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 at 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 al14 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 al15 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 data16 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.
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. Whole blood was collected from 100 patients, CD19+ B cells from 24 patients and minor salivary gland biopsies from 13 patients. As control samples, DNA from whole blood from 400 healthy blood donors from the Uppsala Biobank and CD19+ B cells from 47 donors were analysed. Only controls falling within the main European cluster in our previous study were included. 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. 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, age and sex as covariates was fitted. DMCs with a Bonferroni-adjusted threshold of p<1.3×10^{-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. Reads were mapped with TopHat2 and analysis of differential gene expression was performed using the Cufflinks pipeline.

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. 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<1.24×10^{-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 GTR Base 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. 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 IFIH4L, PARP9, PLSCR1, IFTT1, 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 TREXI. 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).

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 lymphoepitheliomatopic cancer (p=6×10^{-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...
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
Basic and translational research

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).

<table>
<thead>
<tr>
<th>CpG site</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Interferon-induced</th>
<th>CpG island information</th>
<th>Gene property</th>
<th>Mean methylation β in pSS</th>
<th>Mean methylation β in controls</th>
<th>Methylation Δβ*</th>
<th>p Value</th>
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<td>cg21549285</td>
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<td>Yes</td>
<td>Shore</td>
<td>5’UTR</td>
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<td>Gene body</td>
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<td>Gene body</td>
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<td>0.47</td>
<td>0.11</td>
<td>2.4×10−11</td>
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*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-CXCXR3, 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.7 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.5 Methylation levels in blood at cg04864179 were also directly associated with pSS in our EWAS (p=4.8×10−13, 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 al14 in pSS CD4+ T cells and subsequently by Miceli-Richard et al15 in CD19+ B cells, has also been detected in multiple cell types from patients with SLE.36–38 Absher et al19 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...
progenitor cells independent of IFN activity. Coit et al\(^{19}\) 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 states, 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>
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<th>Gene symbol</th>
<th>Gene name</th>
<th>Interferon-induced</th>
<th>CpG island information</th>
<th>Gene property</th>
<th>Enhancer*</th>
<th>Promoter†</th>
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<td>Yes</td>
<td>0.22</td>
<td>3.2×10⁻¹³</td>
<td>-0.09</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*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<1.3×10⁻⁷; Bonferroni corrected).
¶Differential gene expression as log2(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.

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 is implicated in pSS pathogenesis, 46 and the potential role of miRNA expression constitutes another epigenetic mechanism mediating inflammatory cells in 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 promoter 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 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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Competing interests None declared.

Patient consent Obtained.

Ethics approval The study was approved by the Regional Ethics board in Uppsala No. 97358, 217/2006 and 012/2009.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Normalised or raw intensity data of the HM450K BeadChips are available upon request from the authors on a collaborative basis.

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References

Basic and translational research


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


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