation quantitative trait loci (metQTL), was identified for all pSS risk loci (in total 36 679 metQTL). Table 4 shows the metQTL for the pSS associated SNPs.[9] Apart from the *HLA* region, the most significant metQTL was observed for the *IRF5-TNPO3* SNP rs4728142 and CpG site cg04864179, both located in the *IRF5* promoter. Interestingly, the *IRF5-TNPO3* locus is also the non-*HLA* gene locus most significantly associated with pSS.[9] Methylation levels in blood at cg04864179 were also directly associated with pSS in our EWAS ($p=4.8\times10^{-13}$, mean methylation-β difference 0.04).

DISCUSSION

Here we report a comprehensive analysis of DNA methylation in pSS; in whole blood, primary CD19+ B cells and minor salivary gland biopsies. The most prominent finding was hypomethylation of IFN-regulated genes, including multiple associated CpG sites in *MX1* and *IFI44L*, replicating previous reports in pSS.[14 15] Notably, all top DMCs were annotated to IFN-induced genes. We also identified numerous significant, but smaller methylation differences between patients and controls most of which are novel (methylation-β value differences <0.1, online supplementary table S1). We show for the first time that hypomethylation of IFN-regulated genes in pSS CD19+ B cells is correlated with increased gene expression. PSS belongs to the systemic autoimmune diseases, together with systemic lupus erythematosus (SLE) which display an IFN signature, with several possible mechanisms underlying the IFN activation.[32–34] Recently, MX1 has been suggested as a potential biomarker for disease activity and type I IFN bioactivity in pSS,[35] and *IFI44L* is described as an indicator gene of the type I IFN signature.[7] Hypomethylation in *IFI44L*, which was initially reported by Altorok *et al*[14] in pSS CD4+ T cells and subsequently by Miceli-Richard *et al*[15] in CD19+ B cells, has also been detected in multiple cell types from patients with SLE.[36–39] Absher *et al*[37] found hypomethylation of IFN-induced genes in naïve, memory and regulatory CD4+ T cells, CD19+ B cells and CD14+ monocytes from patients with SLE, with either active or quiescent disease. They conclude that epigenetic changes occur in

A

	All associated DMCs	Hypometh. DMCs	Hypermeth. DMCs
CpG Island	**0.29**	**0.49**	**0.08**
Shore	**0.86**	**1.19**	**0.5**
Shelf	**1.2**	1.01	**1.42**
Open Sea	**1.59**	**1.28**	**1.93**

B

	All associated DMCs	Hypometh. DMCs	Hypermeth. DMCs
TSS1500	0.95	**1.2**	**0.67**
TSS200	**0.53**	**0.86**	**0.18**
5'UTR	1.02	**1.19**	**0.83**
First Exon	**0.55**	**0.77**	**0.31**
Gene Body	**1.14**	0.98	**1.32**
3'UTR	**1.42**	0.86	**2.03**
ncRNA	0.97	1.01	0.93
NA	1.04	**0.93**	**1.15**

C

			All associated DMCs	Hypometh. DMCs	Hypermeth. DMCs
Enhancer	H3K4me1	CD19+	0.99	**1.45**	**0.53**
		CD3+	0.96	**1.31**	**0.62**
Enhancer	H3K27ac	CD19+	**0.85**	**1.34**	**0.36**
		CD3+	0.95	**1.27**	**0.63**
Promoter	H3K4me3	CD19+	**0.63**	**1.11**	**0.14**
		CD3+	**0.68**	1.06	**0.3**
Transcribed Gene Body	H3K36me3	CD19+	**1.45**	0.98	**1.91**
		CD3+	**1.31**	0.95	**1.66**
Repressive	H3K27me3	CD19+	**0.4**	**0.6**	**0.2**
		CD3+	**0.26**	**0.37**	**0.15**
Heterochromatin	H3K9me3	CD19+	**0.64**	**0.29**	0.99
		CD3+	0.56	**0.19**	0.92
Open Chromatin	DHS	CD19+	**0.74**	**1.25**	**0.23**
		CD3+	**0.81**	**1.16**	**0.46**

Figure 2 Functional genomic distribution of differentially methylated CpG sites (DMCs) in whole blood from patients with primary Sjögren's syndrome (pSS) and controls. This figure illustrates the functional genomic distribution of all associated autosomal DMCs (first panel), of the hypomethylated DMCs (second panel) and of the hypermethylated DMCs (third panel) annotated (A) in relation to CpG island context, (B) in relation to gene region and (C) in relation to chromatin states in CD19+ B and CD3+ T reference cells. The colour scale indicates fold-enrichment (orange) or fold-depletion (blue) of the DMCs in each functionally annotated region. The bold numbers represent annotations to which the DMCs significantly differ compared with the distribution of probes on the HM450K array (post-QC probe set) (Bonferroni corrected X^2-test p value <0.005). TSS1500, 1500 bp upstream of transcription start site (TSS); TSS200, 200 bp upstream of TSS; ncRNA, non-coding RNA; NA, probe not annotated to a defined gene property.

progenitor cells independent of IFN activity. Coit *et al*[39] reported hypomethylation of IFN-induced genes in neutrophils from patients with SLE and speculate that the exposure to IFN during the disease course may induce methylation differences that will increase the responsiveness to IFN. Taken together, epigenetic changes in different cell types in pSS and SLE makes them poised for type I IFN expression, although the exact mechanisms remain to be elucidated. In concordance with others, we found that the hypomethylation of IFN-induced genes is largely driven by the antibody-positive patients.[15] Our pathway analysis of DMCs also revealed IFN signalling among the top associated pathways. Monoclonal antibodies interfering with the IFN signalling pathway currently under development in SLE might be of interest in future clinical trials in selected patients with pSS.

Polymorphisms in genes of the type I IFN system have shown associations with pSS.[9 10 40] We therefore investigated whether genetic variants in known pSS associated loci mediate disease risk by influencing methylation levels at target CpG sites. We identified significant associations of genetic variants in *DDX6-CXCR5*, *FAM167A-BLK*, *IL12A*, *IRF5-TNPO3*, *STAT4*, *TNIP1*, and within the *HLA* region with DNA methylation, indicating that pSS GWAS risk alleles have the potential to affect DNA methylation levels.

For insights into functional mechanisms of DMCs in pSS we studied the regional genomic distribution of associated sites. We found distinct enrichment patterns for overlap with chromatin marks, where hypomethylated DMCs were enriched for enhancer marks and regions of open chromatin, while hypermethylated DMCs were highly under-represented in these functional regions. Hypomethylated CpG sites were also more prominent in promoter regions, indicating a putative transcriptional activation of genes with hypomethylation in these regions.

Table 3 Top DMCs and the corresponding differential gene expression in CD19+ B cells in patients with primary Sjögren's syndrome (pSS) and healthy controls

CpG site	Gene symbol	Gene name	Interferon-induced	CpG island information	Gene property	Enhancer*	Promoter†	Methylation Δβ‡	Methylation p value§	Expression log2 (FC)¶	Expression p value**
cg21549285	*MX1*	MX dynamin-like GTPase 1	Yes	Shore	5′UTR	No	Yes	−0.34	2.3×10^{-11}	1.97	$<5\times10^{-5}$
cg05696877	*IFI44L*	Interferon-induced protein 44-like	Yes	Open sea	5′UTR	No	No	−0.33	3.8×10^{-20}	3.79	$<5\times10^{-5}$
cg03607951	*IFI44L*	Interferon-induced protein 44-like	Yes	Open sea	TSS1500	Yes	Yes	−0.30	2.6×10^{-18}	3.79	$<5\times10^{-5}$
cg23570810	*IFITM1*	Interferon-induced transmembrane protein 1	Yes	Shore	Gene body	Yes	No	−0.26	1.3×10^{-10}	1.63	$<5\times10^{-5}$
cg05552874	*IFIT1*	Interferon-induced protein with tetratricopeptide repeats 1	Yes	Open sea	Gene body	Yes	Yes	−0.26	2.1×10^{-16}	4.44	$<5\times10^{-5}$
cg06981309	*PLSCR1*	Phospholipid scramblase 1	Yes	Shore	5′UTR	No	Yes	−0.25	2.3×10^{-12}	1.64	$<5\times10^{-5}$
cg14293575	*USP18*	Ubiquitin-specific peptidase 18	Yes	Shelf	5′UTR	No	No	−0.25	9.7×10^{-14}	3.27	$<5\times10^{-5}$
cg03038262	*IFITM1*	Interferon-induced transmembrane protein 1	Yes	Shore	3′UTR	Yes	No	−0.25	2.9×10^{-10}	1.63	$<5\times10^{-5}$
cg22930808	*PARP9*	Poly (ADP-ribose) polymerase family, member 9	Yes	Shore	5′UTR	Yes	No	−0.24	2.4×10^{-10}	1.89	$<5\times10^{-5}$
cg00959259	*PARP9*	Poly (ADP-ribose) polymerase family, member 9	Yes	Shore	5′UTR	Yes	No	−0.24	1.3×10^{-13}	1.89	$<5\times10^{-5}$
cg22862003	*MX1*	MX dynamin-like GTPase 1	Yes	Shore	TSS1500	Yes	Yes	−0.22	5.8×10^{-12}	1.97	$<5\times10^{-5}$
cg10549986	*RSAD2*	Radical S-adenosyl methionine domain containing 2	Yes	Open sea	First exon	Yes	Yes	−0.21	1.4×10^{-17}	3.19	$<5\times10^{-5}$
cg16810031	*ZPBP2*	Zona pellucida binding protein 2	No	CpG island	TSS1500	Yes	No	0.21	7.4×10^{-8}	n.a.	n.a.
cg25330422	*STAT3*	Signal transducer and activator of transcription 3	Yes	Shelf	3′UTR	Yes	Yes	0.22	3.2×10^{-13}	-0.09	n.s.

*Genomic location of differentially methylated CpG site (DMC) overlapping H3K4me1 (poised enhancer mark) and/or H3K27ac (active enhancer mark) peak in reference CD19+ B cells.
†Genomic location of DMC overlapping H3K4me3 (active promoter mark) peak in reference CD19+ B cells.
‡Methylation Δβ refers to the difference in mean methylation β (β-values) between patients with pSS and healthy controls. A negative Δβ represents hypomethylation in the patients.
§p Value of the association between CpG methylation and pSS (significance threshold $p<1.3\times10^{-7}$; Bonferroni corrected).
¶Differential gene expression as $\log_2$(FC) between patients with pSS and healthy controls.
**p Value of differential gene expression between patients with pSS and healthy controls.
FC, fold change; n.a., not available; n.s., not significant; TSS, transcription start site.

Table 4 Methylation quantitative trait loci (metQTL) of genetic variants associated with primary Sjögren's syndrome (pSS) at genome-wide significance and the DNA methylation association of the most significant metQTL CpG site

Gene locus	pSS associated SNP*	Top CpG site	metQTL p Value	pSS EWAS p Value
DDX6-CXCR5	rs4936443	cg09144398	2.5×10^{-3}	9.9×10^{-4}
FAM167A-BLK	rs2736345	cg21775007	2.1×10^{-9}	0.02
IL12A	rs485497	cg25829945	0.02	0.03
IL12A	rs583911	cg07906551	0.01	4.5×10^{-8}
IRF5-TNPO3	rs4728142	cg04864179	4.8×10^{-9}	4.8×10^{-13}
IRF5-TNPO3	rs17339836	cg04864179	4.6×10^{-4}	4.8×10^{-13}
IRF5-TNPO3	rs10954213	cg04864179	6.9×10^{-6}	4.8×10^{-13}
STAT4	rs13426947	cg15325732	0.01	0.03
TNIP1	rs6579837	cg19084508	0.03	2.5×10^{-3}
TNIP1	rs7732451	cg15989436	0.04	1.5×10^{-13}
HLA-DRA	rs3135394	cg25645491	8.2×10^{-13}	2.0×10^{-3}
HLA-DQB1	rs115575857	cg07180897	3.0×10^{-70}	8.9×10^{-7}
HLA-DQB1	rs3129716	cg07180897	3.0×10^{-70}	8.9×10^{-7}
HLA-DQA1	rs9271588	cg08269402	4.1×10^{-51}	5.0×10^{-4}

*Single nucleotide polymorphism (SNP) associated with pSS at genome-wide significance reported by Lessard *et al*.[9]
EWAS, epigenome-wide association study.

Interestingly, distinct hypomethylation and increased gene expression of the *PARP9* (*BAL1*) gene were found in CD19+ B cells. Whereas *BAL1* has been shown to be overexpressed in diffuse large B cell lymphomas,[41] none of our patients sampled for CD19+ B cells had a previous lymphoma. Disease annotation analysis of the top DMCs in whole blood also pointed to an extensive enrichment of genes associated with lymphoproliferative disorders, including B cell non-Hodgkin's lymphoma. This is intriguing since pSS is the autoimmune disease which displays the highest risk for lymphoma development.[42] Whether aberrant methylation in target genes contributes to lymphoma development is an important topic for future studies.

The strongest genetic association for pSS is found in the *HLA* region.[9] Although none of the most significantly associated DMCs were annotated to *HLA* genes, we nonetheless identified a considerable number of significant signals within this region with the majority being hypomethylated in patients with pSS, possibly pointing to an increased expression of alternatively spliced antigenic transcripts in pSS.[43 44] Analysing X chromosomal DNA methylation we observed several DMCs in genes with central roles in connecting innate and adaptive immunity, such as *TLR8* and *CD40L*, as well as in miRNAs.[45] Altered miRNA expression constitutes another epigenetic mechanism implicated in pSS pathogenesis,[46] and the potential role of *miRNA-223* hypomethylation in pSS pathogenesis warrants further investigation.

In our study we used a well known reference based method for cell type estimation of whole blood samples.[23 24] In the purified CD19+ B cells compared with the whole blood analysis, we noted a larger mean difference in methylation levels between cases and controls for many associated CpG sites, including at *MX1* and *IFI44L*, perhaps indicating the advantage of a single cell type in the analysis. However, the smaller number of individuals from which purified cells could be obtained, compared with DNA extracted from whole blood, still meant that fewer DMCs were detected studying B cells. Due to the low absolute number of B cells in serum from patients with pSS we were not able to assess methylation patterns in different B cell subtypes. Minor salivary gland biopsies consist of different cell types including epithelial and acinar cells in both patients with pSS and controls, but also inflammatory cells in the patients' biopsies. Therefore we cannot deduce the cell types that are responsible for the difference in methylation between diseased and normal glands, and the results must be interpreted with caution. Nevertheless, it is noteworthy that the strongest association was found in *OAS2*, an IFN-induced gene involved in the innate immune response.[31] The minor salivary glands are known targets for IFN where both increased IFN-α levels and an IFN signature have been demonstrated.[3–5]

In conclusion, our study of epigenetic profiles in multiple tissues in pSS using a large collection of patients and controls has replicated the previously reported hypomethylation of IFN-regulated genes in pSS and identified numerous new associations. We report hypomethylation in regulatory enhancer and promotor regions and show for the first time that hypomethylation of IFN-regulated genes in B cells corresponds to an increase in gene expression. Evidence for genetic control of methylation levels at known pSS risk loci is presented. Independent replication in cells from patients with pSS and controls will be required to confirm these novel findings. Studying the epigenetic basis of pSS will hopefully increase our understanding of the disease mechanisms and guide the search for novel and more specific therapeutic targets.

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Contributors JI-K, JKS, KBN, RO, A-CS and GN designed the study; LS, LR, M-LE and GN collected patient and control material and clinical data; JI-K and JKS performed the experiments; JI-K, JKS, JCA and JN analysed the data; JI-K, JKS and GN drafted the manuscript and all authors read and accepted the final version of the manuscript. JI-K and JKS contributed equally to the study.

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