

ORIGINAL RESEARCH ARTICLES

EPIGENETIC MODIFICATIONS AFFECT CYTOKERATIN 19 EXPRESSION IN SALIVARY GLANDS FROM PATIENTS WITH SJÖGREN'S SYNDROME

O.D. Konsta^{1,2*}, A. Charras^{1*}, C. Le Dantec¹, E. Kapsogeorgou², A. Bordron¹, W.H. Brooks³, A.G. Tzioufas², J.O. Pers¹, Y. Renaudineau^{1,4}

¹Research Unit EA2216 Immunology, Pathology and Immunotherapy, SFR ScinBios and Labex Igo "Immunotherapy Graft, Oncology", Réseau épigénétique du canceropole Grand Ouest, European University of Brittany, Brest, France ²Department of Pathophysiology, School of Medicine, National University of Athens, 75 Mikras Asias street, Athens 11527, Greece ³Department of Chemistry, University of South Florida, Tampa, Florida, USA ⁴Laboratory of Immunology and Immunotherapy, Brest University Medical School, CHU Morvan, Brest, France

*Have contributed equally to this work

Abstract

Primary Sjögren's syndrome (pSS) is a chronic autoimmune epithelitis. Several lines of experiments indicate that multiple factors contribute to salivary gland epithelial cell (SGEC) dysfunctions in SS including a combination of environmental factors, lymphocytic infiltrations, genetic predispositions as well as epigenetic defects. The epigenetic origin is reinforced by the observation that global DNA methylation (^{5mC}) is altered in minor salivary glands from pSS patients and that such defect is associated with cytokeratin 19 (KRT19) overexpression. An epigenetic deregulation of the KRT19 gene was further tested by treating the human salivary gland (HSG) cell line with the DNA demethylating agent 5-azacytidine, and with the histone acetylase inhibitor trichostatin A. Blocking DNA methylation, but not histone acetylation, with 5-azacytidine was associated with KRT19 overexpression at both the transcriptional and protein level. Next, analysis of the CpG genome-wide methylome array in the KTR19 locus from long-term cultured SGEC obtained from 8 pSS patients revealed more reduced DNA methylation levels in those patients with defective global DNA methylation. Altogether, our data suggest that alteration of DNA methylation and, in turn, altered KRT19 expression in SGEC may contribute to pathophysiology thus opening new perspectives in pSS.

Key words

Sjögren's syndrome, DNA methylation, cytokeratin 19, epithelial cells

Introduction

Primary Sjögren's syndrome (pSS) is a chronic and systemic autoimmune epithelitis, affecting exocrine glands, such as salivary and lacrimal glands, plus parenchymal organs (1). As a consequence, the clinical manifestations include dry mouth (xerostomia), dry eyes (keratoconjunctivitis sicca) and systemic features. Patients with pSS have a 20-40 fold increased risk of developing lymphoma (2, 3). Furthermore, SS is characterized by abnormal salivary glands, altered blood B cell distribution, and the presence of circulating autoantibodies (Ab) against the sicca syndrome (SS)A/Ro and SSB/La ribonucleoprotein particles (4-8).

The mechanisms leading to the systemic autoimmune epithelitis are multifactorial and include a combination of genetic predispositions, environmental factors, and epigenetic factors (9-11). Epigenetics can be defined as inheritable modifications in gene expression that do not entail changes in the DNA sequence (12). Epigenetics can also explain how cells can differentiate into alternative cell types and how a phenotype can be passed from one cell to its daughter cells. Epigenetic

mechanisms are important controlling the pattern of gene expression during development, the cell cycle, and in response to biological or environmental changes. Epigenetic dysregulations have been linked with a long list of autoimmune diseases (AID) including SS (13, 14). A close association has been further established between epigenetic and genetic risk factors in AID in specific cellular subsets as observed in SS (15, 16).

Recently, we have observed that global DNA methylation was reduced in minor labial salivary glands (MSG) from pSS patients when comparing 5 methylcytosine (^{5mC}) staining to controls (17). The defective DNA methylation process was related to an abnormal PKC-delta/Erk/DNMT1 pathway, which was conserved after long-term growth of a primary culture of salivary gland epithelial cells (SGEC) (18). The contribution of DNA demethylation in SSB overexpression and in turn to the production of anti-SSB Ab was further tested and confirmed by comparing MSG from anti-SSB positive to anti-SSB negative pSS patients, on the one hand, and by treating with 5-aza-2'-deoxycytidine (5-Aza) the human salivary gland (HSG) cell line to overexpress SSB, on the other hand (19). As part of this latter study, it was observed that cytokeratin (KRT)19 was overexpressed in those patients with major DNA methylation defects, suggesting control by DNA methylation. Further investigation of this control is the aim of the present work.

Material and methods

Patients and controls

MSG biopsies were obtained from 13 individuals fulfilling the criteria for pSS according to the revised American-European SS classification criteria (7, 20) including a biopsy focus score ≥ 1 (lymphocytic foci/4 mm² of glandular tissue). SG specimens were collected from 4 patients who did not meet the criteria for SS, although they had described sicca symptoms and, as such, had undergone an SG biopsy. The institutional review boards at the University of Athens approved this study, and participants signed an informed consent statement.

Immunofluorescence staining of tissue sections

Biopsy specimens were embedded in optimal cutting temperature (OCT)-compound, snap frozen in isopentane, cut into thick tissue sections, and mounted into poly-L-lysine coated

slides. For global DNA methylation (anti-^{5mC}), serial sections were fixed in 4% cold paraformaldehyde for 10 min. After extensive washing, sections were treated with HCl 2N for 30 min, washed and incubated with a mouse anti-^{5mC} monoclonal (m)Ab (clone 33D3; Abcam, Cambridge, UK) diluted at 1:50 in PBS-BSA 2% Triton 0.3%. After 16h at 4°C, the slides were washed and incubated with a rhodamine red-X (RRX)-goat anti-mouse IgG Ab (Jackson ImmunoResearch, West Grove, PA) diluted at 1:200 for 40 min. The images were acquired by a Zeiss Axioplan confocal laser scanning microscopy (Zeiss, Oberkochen, Germany). For the anti-KRT19 protocol, sections were fixed in 4% cold paraformaldehyde for 10 min. After extensive washing, the sections were incubated with 5% donkey serum (Sigma-Aldrich, St Louis, MO) to reduce background, and then incubated with the monoclonal rabbit anti-KRT19 Ab (Clone EP1580Y, Abcam) diluted at 1:100 in PBS for 4h at room temperature. After 3 washes, a fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (Jackson ImmunoResearch) diluted at 1:200 was applied to the sections. The images were acquired by an Olympus confocal laser scanning microscopy (Hamburg, Germany). The intensity of ^{5mC} and KRT19 staining were quantified by Image J software (NIH image, Bethesda, MD) and results expressed as arbitrary units (AU).

Epithelial cell cultures

In order to obtain non-neoplastic long-term cultured SG epithelial cell lines (SGEC) from biopsies, SGEC were established by standard technique (21). Cells were incubated at 37°C and 5% CO₂. The Human Salivary Gland (HSG) cell line established from irradiated neoplastic epithelial duct cells was suspended in Dulbecco's Modified Eagle's Medium (DMEM; Lonza Inc., Allendale, NJ) supplemented with 10% heat-inactivated fetal calf sera (FCS; Eurobio, Les Ulis, France), 2mM L-glutamine (Gibco BRL, France), non-essential amino acids 1% (Sigma-Aldrich) and antibiotics (Panpharma SA, Fougères, France). HSG cells were seeded at 7×10⁵ cells per well in a 6 well/plate (Nunc, Kamstrup, DK), and at 80% confluence the cells were treated (or not) with 10 μM, 25 μM, and 50 μM 5-Aza (Sigma-Aldrich), a selective inhibitor of DNMTs, or with 5 μM, 10 μM, and 25 μM of trichostatin A, a histone deacetylase inhibitor, for 48h at 37 °C and 5% CO₂. After treatment, cellular via-

bility was estimated by flow cytometry using FITC-conjugated annexin-V (AV) and propidium iodide (IP) (Beckman Coulter), and was over 97%.

RNA isolation and real time quantitative PCR

Total mRNA was extracted using the RNAble method (Eurobio, Les-Ullis, France), and cDNA synthesized by reverse transcription in 20µl volume with Superscript™II RNase reverse transcriptase (Invitrogen Life Sciences, Carlsbad, CA). Quantitative RT-PCR was carried out in 20µl mixtures containing 50ng template cDNA, 1X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 250nM of each primer. The following primers were used for real time quantitative PCR: KRT19 forward (Fw) 5'-CC-CGCGACTACAGCCACTA-3' and reverse (R) 5'-GCTCATGCGCAGAG-CCT-3'; and GAPDH Fw 5'-TGCCCTCAACGA-CCACTTT-3' and R 5'-GGTCCAGGGGTCTTACTCCTT3'. All assays included a negative control, which consisted of the reaction mixture with no template. Comparison of cycle thresholds was completed with the $2^{-\Delta Ct}$ method using GAPDH as an endogenous control.

Western blot analysis

Whole cell extracts were isolated using lysis buffer containing 5M NaCl, 1M Tris-HCl, 100mM NaF, 0.1M EDTA and supplemented with protease inhibitors. The concentration of the isolated proteins was determined using the micro BCA Protein Assay Reagent (Pierce, Rockford, ILL). The lysates were separated in 10% SDS-PAGE, transferred onto PVDF membrane, and probed with rabbit specific Abs from Abcam: anti-KRT19, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 1% skim milk in TBS/0.1% Tween-20, overnight at 4°C, followed with the appropriate HRP-conjugated IgG antibodies (GE Health Care Limited, UK). Signals were visualized by Luminata Forte Western HRP Substrate (Millipore, Billerica, USA), a premixed, ready-to use reagent for chemiluminescent detection in Western blotting applications that employ horseradish peroxidase (HRP)-conjugated Ab. The results were expressed as ratios of optical density (OD) bands to those of GAPDH bands used as loading controls.

DNA methylome analysis for KRT19

DNA samples (500 ng) from long-term cultured

SGEC were submitted to the NXT-Dx service facility (Belgium) for bisulphite conversion (EZ-96 DNA methylation kit, Zymo Research) and DNA methylation profiling using the Illumina Human Methylation 450k platform to analyze the methylation status of 485,577 single CpG sites. The SWAN normalization method was used to normalize the data (22), and results were expressed in beta value and compared to the beta reference values. The beta value is the ratio of the methylated probe intensity and the overall intensity (sum of methylated and unmethylated probe intensity). A value of 0 indicates that all copies of the CpG site in the sample are unmethylated, while a value of 1 indicates that all copies are methylated which can be interpreted as the approximation of the percentage of methylation for the population of a given CpG site in the sample.

Statistical analysis

The results are expressed as arithmetic means with standard error of the mean (SEM). Data were compared using the non-parametric Mann-Whitney U-test for unpaired data, using the GraphPad-Prism 6.0 software. Significance was assessed as $P < 0.05$.

Results

In patients with pSS, DNA methylation is associated with cytokeratin 19 overexpression

In order to extend our previous studies that have established a defective global DNA methylation ($5mC$) in MSG epithelial cells from pSS (17, 19), samples from thirteen pSS patients were stained with an anti- $5mC$ mAb and with an anti-KRT19 mAb (Figure 1A), revealing for patient with low levels of $5mC$ high amounts of KRT19 (AU: 3.0 ± 0.4 in $5mC$ low pSS patients versus 1.8 ± 0.3 in $5mC$ high pSS patients, $P=0.04$) (Figure 1B). As a consequence, this result raises the question of an epigenetic deregulation of the KRT19 gene in pSS patients.

DNA methylation controls KRT19 expression in human salivary glands

Next, and to test whether KRT19 expression is regulated at the epigenetic level, the HSG cell line was selected and cultured 48h using either the DNA methyl transferase (DNMT) inhibitor 5-Aza, or the histone acetylase inhibitor trichostatine A. Inhibition of DNMTs by 5-Aza was effective, in a dose-effect relations-

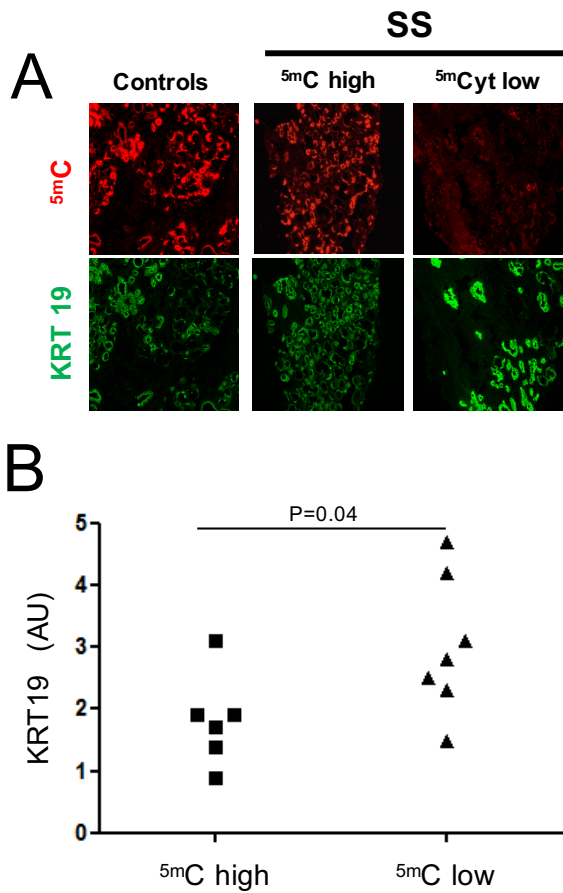


Figure 1. Global DNA methylation (5mC) and cytokeratin (KRT)19 analysis in minor salivary glands (MSG) from patients with primary Sjögren's syndrome (pSS). A- Photomicrographs showing global DNA methylation and KRT19 staining in MSG from a representative control and two pSS patients. The intensities of 5mC and KRT19 staining from 13 pSS patients were quantified by image J software and patients were subdivided according to the level of 5mC in two groups (5mC high n=6, and 5mC low n=7). B- Patients with reduced 5mC Ab staining have high levels of KRT19 staining. Differences were considered positive and indicated when $P < 0.05$.

hip, to increase the level of KTR19 at the protein level (Figure 2A) as observed by Western blot (KRT19/GAPDH protein (OD): 7.6 ± 10.3

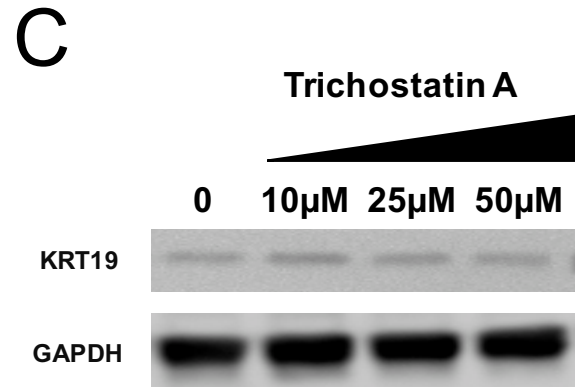
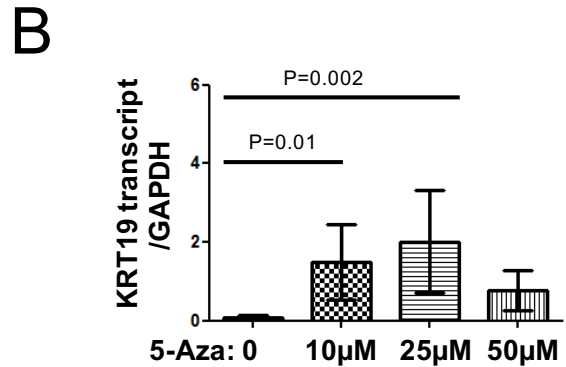
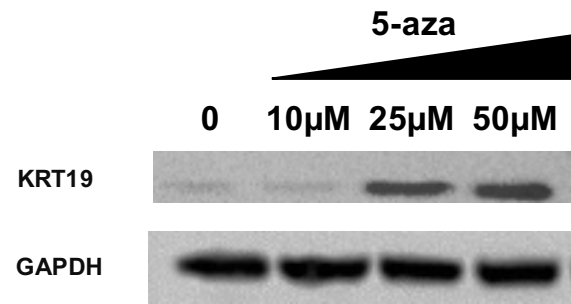
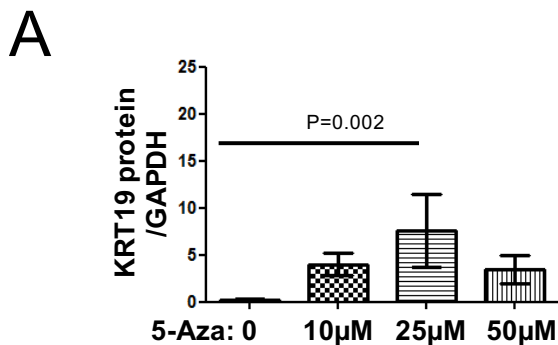


Figure 2. DNA methylation but not histone deacetylation controls KRT19 expression in the human salivary gland (HSG) cell line. The HSG cell line was treated 48h with increasing amounts of the DNA demethylating drug 5-azacytidine (5-Aza) or with the histone acetylase inhibitor Trichostatin A. A- Western blot analysis testing the effects of 5-Aza in HSG on KRT19. Results, from 8 experiments, are expressed as arbitrary units (AU) after normalization to GAPDH (AU=1). Left panel a representative experiment is presented. B- Real time quantitative PCR testing KRT19 gene expression, eight experiments. C- Representative Western blot analysis testing the effects of Trichostatin A. Differences are indicated when $P < 0.05$.

with 25µM 5-Aza versus 0.24 ± 0.09 without 5-Aza, $P = 0.002$), and at the transcriptional level (Figure 2B) as seen by RT-qPCR (KRT19/GAPDH transcript: 2 ± 3.2 with 25µM 5-Aza versus 0.1 ± 0.05 without 5-Aza, $P = 0.002$). In contrast, when using trichostatin A, no effect on KRT19 expression was observed at the

transcriptional level (data not shown), and at the protein level (Figure 2C). Altogether, these results indicate that DNA methylation, but not histone acetylation, controls KRT19 gene expression in HSG.

Methylome analysis confirmed KRT19 DNA demethylation in those patients with reduced global DNA methylation

In addition, as KRT19 expression was increased at transcriptional level in long-term cultured SGENC from pSS patients (KRT19/GAPDH transcript: 2.6 ± 0.6 in pSS patient versus 0.6 ± 0.2 in controls, $P=0.004$; data not shown), DNA methylation at the KRT19 locus was assessed in long-term cultured SGENC from 8 pSS patients using the human methylation 450K array from Illumina. As reported in Table 1, 14 probes spanning the KRT19 locus were selected and annotation was performed

using a combination of two tools based on information from the encyclopedia of DNA elements and the Roadmap Epigenome projects (<http://Ensembl.org> and <http://RegulomDB.org>) to determine whether CpG probes are likely to be within enhancers (probes 1-3 and 13-14), promoters (5-6), insulators (7-8), and/or repressors (9-12). The recruitment of RNA polymerase 2 (Pol2) was used to define promoters, enrichment of the histone H3k27Ac in the absence of Pol2 for enhancers, the DNA regulatory element CTCF for insulators, and enrichment of silencing histone marks (H3K-27me3 and H3K36me3) for repressors. Using the Illumina's reference β value as cut-off to discriminate probes with low methylation levels (β value < reference β value), it was observed that probes located within promoter area (probes 3-6) and probes located within distal regulatory area (probes 11-14) were di-

Number	CpG probe	Genomic probe location (hg19)	Gene location	ENCODE analysis	Beta value (cut-off)
1	cg12491114	chr 17:39685524-39685573	5'UTR	Enhancer (rs138464215)	0.09
2	cg03550233	chr17:39685434-39685483	5'UTR	Enhancer (TP53, rs4616333)	0.28
3	cg16585619	chr 17:39685250-39685299	5'UTR	Enhancer (rs28464674)	0.52
4	cg02416424	chr17:39684825-39684874	5'UTR	Promoter (SP1, TAF, rs143519434)	0.11
5	cg02893823	chr17:39684496-39684545	Exon 1	Promoter (SP1, TAF, rs372117019)	0.14
6	cg22379915	chr 17:39684239-39684288	Exon 1	Promoter (TAF, EZH2, rs145569885)	0.05
7	cg11462865	chr 17:39684076-39684125	Exon 1 / Intron 1-2	Insulator (CTCF, rs200315408)	0.09
8	cg17231690	chr 17:39684038-39684087	Intron 1-2	Insulator (CTCF, rs569080949)	0.04
9	cg13002493	chr 17:39683800-39683849	Intron 1-2	Repressor (H3K-27me3)	0.1
10	cg05396212	chr17:39683705-39683754	Intron 1-2	Repressor (H3K-27me3)	0.07
11	cg12871463	chr 17:39680662-39680711	Exon 4	Repressor (H3K36me3, rs148803515)	0.49

Number	CpG probe	Genomic probe location (hg19)	Gene location	ENCODE analysis	Beta value (cut-off)
12	cg27608102	chr 17:39680504-39680553	Exon 5	Repressor (H3K-36me3)	0.09
13	cg19051042	chr 17:39680271-39680320	Intron 5-6	Enhancer (STAT3, AP1, GATA, E2F, rs145636969)	0.13
14	cg09692449	chr17:39680217-39680266	Exon 6	Enhancer (STAT3, AP1, GATA, E2F, rs145232704)	0.09

Table 1.Characteristics of the 14 CpG probes used in the 450k bead chip from illumina spanning the human cytokeratin (KRT)19 gene. CpG probe annotation was performed using the two ENCODE web tools available at ensembl.org and RegulomeDb.org. Collected information included KRT19 genes location, regulatory function, transcription factor or CTCF binding and the presence of single nucleotide polymorphisms.

fferentially methylated. As expected and presented in Figure 3, those patients with reduced global DNA methylation were those with higher demethylated probes, confirming the link between global DNA demethylation, as determined by confocal microscopy in MSG, and KRT19 promoter demethylation in long-term cultured SGEC from pSS patients.

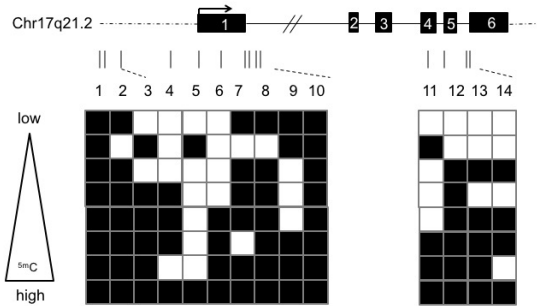


Figure 3. KRT19 gene DNA methylation in long term cultured salivary gland epithelial cells (SGEC) from primary Sjögren's syndrome (pSS) patients. KRT19 locus (chr 17: 39,679,869-39,684,560; GRCh 37/hg19) is shown depicting the location of the 14 CpG probes evaluated in this study. We identified two main hypomethylated CpG clusters that are present in the promoter area of KRT19 (3-5) and one present in the distal part of KRT19 (11-14). White boxes indicate CpG sites with a β value below the reference (see table 1).

Discussion

Several arguments, including our own studies, support a role for DNA methylation in pSS. Indeed, hydralazine and procainamide, two drugs known to act as DNA methylation inhibitors, induce SS with immunological features of systemic lupus erythematosus (SLE) both

in humans and rodents (23, 24). Interestingly, such effect disappeared after discontinuation of the drug and variations were observed depending on animal age, sex, and strain. In addition, an overexpression of DNA methylation sensors, such as human endogenous retrovirus genes, are reported in MSG from pSS patients (25). HERVs are believed to play a role in the pathophysiology of AID such as SS, SLE and rheumatoid arthritis (RA) through the production of anti-HERV autoantibodies or by interfering with neighboring genes like CD5 in SLE B cells (26-29). Similarly, DNA methylation changes in pSS are associated with the expression of micro-RNAs and non-coding regulatory RNAs (30). Moreover epigenetic histone mark modifications are immunogenic leading to autoantibody production (31). In normal salivary glands, KRT19 is consistently expressed in epithelial cells with higher expression reported in ductal than in acinar cells (32). KRT19 is also described as an epigenetically regulated tumor and metastasis suppressor gene (33). As a consequence KRT19 promoter demethylation is reported in renal cell carcinoma (34), while KRT19 promoter hypermethylation was associated with transcriptional repression in a wide variety of tumor cells such as neuroblastomas, squamous cell carcinomas and uterine leiomyoma (35). In addition, treating tumor cells with the DNA demethylating inhibitor 5-Aza is effective for increasing KRT19 expression (36). Our study confirms the link between epigenetic DNA modifications and KRT19 gene expression in SG by testing the KRT19 DNA methylation status, and by demonstrating the effects

of 5-Aza on mRNA and protein expression. The Microarray-based Illumina Infinium 450K methylation array is characterized by its high resolution, quantitative measurements, accuracy, small sample requirement and a relatively low cost (37). In addition, the methylation status at the interrogated CpG sites could be investigated by comparing the measured intensities (38). Applied to SGEC from pSS patients and among the 14 KRT19 CpG probes tested, there were 12 methylation sites below the cut-off β value and in agreement with previous reports, variations occurred predominantly within the promoter region of KRT19. Interestingly, our results also suggested that a distal repressor/enhancer is present in the distal part of KRT19. Such hypothesis needs further exploration in order to test whether, or not, this element is effective in modulating KRT19 expression.

Genome-wide analysis of DNA methylation was reported in naïve CD4+ CD45RA+ T cells, and B cells purified from patients with pSS, highlighting DNA methylation changes with the interferon pathway, disease activity, and anti-SSA/SSB Ab production, and an overlap was observed between DNA methylation probes and genetic risk-factors (39, 40). In line with these observations we have recently reported a link between defective global DNA methylation in SGEC and anti-SSB Ab production in pSS patients, and cross-talk between epigenetic factors and pSS-associated risk factors in B cells (9, 16). Genome analysis of the gene promoter DNA methylation status in CD4+ T cells has further revealed a DNA promoter demethylation at lymphotoxin alpha (LTA) and CD70 (TNSF7) genes (39, 41), and a DNA hypermethylation profile at the Treg marker forkhead box P3 (FoxP3) promoter coupled with a significant decrease in FoxP3 expression (42). In contrast, authors failed to demonstrate an epigenetic regulation for interferon regulatory factors (IRF5) and CD40L in pSS (43, 44). In SGEC, DNA methylation is suspected of controlling important functions, such as salivary flux, based on the observations that 5-Aza is effective in inducing the expression of the aquaporin 5 (AQP5) gene in the human salivary gland ductal cell line NS-SV-DC (45), on one hand, and to the increased fluid secretion in the murine aging model C57Bl/6CrSkc on the other hand (46). Similarly, changes in the expression of the type I hemidesmosome BP230 protein in labial SG from pSS patients resulted from BP320 gene hypermethylation (47)

In conclusion, the increase in KRT19 mRNA levels in SGEC from pSS patients may be explained by DNA methylation changes. Therefore, future studies are mandatory to characterize and determine the functional consequences of DNA methylation and KRT19 changes observed in SGEC from pSS patients and will undoubtedly provide a better comprehensive understanding of the pSS pathogenesis with clinical and therapeutical applications in this disease (48).

Conflicts of Interest: None

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