EPIGENETIC MECHANISMS IN SJÖGREN'S SYNDROME

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ABSTRACT

Primary Sjögren's syndrome (pSS) is a systemic autoimmune epithelitis and recent advances in our comprehension of its pathophysiology strongly suggest a multi-step process that involves environmental factors (e.g. chronic viral infection, drugs), followed by deregulation of the epigenetic machinery (e.g. DNA demethylation, histone modifications, microRNAs), which in turn specifically affects lymphocytes and epithelial cells leading to an aberrant inflammation. This process is amplified in the case of genetic mutations. As a consequence, autoreactive lymphocytes and autoantigens are produced leading to the development of autoantibodies. Moreover, it was observed that epigenetic modifications in pSS could be reversed, thus providing arguments to suggest that therapeutic strategies targeting the epigenetic deregulation and in particular the PKC-delta/Erk/DNMT1 pathway would be effective in pSS.

Keywords: Sjögren's syndrome (SS), epigenetics, DNA methylation, histone acetylation, endogenous retrovirus.

INTRODUCTION

Sjögren's syndrome (SS) is a chronic and systemic autoimmune epithelitis, primarily affecting exocrine glands such as the salivary and lacrimal, and leading to dry mouth (xerostomia), and dry eyes (keratoconjunctivitis sicca).[1] Moreover, in one-third of SS cases, patients have systemic features that could affect the kidney, lung, liver, and thyroid. Although primary Sjögren's syndrome (pSS) is typically reported in women (sex ratio 9:1) during their fourth or fifth decade, the disease also occurs in both sexes and at any age.[2] SS is slowly progressive with benign evolution, except in up to 5% of patients that develop a lymphoma, usually B cell lymphoma.[3] In half of the patients, SS disease is a unique disease and referred to as pSS, while in the other half, SS is secondary and coexists with another autoimmune disease (AID) such as systemic lupus erythematosus (SLE), rheumatoid arthritis, primary biliary cholangitis, and thyroiditis.

Table 1: Epigenetic modifications in salivary gland epithelial cells from patients with primary Sjögren's syndrome.

	Primary Sjögren's syndrome
Environmental factors	Drugs (procainamide, isoniazide) virus, stress[9] HERV-E (MSG)[20,14]
Retrotransposons DNA methylation	Decreased PKC-delta-Erk SGEC)[21] Decreased *DNMT1* (SGEC)
microRNA Methylation sensitive genes Reversibility	Increased GADD45A Increased *miR-146, -574, -768*[59] *SSB/La, KRT19*,[52] *aquaporin 5*[21] Anti-CD20[21]

HERV-E: human endogenous retroviruses; MSG: minor salivary glands; SGEC: salivary gland epithelial cells; GADD45A: growth arrest and DNA damage inducible alpha; DNMT1: DNA methyltransferase; SBB/La: Sjögren's syndrome type B antigen; KRT19: Keratin 19.

Focal lymphocytic infiltration of tissues characterises pSS with typically an initial T cell predominance with an elevated CD4/CD8 ratio, while progressively B cells become preponderant and may be organised in germinal centre-like structures.[4] Antigen presenting cells have been described in heavy infiltrates. B cell hyperactivity characterises pSS patients highlighted by the presence of circulating autoantibodies (AAB) against the sicca syndrome A/Ro and B/La ribonucleoprotein particles, detection of rheumatoid factors, hyperglobulinaemia, and hypocomplementaemia.[5] It is suspected that an altered peripheral blood B cell distribution reflects B cell infiltration of the tissues.[6-8]

ENVIRONMENTAL FACTORS

As reported in Table 1, multiple lines of evidence support a role for environmental factors in pSS altering the epigenetic machinery[9] and this is supported by reports that drugs such as hydralazine and procainamide, two drugs known to interfere with the main epigenetic event, DNA methylation, induce SS in association with SLE in both humans and mice.[10-12] This hypothesis is reinforced further by observations that other factors effective in inducing important epigenetic changes have been associated with pSS such as ultraviolet light, smoking, and chemicals.[13] Other arguments are related to the disease concordance rates for monozygotic twins, revealing a rate of 15–25% for pSS which signifies a complex process involving more epigenetic than genetic components.[14] In addition, SS geoepidemiology distribution reveals that the highest rates are reported in Northern countries[15] and epidemiological studies indicate associations between SS and viruses and between SS and psychological stress. As a whole, these observations strongly suggest a major role played by epigenetic mechanisms in the development of pSS.[16]

GLOBAL DNA METHYLATION ANALYSIS

DNA is Demethylated in Primary Sjögren's Syndrome

DNA methylation is a heritable and reversible epigenetic mark involving the covalent transfer of a methyl group to the fifth carbon of the cytosine pyrimidine ring (^{5me}C) in a CpG dinucleotide by DNA methyltransferases (DNMTs).[17,18] Recent research has also revealed the presence of non-CG methylation in embryonic and pluripotent mammalian stem cells.[19] In mammals, most CpG dinucleotides are methylated with the notable exception of the active proximal and long range gene regulatory sequences (e.g. promoter, enhancer, insulator) that tend to be protected from methylation. When methylated, the DNA alone can physically prevent binding of transcriptional regulators to the gene, and more importantly, methylated DNA participates in chromatin compaction. The overall degree of genome DNA methylation can be easily estimated, usually around 70–80%, and for determining the amount, several approaches exist such as high performance liquid chromatography and immunological techniques (immunohistochemistry, enzyme-linked immunosorbent assay) based on utilisation of specific monoclonal antibodies such as the murine anti-^{5me}C clone 33D3.

In AID, DNA demethylation is the most studied epigenetic hallmark with defects arising mainly from loss of maintenance DNA methylation at repeat elements which are normally heavily methylated, including satellites and retrotransposons (as discussed later). Such an effect is cell-specific and associated with defective DNMT1 activity, with a stronger effect reported in lymphocytes from SLE patients, synoviocytes in rheumatoid arthritis, and cells within the brain in multiple sclerosis.[10,20-23] Regarding pSS, we have recently reported that global DNA methylation was altered in epithelial cells when analysing minor salivary gland (MSG) biopsies.[24,25] Interestingly, and because of DNMT1 downregulation, global DNA demethylation was conserved after long-term culture of salivary gland epithelial cells (SGEC), thus confirming the heritability of the process. In contrast, no differences relative to controls were reported when analysing peripheral blood T and B cells from pSS patients for global DNA methylation,[24,26,27] indicating that global DNA demethylation is altered primarily in epithelial cells.

Crosstalk Between Epithelial Cells and Lymphocytes

Clinical and biological factors associated with epithelial cell DNA demethylation in MSG biopsies were further explored, revealing that global DNA methylation was inversely correlated with the level of lymphocyte infiltration and anti-sicca syndrome B/La AAB detection but surprisingly, not with anti-sicca syndrome A/Ro AAB and the clinical

index designed to measure activity in pSS, the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI).[25] One further step was to demonstrate lymphocyte and B cell involvement, which was done by conducting co-culture experiments between the human salivary gland (HSG) epithelial cell line and B cells to reveal that B cells were effective to control within HSG cells global DNA methylation, through the control of DNMT1, in a time and dose-effective fashion.[24]

Reversibility and PKC-delta /Erk/DNMT1 Pathway

At the molecular level, DNMT1 activity in SGEC is regulated upstream by the PKC-delta/Erk pathway. Direct contact between epithelial cells and B cells, but not with T cells, is effective in promoting PKC-delta degradation and downstream DNMT1 repression in SGEC.[28] As a consequence it was not surprising to observe that global DNA methylation was restored in MSG from pSS treated with an anti-CD20 monoclonal antibody (mAb) therapy, rituximab.[24] Thus part of the clinical activity of the B cell-depleting agent rituximab in pSS may be attributed to an epigenetic effect.[29-34] Similarly, in SLE a defective Erk/DNMT1 pathway leading to DNA methylation is reported in SLE B cells and this pathway could be restored by utilisation of another mAb, the anti-interleukin (IL)-6 receptor mAb itolizumab.[35,36]

WHOLE METHYLOME ANALYSIS

Infinium Technology

Several strategies have been developed for whole genome DNA methylation profile determination.

Table 2: Genome-wide DNA methylation patterns in primary Sjögren's syndrome.

	Whole blood[36]	B cells (CD19+)[36]	B cells (CD19+)[23,24]	All T cells (CD4+)[23,24]	Naïve T cells (CD4+, CD45, RA+)[35]
Patients (controls)	100 (400)	24 (47)	26 (22)	26 (22)	11 (11)
Differentially methylated CpG Increased methylation Decreased methylation	11,785 6,171 (52.4%) 5,614 (47.6%)	453 98 (21.6%) 355 (78.4%)	6,707 2,953 (44%) 3,754 (56%)	119 54 (45.4%) 65 (54.6%)	753 200 (26.6%) 553 (73.4%)
Differentially methylated genes Hypermethylated Hypomethylated	5,623 *EBF4* *MX1, IFI44L, PARP9, IFIT1*	303 *ZPBP2, STAT3* *MX1, IFI44L, IFITM1, PARP9*	3,619 *IRF5* *IFITM1, IFI44L*	74 - -	426 *RUNX1* *LTA, STAT1, IFITM1, IFI44L, CD247*
Pathways Lymphocyte activation Interferon pathway Antigen presentation	- Yes Yes	- Yes -	Yes Yes -	- - -	Yes Yes Yes
Association with clinical data SSA/SSB AAB Disease activity (ESSDAI)	- -	Yes -	Yes Yes	- -	Unknown -
Association with risk factors	-	*HLA, IRF5 CXCR5, BLK*	*HLA, IRF5 CXCR5, BLK*	-	-

ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; HLA: human leukocyte antigen; IRF5: interferon regulatory factor 5; CXC: chemokine; BLK: B lymphocyte kinase; EBF4: early B cell factor 4; MXI: myxovirus resistance 1; IFI44L: interferon induced protein 44 like; IFIM1: interferon induced transmembrane protein 1; IFIT1: interferon induced protein with tetratricopeptide repeats 1; PARP9: poly(ADP-ribose) polymerase family member 9; ZPBP2: zona pellucida binding protein 2; STAT3: signal transducer and activator of transcription 3; RUNX1: runt-related transcription factor 1; LTA: lymphotoxin-alpha; CD247: cluster of differentiation 247; AAB: autoantibodies.

The most utilised of these, which is cost-effective and requires only a small amount of DNA, is the Infinium® methylation BeadChips developed by Illumina®.[37] This technology interrogates genomic DNA after bisulphite conversion (unmethylated cytosine is converted to uracil) using two 50-mer probes per CpG locus, one methylated and one unmethylated. The methylation state at each probe is calculated by determining the fluorescence ratio between the two beads. The human methylation 450K (HM450K) covered 2% of the 28 million CpG sites present in the human genome, 96% of the CpG islands, and 99% of the genes with an average of 17 CpG sites per gene.

Whole Blood, B cells, and T cells

To date, genome-wide DNA methylation profiles were conducted with the HM450K beads in pSS by three groups using peripheral blood mononuclear cells, T cells (whole and naïve), and B cells.[26,27,38,39] Although methylation analysis was not conducted in the same manner in the studies, several important points could be raised and they are summarised in Table 2. First, and irrespective of the lymphocyte subset, analysis of the hypermethylated genes highlighted as hot spots are the human leukocyte antigen regions (HLA I/II) and Type I interferon (IFN)-regulated/induced genes (*IFI44L, IFITM1*). Second, specific pathways were characterised including solute carrier proteins and the RUNX2 transcription factor for T cells and B cell receptor signalling and development genes for B cells. Third, between B cells and T cells, up to 50-times more DNA methylation changes are observed in B cells thus explaining that DNA methylation alterations in peripheral blood are predominantly related to B cells.

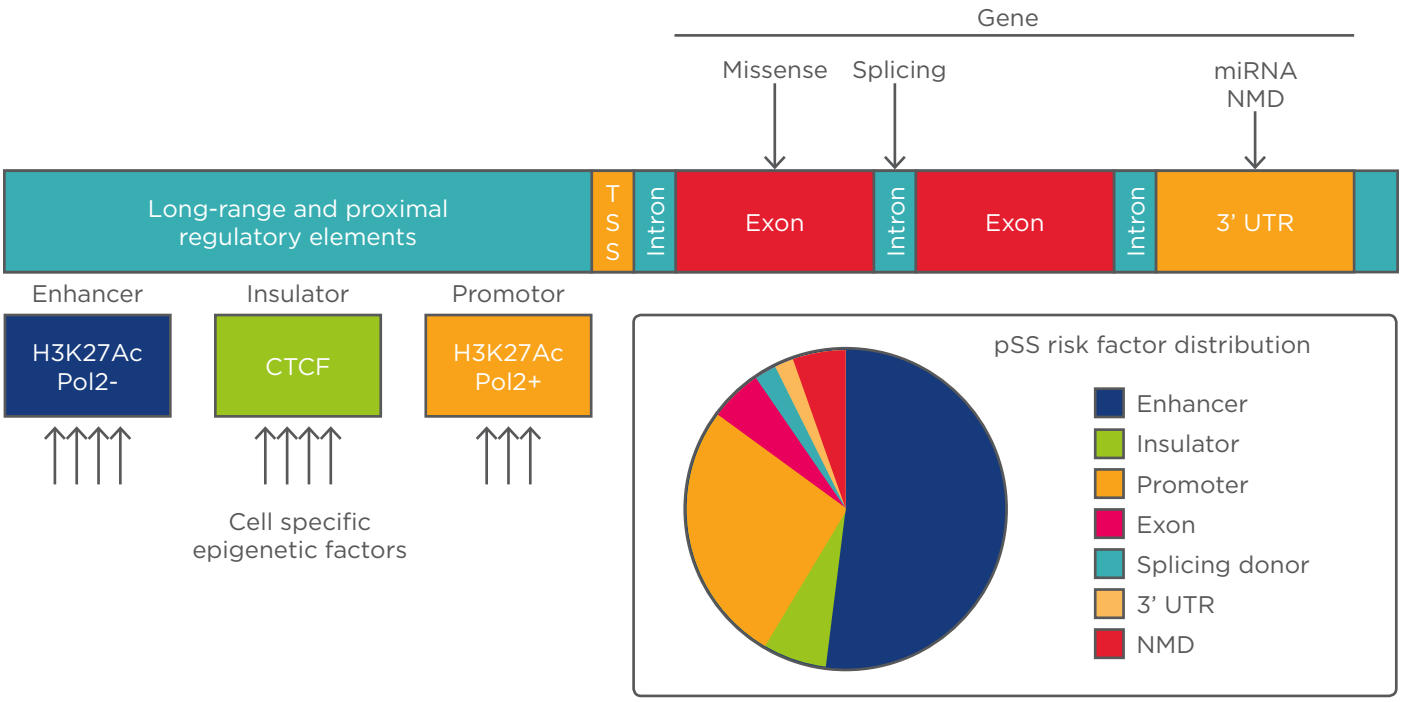

Figure 1: A schematic of the different locations in which causal variants can occur and the distribution that is observed.

Detailed mapping of causal variants in primary Sjögren's syndrome reveals that they map predominantly to regulatory elements (93.1). These elements are cell-type specific and hot spots for epigenetic factors (H3K27Ac for enhancers, H3K27Ac plus polymerase 2 (Pol2) for promoters, and the nuclear protein CCCTC-binding factor (CTCF) that prevent crosstalk between active and inactive chromatin for insulators. The other risk factors are related to mutations within the TSS, coding regions (silent, missense and nonsense mutations), splicing mutations, nonsense-mediated messenger RNA decay triggered by polymorphism, and miRNA targets in the 3'UTR.[55,59]

pSS: primary Sjögren's syndrome; TSS: transcription start site; 3'UTR: three prime untranslated region; TSS: toxic shock syndrome; CTCF: CCCTC-binding factor; Pol2: polymerase 2; NMD: nonsense-mediated messenger RNA decay.

Minor Salivary Glands

So far, only one study has applied the HM450K beads to MSG obtained from pSS patients.[39] Moreover, such a study is limited because the of the small sample size (N=15) and because MSG consist of multiple cell types including epithelial and acinar cells in pSS patients and controls, but also immune cells in pSS patients. Despite these limitations, the IFN induced gene *OAS2* was shown to be demethylated in MSG from pSS patients. Again the IFN pathway is highlighted and this is in agreement with histological and gene expression studies, conducted in MSG from pSS patients, which have demonstrated increased levels of IFN-α and altered interferon gene expression.[31,40]

Another way to test the influence of DNA methylation in pSS is to use DNMT inhibitors. In this manner, treatment with 5-aza-2'-deoxycytidine (5-Aza) results in expression of the *SSB/La autoantigen* and *cytokeratin 19* genes in the HSG cell line,[25,41] *aquaporin 5* gene expression in the HSG ductal cell line NS-SV-DC,[42] alteration in the control of Type I hemidesmosome components,[43] and to the increased fluid secretion in the murine ageing model C57Bl/6Cr Skc. In contrast, 5-Aza was ineffective in inducing *IRF5* and *CD40L* upregulation in $CD4^+$ T cells from pSS women.[44,45]

EPIGENETIC REPROGRAMMING AND RETROTRANSPOSONS

Interspersed and comprising 8% of human genome, human endogenous retroviruses (HERV) are controlled at the epigenetic level by DNA methylation.[20] When such control is impaired, HERV can produce different effects. One example is related to the human T cell leukaemia related endogenous retrovirus (HRES-1) that is inserted in the long arm of chromosome 1 at position 1q42 and controlled by DNA methylation,[46] when expressed the HRES-1 provirus produces a p38 gag protein that can induce AAB development as observed in 10% of patients with pSS in contrast to 1.5% in healthy donors.[47] An association between SLE and HRES-1 polymorphisms is also described.[48] Another example is HERV-CD5 that is integrated into chromosome 11 upstream of the host *cd5* gene exon 1 and downstream of the *cd6* gene.[6] This integration occurred just prior to the divergence of hominoids from old world monkeys 25 million years ago.[49] Defective DNA methylation at the HERV-CD5 promoter introduces an alternative promoter for the *cd5* gene, and enables transcription of a fusion transcript with the consequence of an intracellular variant of CD5,[35,50] which can in turn promote B cell autoreactivity as observed in SLE B cells.[51,52]

In MSG from pSS patients and in addition to HRES-1,[53] other HERV were reported to be over-expressed including HERV-K113 and HERV-5,[54] and HERV-E elements.[14]

GENETIC FACTORS AND CROSSTALK WITH EPIGENETIC FACTORS

Genetic Risk Factors Associated with Primary Sjögren's Syndrome

Up to 40 non-HLA genetic risk-factors are characterised in pSS[55] and the recent development of genome-wide association studies conducted in Caucasian and Han Chinese pSS patients have contributed to improve this list.[55,56] Genetic loci associated with pSS are associated with key pathological pathways including antigen presentation (HLA I/II), Type I IFN-regulated/induced genes (*IRF5, STAT4*), NF-κB pathway (*TNIP1, TNFAIP3*), and B cell activation (*CXCR5, BLK*).[57] Interestingly, it has been demonstrated for some of these risk-factors that they are associated with gene expression as observed with *HLA* and *BLK-FAM167* genes.[58] Another observation is that parts of them (~50%) are shared with SLE and other AID, and related to important immune components crucial in the development of AID such as cytokines (IL-10, IL-21), receptors (TNFSF4/CD252), transcription factors (*IRF5*), and B cell signalling proteins (*BLK, BANK1*). However, and with the exception of the *HLA I/II* genes that have a significant odds ratio ([OR]; usually above 2), the other risk factors are usually modest (1.1<OR<1.8) and for half of the patients no risk factors were reported.

Genetic Risk Factor Annotation

The vast majority of genetic factors associated with pSS are excluded from protein coding regions (5.6%), and as described in Figure 1, are in regulatory areas (93.1%).[55,59] Regulatory pSS risk factors are suspected of controlling genes through an effect on: i) the transcriptional machinery when present within cell specific regulatory regions referred to as promoters (29.2%), enhancers (56.9%), and insulators (6.9%) (an insulator is a gene regulatory element that blocks interactions between enhancers and promoters); ii) the spliceosome complex formation that controls intron

excision (2.4%); iii) the activation of messenger RNA non-sense mediated decay (5.6%), and; iv) the control of messenger RNA stability through microRNA binding at three prime untranslated regions (2.4%). Such a breakthrough in our understanding regarding AID-genetic risk factor functions was possible due to recent development of the ENCODE (Encyclopedia of DNA elements) and Roadmap Epigenomics programmes.[60]

Genetic Risk Factors and Cellular Specificity

As the primary role of the epigenome is to regulate transcription in a cell-specific manner, recent research demonstrated whether or not genetic variants are enriched in tissue-specific epigenomic marks and whether or not they can influence autoreactivity.[61,62] In line with this hypothesis, a B cell signature was observed in nearly all of the pSS-associated risk-factors when considering the active histone mark H3K27Ac, expression quantitative trait loci, and differentially demethylated CpGs.[55] The salivary gland epithelial cell signature remains to be demonstrated, and we can postulate that both signatures overlap partially. Moreover, it was also reported that binding sites for SS risk variants were enriched within binding sites for immune-related transcription factors such as NF-κB, STATs, and PU1; and some common pathways previously described to be affected in pSS (Type I IFN, NF-κB).

CONCLUSIONS

The information presented here indicates that DNA demethylation confers a risk for pSS, providing a strong argument for epigenetic causality in genetically predisposed individuals. Although not reported in this review, DNA methylation defects in pSS are associated with other epigenetic modifications such as histone modifications and miRNA overexpression.[63] Another important point is related to the cellular specificity, which concerns mainly B cells and epithelial cells. However, it was also observed that the process was reversible and that DNA demethylating drugs are related to a defective PKC-delta/Erk/DNMT1 pathway. As a consequence, it can be postulated that drugs controlling this pathway would undoubtedly have benefits for pSS prevention and treatment.[18,64]

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