RESEARCH ARTICLE

Methylation profile of the promoter region of IRF5 in primary Sjögren's syndrome

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ABSTRACT. The transcription factor interferon regulatory factor 5 (*IRF5*), in the type I interferon pathway is involved in the genetic susceptibility to various autoimmune diseases. A 5-bp insertion/deletion (CGGGG indel) polymorphism in the promoter region of *IRF5* associated with primary Sjögren's syndrome (pSS) could be epigenetically deregulated in this condition. Therefore, we investigated DNA methylation patterns of the promoter region of *IRF5* to determine whether its epigenetic deregulation could explain the increased expression of *IRF5* mRNA in pSS patients, along with the risk of pSS induced by the genetic polymorphism. DNA extracted from total peripheral blood mononuclear cells, isolated $CD4^+$ T cells, B lymphocytes and monocytes from 19 pSS patients and 24 healthy controls underwent methylation analysis by pyrosequencing. Salivary gland epithelial cells (SGECs) were cultured from minor salivary glands. Regions of interest in the CGGGG repeat and ATG initiation codon region were amplified by PCR and analysed by pyrosequencing. The effect of the demethylating agent 5-AzaC on *IRF5* mRNA expression in controls was quantified by RT-PCR. Among the healthy controls, the mean methylation of the nine CpG pairs of the CGGGG repeat region and the 18 CpG pairs of the ATG region was $< 15\%$ in $CD4^+$ T cells, B lymphocytes, monocytes and SGECs. Patients and controls did not differ in methylation profiles as regards $CD4^+$ T cells and B lymphocytes. *IRF5* mRNA expression did not differ with or without 5-AzaC in controls. The absence of aberrant DNA methylation profiles for the putative regulatory regions of *IRF5* in $CD4^+$ T cells, B lymphocytes, and monocytes from patients with pSS, does not support the hypothesis that epigenetic deregulation in combination with the genetic polymorphism explains the increase in *IRF5* mRNA levels in pSS patients.

Key words: IRF5, Sjögren's syndrome, DNA methylation, epigenetics, autoimmune diseases

Autoimmune diseases affect about 8% of the population [1], result from the interaction between genetic and environmental factors, and involve both innate and adaptive immunity. Sjögren's syndrome (SS), also referred to as autoimmune epithelitis, is a prototypic, systemic, autoimmune disease because it can be primary (pSS) or secondary to other systemic, connective tissue diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, scleroderma). After rheumatoid arthritis, pSS is the most common systemic, autoimmune disease, with a prevalence of 0.1% to 0.6% in the general population [2]. Lymphoid infiltration of lacrimal and salivary glands leading to xerophthalmia and xerostomia, as well as enhanced activation of polyclonal B lymphocytes, represent the hallmarks of the disease. The disease mainly affects exocrine glands (particularly salivary and lacrimal glands), but can also present as a systemic disease with musculoskeletal (arthralgia, arthritis, myalgia), gastrointestinal, pulmonary, dermatological, hematological, neurological and renal manifestations.

Until 2007, the most important genetic factors associated with pSS were alleles of the major histocompatibility complex, specifically the ancestral haplotype *HLA-A1-B8-DR3-DQ2,* in patients with autoantibodies [3, 4]. More recent data elucidating the pathogenic mechanisms involved in pSS, support the role of the interferon (IFN) pathway through an IFN signature, both in peripheral blood mononuclear cells (PBMCs) and in salivary glands [5, 6]. Thus, research has mainly focused on genes involved in innate immunity and the IFN pathways. These approaches have successfully demonstrated the role of two crucial genes: signal transducer and activator of transcription 4 (*STAT4*, a gene involved in T_H1 differentiation [7, 8] and interferon regulatory factor 5 (*IRF5*), a gene implicated in type I IFN secretion after stimulation of innate immunity, and in type I IFN signal transduction [9]. The most

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strongly associated genetic polymorphism within the *IRF5* gene is a 5-bp insertion/deletion (CGGGG indel) polymorphism located 64 bp upstream of the first untranslated exon (exon 1A) within the promoter region of *IRF5* [8, 10]. This 5-bp CGGGG indel polymorphism has also been significantly associated with other autoimmune diseases such as inflammatory bowel diseases (IBD) [11] and systemic lupus erythematosus (SLE) [12].

The transcription factor specificity protein 1 (Sp1) is widely expressed in tissues, and binds GC box motifs in promoters. The 3×CGGGG repeat of the *IRF5* promoter contains two Sp1 binding sites, whereas the 4×CGGGG repeat, the risk-conferring allele for the associated autoimmune diseases, provides an additional Sp1 binding site, which might lead to increased fixation of Sp1 [12]. We recently described the functional consequences of the risk allele on *IRF5* mRNA expression in pSS patients [8].

DNA methylation occurring on the 5 position of the pyrimidine ring of cytosines in the context of the dinucleotide sequence CpG, forms one of the multiple layers of epigenetic mechanisms controlling and modulating gene expression through chromatin structure. Aberrant DNA methylation changes have been detected in several diseases, particularly cancer, where genome-wide hypomethylation coincides with gene-specific hypermethylation. Hypomethylation of gene regulatory sequences has also been found to induce overexpression of the genes associated with autoimmune diseases, as demonstrated recently for *CD40L* and *CD70*, expression of which is increased, through DNA hypomethylation in T-cells from patients with systemic lupus erythematosus compared with healthy controls [13, 14]. Epigenetic deregulation of *IRF5* in pSS has not been assessed.

Interestingly, the 5-bp CGGGG indel sequence lies within a GC-rich region (69%) meeting the criteria of a CpG island. This region is susceptible to epigenetic deregulation, as was recently shown by microarray analysis of methylated genes in hepatocellular carcinoma, with *IRF5* frequently found to be methylated [15]. *IRF5* methylation was associated with low expression or silencing of IRF5 mRNA. *IRF7*, another IRF family member, is also epigenetically silenced by DNA methylation in Li-Fraumeni fibroblasts [16].

We hypothesized that epigenetic deregulation could combine with the genetic polymorphism to explain the increased *IRF5* mRNA expression in pSS patients. We assessed the *IRF5* methylation profile in cell types from pSS patients and healthy controls: B lymphocytes, T lymphocytes, monocytes, and salivary gland epithelial cells (SGECs), the target of autoimmunity in pSS.

PATIENTS AND METHODS

Patients

We included 19 unrelated females with pSS (16 positive for anti-SSA and/or anti-SSB antibodies), fulfilling the European American consensus group criteria for pSS [17] (mean age 57.6 ± 15.2 years) and 24 healthy controls (23 females) (mean age 43.6 ± 13.1 years). All patients and controls were Caucasians. Controls had sicca symptoms without any features of autoimmunity and were referred to the Rheumatology Department of Bicêtre Hospital for a diagnostic procedure. All patients underwent the same clinical, biological and immunological screening. The study received approval from the local ethics committee, and informed consent was obtained from all subjects.

Isolation of cell populations

PBMCs were isolated from subjects by density-gradient centrifugation. $CD4^+$ T cells, B lymphocytes and monocytes were isolated by positive selection (Miltenyi Biotec, Paris, France). $CD4^+$ T cells were stained with CD3-FITC and CD4-APCH7, B lymphocytes with CD19-PE and monocytes with CD14-PE (BD Biosciences, Le Pont de Claix, France). All cell fractions were analyzed using BD FACSCanto (BD Biosciences). The cell purity for all cell fractions was >95%.

Cell culture

We established primary cultures of SGECs from minor salivary glands as described [18]. DMEM, Ham's F-12 and DMEM/F-12 were from Invitrogen (Cergy Pontoise, France), penicillin and steptomycin were from PAA (France), fetal bovine serum (FBS) and 0.125% trypsin-EDTA were from Seromed (Berlin), hydrocortisone was from Pharmacia (Guyancourt, France), insulin was from Novo Nordisk A/S (Denmark) and epidermal growth factor (EGF) was from BD Biosciences. Briefly, each lobule was cut into small fragments and set in six 75-cm^2 flasks with basal epithelial medium (a 3:1 mixture of Ham's F-12 and DMEM) supplemented with 2.5% FBS, EGF (10 ng/mL), hydrocortisone (0.4 μg/mL), insulin (0.5 μg/mL), penicillin (100 IU/mL) and streptomycin (100 μg/mL), and incubated at 37 °C with 5% CO_2. After four to five weeks of culture, cells at 70%-80% confluence were dissociated using 0.125% trypsin-EDTA.

$CD4^+$ T cells were stimulated with phytohemagglutinin (PHA) (5 μg/mL, Sigma-Aldrich), then interleukin 2 (IL-2) (20 U/mL, Roche Diagnostics, Meylan, France) for 72 h, and treated or not with 5-azacytidine (5-AzaC) (Sigma-Aldrich, Saint Quentin Fallavier, France) at 1 μM. Cell division and apoptosis were controlled by successive halving of the fluorescence intensity of 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (Sigma-Aldrich) with 7-aad (eBiosciences, France). We analyzed the methylation profile of a control, CD40L, located on X chromosome, for methylation of one random allele in healthy women. The mean percentage methylation of the CpG studied within the promoter region of CD40L was analyzed by pyrosequencing in T-cell cultures from healthy women, with or without 5-AzaC treatment. Similarly, CD40L mRNA expression was compared with or without 5-AzaC treatment. 5-AzaC significantly reduced the methylation levels of the CpG studied and significantly increased CD40L mRNA expression (data not shown), which demonstrates the effectiveness of 5-AzaC as a demethylating agent.

Cell media were RPMI 1640 glutamax Gibco supplemented with 10% SVF (Dutscher), penicillin (100 U/mL), streptomycin (100 μg/mL), HEPES buffer (10 mM), sodium pyruvate (1 mM) and amino acids (Invitrogen).

Real-time PCR and RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). cDNA synthesis involved use of the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich). *IRF5* and β-actin mRNA levels were detected using a LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) in Light Cycler-based kinetic quantitative RT-PCR (Roche Diagnostics). Amplification involved primers for *IRF5*, (forward) 5'-CAT TAC TGT ACA GGT GGT GC-3' and (reverse) 5'- AGA TGT GAT GGA GCT CCT TG -3'; and β-*actin*, (forward) 5'-GCT GTG CTA CGT CGC CCT-3' and (reverse) 5'-AAG GTA GTT TGG TGG ATG CC-3'. Primers for *IRF5* were specific to exons 8 and 9, which allowed for amplification of all *IRF5* transcripts containing exon 1A. Each sample was processed in duplicate, with initial incubation at 96 °C for 10 min, then 40 cycles at 95 °C for 10 sec, 63 °C for 10 sec, and 72 °C for 10 sec. Variations in mRNA recovery and reverse transcription yield were normalized to that of β-actin.

DNA analysis

DNA from $CD4^+$ T cells, B lymphocytes, monocytes and SGECs was isolated using the QIAamp® DNA Mini Kit, and bisulfite-treated with the EpiTect® Bisulfite Kit (both Qiagen). Regions of interest, encompassing 66 bp and 132 bp containing the CGGGG repeat and the ATG initiation codon region, respectively, were amplified by the HotStar Taq DNA polymerase method (Qiagen). DNA methylation patterns were analyzed by pyrosequencing [19]. The following primers were used for amplification and pyrosequencing: IRF5 CGGGG region, (forward) 5'-TTT TGT TAT TTT AGA TTG TTA AAA GAG TTA-3', (reverse) 5'-Biotin-CCA AAC TAA ACT CTA CCC AAA CTA C-3' and (sequencing primer) 5'-GGA TTT GTA GTT GTT AGG TT-3'; IRF5 ATG region, (forward) 5'-GTT TAG GTT TAG ATT GGG TTT-3', (reverse) 5'-Biotin-CCT AAA TCA CTA AAC TCC CC-3', (sequencing primer 1) 5'-TTC GCG TTT TTT AGG TA-3', (sequencing primer 2) 5'-GGG ATG AAG ATT GGA GTA-3'; (sequencing primer 3) 5'-GGG GGG GTG TTT ATA GTA-3' and (sequencing primer 4) 5'-GGT TTG GGA TTT TTA AAG-3'. PCR protocols were initial incubation at 96 °C for 10 min, then 50 cycles at 95 °C for 10 sec, Δ for 10 sec, and 72 °C for 10 sec (Δ is 62 °C for CGGGG region and 60 °C for ATG region). Methylation data were analyzed using Pyro Q-CpG (Qiagen). The degree of methylation at each CpG was expressed as percentage of methylated cytosines compared to the sum of methylated and unmethylated cytosines at the respective CpG. We used non-CpG cytosines as a control to verify completeness of the bisulfite conversion. Each sample was processed in duplicate.

Statistical analyses

Data are expressed as mean±SD percentage (range). Statistical analyses involved the use of GraphPad Prism 5. *IRF5* mRNA expression and DNA methylation level were analyzed using the non-parametric Mann Whitney test. $P<0.05$ was considered statistically significant.

RESULTS

IRF5 DNA methylation patterns

We examined the methylation patterns of nine CpGs in the region encompassing the 5-bp CGGGG indel polymorphism and 18 CpGs surrounding the start site (ATG codon) of *IRF5* in PBMCs isolated from four pSS patients and various cell sub-populations isolated from the 24 healthy controls and 19 pSS patients (*figure 1*).

In assessing global DNA methylation, both CGGGG and ATG regions in PBMCs from the four pSS patients were largely unmethylated: mean 4.6% (range 4.2-5.0) and 4.8% (range 3.4-5.6), respectively. Methylation profiles were similar for patients carrying the 3×CGGGG repeat or the 4×CGGGG repeat (data not shown).

Because we hypothesized that *IRF5* could be demethylated among pSS patients as compared with controls, we assessed whether both regions had high levels of

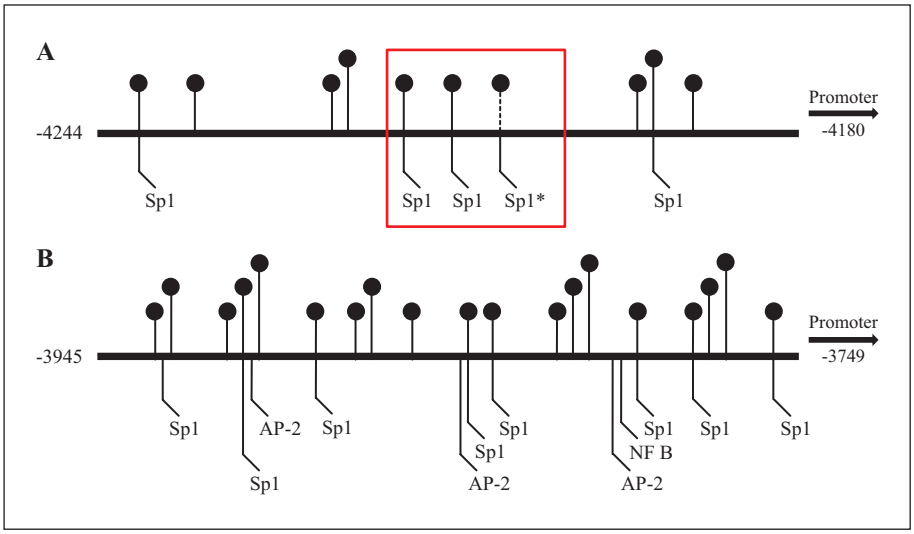

Figure 1

Representation of nine CpG dinucleotides studied from the CGGGG region and 18 from the ATG region of *IRF5*. The axis numbering corresponds to the base pairs from the ATG starting site. For **(A)** and **(B)**, putative Sp1 or alternative transcription factors binding sites (NFκB or AP-2) are represented by bent lines. Balloons correspond to the CG pairs studied. The box shows *IRF5* CGGGG repeat. * corresponds to the additional Sp1 binding site with 4×CGGGG carriage.

methylation in control mononuclear cell subpopulations ($CD4^+$ T cells [n = 21], B lymphocytes [n = 15], monocytes [n = 4]), and SGECs (n = 2). The CGGGG repeat region was largely unmethylated in all studied control cell populations: 6.9% (2.8-11.9; n = 9), 9.9% (6.3-12.2; n = 5), 4.5%, (3.6-5.7; n = 4), and 6.5% (6.2-6.9; n = 2), respectively (*figure 2A*). As well, the ATG codon region was largely unmethylated in controls: 6.1% (3.3-12.3; n = 15), 5.5% (3.8-6.5; n = 10), 5.6%, (3.8-6.7; n = 4), and 5.9% (5.7-6.2; n = 2), respectively (*figure 2B*).

We studied methylation profiles of both regions in sorted $CD4^+$ T cells (n = 14) and B lymphocytes (n = 11) from pSS patients. Within the CGGGG region, unmethylation patterns were 7.1% (1.5-12.2; n = 10) and 6.7% (3.2-11.7; n = 7), respectively, and within the ATG region, 8.0% (5.7-14.3; n = 5) and 7.1% (5.8-9.9; n = 5), respectively.

Thus, all studied cell populations showed unmethylated profiles, with no significant differences between patients and controls in $CD4^+$ T cells or B lymphocytes in the CGGGG region (*figures 3A-B*) or ATG region (*figures 4A-B*).

IRF5 mRNA expression on culture with 5-AzaC

IRF5 might be subjected to methylation in alternative regulatory regions among controls, with aberrant demethylation occurring in pSS patients. We thus further examined whether 5-AzaC, an irreversible DNA methyltransferase inhibitor, could increase *IRF5* expression in healthy controls. To maximize the demethylating effect of 5-AzaC requiring cell divisions, T cells were cultivated under stimulating conditions (PHA/IL-2), with or without 5-AzaC (1 μM) for 72 h. *IRF5* mRNA level did not differ with or without 5-AzaC (0.51 ± 0.6 versus 0.30 ± 0.23).

DISCUSSION

This study is the first to investigate the DNA methylation patterns of regulatory regions of *IRF5* in pSS, and to reveal no aberrant demethylation profile in the region encompassing the 5-bp CGGGG indel polymorphism or that surrounding the ATG start site of *IRF5* in pSS. Our functional experiments with 5-AzaC do not support other, highly methylated sequences in controls that could be subject to demethylation in pSS patients.

The transcription factor IRF5 plays a key role in bridging innate and adaptive immune responses: it contributes to B-lymphocyte activation [20, 21], dendritic cell differentiation [22], and polarization of T cells toward a T_H1 phenotype [23] and participates in the IFN type I signature that characterizes pSS. Besides being involved in pSS genetic susceptibility, *IRF5* has been associated with a broad spectrum of other autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis, inflammatory bowel diseases and rheumatoid arthritis [11, 24-26]. Dissecting the mechanisms of *IRF5* mRNA expression is thus a key issue in many autoimmune diseases.

One of the most important *IRF5* risk-conferring alleles associated with autoimmune diseases is a CGGGG repeat lying within a CpG-rich region, possibly associated with differential patterns of DNA methylation. Hypomethylated profiles among pSS patients could have amplified the functional effect of the genetic polymorphism, for the addition of an Sp1 binding site. Moreover, methylation of an Sp1 consensus sequence can alter Sp1 binding [12], which could support a possible interplay between genetics and epigenetics in the regulation of *IRF5* expression.

Nevertheless, we did not find any evidence of alterations in DNA methylation patterns of the region encompassing the 5-bp CGGGG indel polymorphism in $CD4^+$ T cells,

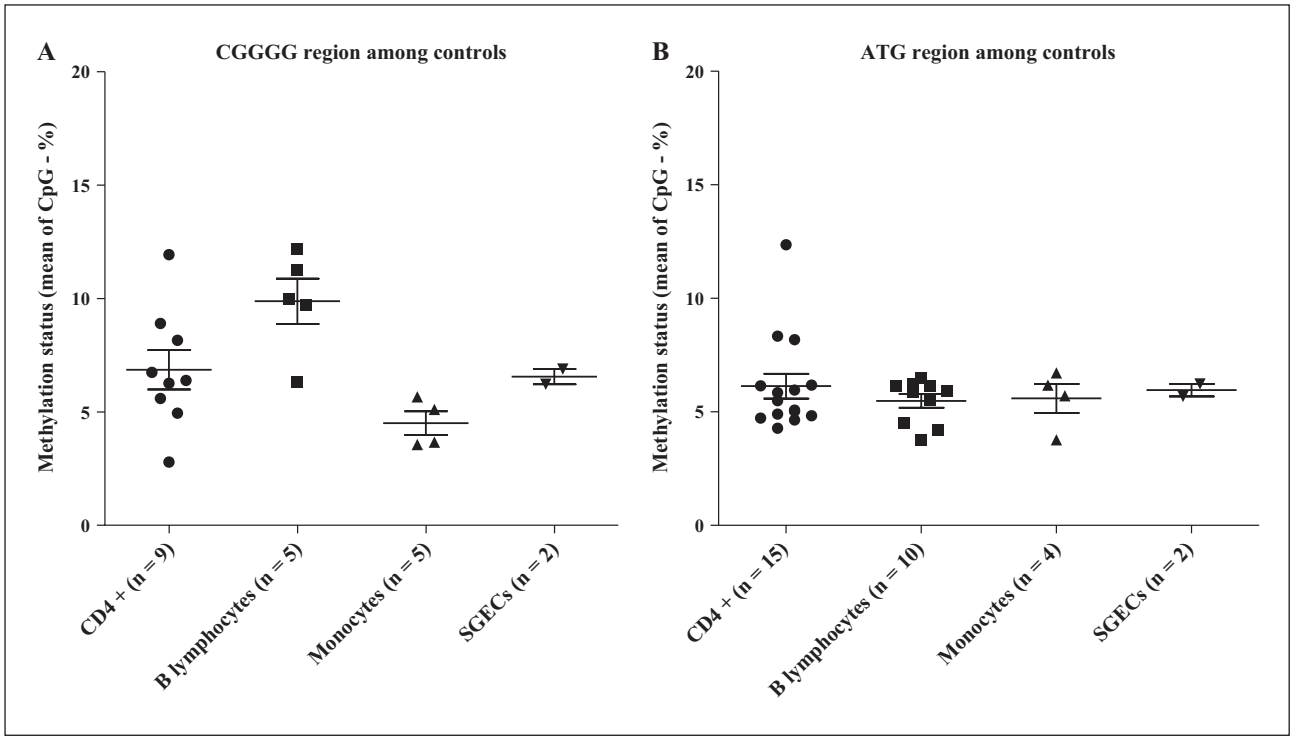

Figure 2
Mean ± SD percentage methylation for all CpGs analyzed in *IRF5* CGGGG **(A)** and ATG **(B)** regions in different cell types from healthy controls: $CD4^+$ T cells, B lymphocytes, monocytes, and salivary gland epithelial cells (SGECs; n = 2).

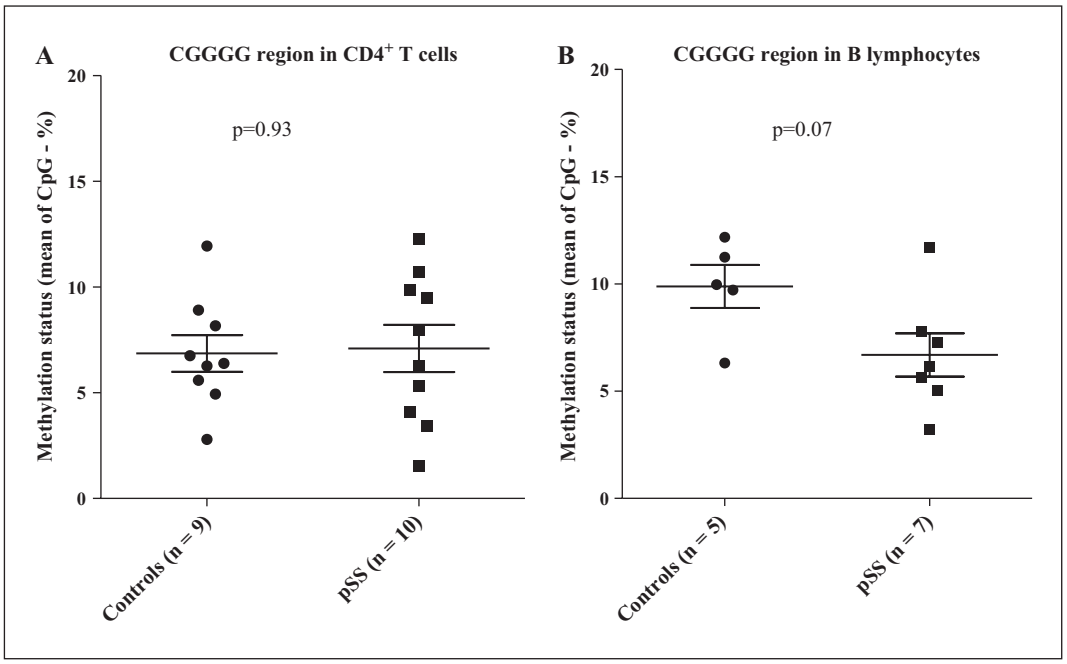

Figure 3
Mean±SD percentage methylation for all CpGs analyzed in *IRF5* CGGGG region in CD4+ T cells **(A)** and B lymphocytes **(B)** from pSS patients and controls.

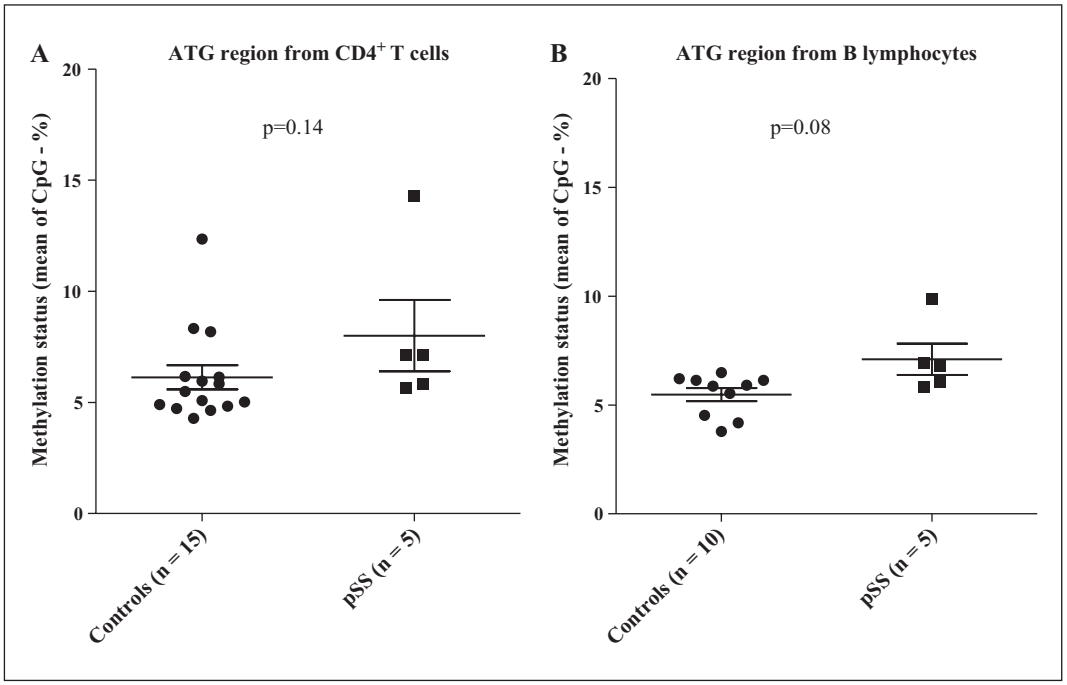

Figure 4
Mean±SD percentage methylation for all CpGs analyzed in *IRF5* ATG region in CD4+ T cells **(A)** and B lymphocytes **(B)** from pSS patients and controls.

B lymphocytes, monocytes or SGECs from healthy controls. Findings were similar in PBMCs from pSS patients, regardless of the number of CGGGG repeats they carried. As well, we found no methylation and no difference between healthy controls and pSS patients in methylation of the ATG region in CD4+ T cells and B lymphocytes. Moreover, functional experiments with 5-AzaC did not highlight a significant increase in *IRF5* mRNA expression in healthy controls, which suggests that IRF5 mRNA expression is not methylation-sensitive.

Consequently, our results do not support an epigenetic deregulation of *IRF5* in pSS by aberrant hypo- or hypermethylation of CpG dinucleotides located within the most important regulatory regions of the gene. This work agrees with recent data demonstrating that the methylation patterns of the *IRF5* promoter are not affected by the presence of the 5-bp CGGGG insertion in inflammatory bowel disease [27]. Nevertheless, this latter work did not analyze the region surrounding the start site of *IRF5*, nor analyze the different cell subtypes in PBMCs.

Thus, the trend toward a higher *IRF5* mRNA level in pSS patients than in healthy controls is probably mainly attributable to the genetic consequences of an overrepresentation of 4×CGGGG repeats of *IRF5* among pSS patients. This hypothesis is suggested by minigene constructs of promoters cloned from individuals homozygous for the 4×CGGGG or 3×CGGGG repeat [12] and our previous work demonstrating that 4×CGGGG-repeat carriage is associated with increased *IRF5* mRNA level in PBMCs from pSS patients [8].

The current study does not allow for excluding all potential epigenetic deregulation paths of *IRF5* in pSS, because DNA methylation is not the only mechanism of epigenetic regulation. The other main mechanisms involve histone acetylation and methylation and regulation through micro-RNAs (miRNAs). Recently published data demonstrated increased expression of miR-21 [28] and decreased expression of miR-146a in patients with systemic lupus erythematosus [29]. Interestingly, transfection of miR-146a into 293T cells consistently reduced the expression of *IRF5* [29]. Therefore, miR-146a could be an efficient, negative regulator of *IRF5*, and its reduced expression in SLE could participate in *IRF5* overexpression. A similar mechanism modulating *IRF5* mRNA expression could be involved in pSS.

New techniques have become available for searching for hundreds or thousands of genes differentially methylated in controls and patients. Comparison of genome-wide DNA methylation patterns in $CD4^+$ T cells from SLE patients and healthy controls identified numerous hypomethylated or hypermethylated CG sites, which confirms the widespread DNA methylation changes found in T cells in systemic lupus erythematosus [30]. A similar approach is also a promising strategy for identifying novel targets of epigenetic deregulation in pSS.

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REFERENCES

1. Cooper GS, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun* 2009; 33: 197-207.
2. Bowman SJ, Ibrahim GH, Holmes G, Hamburger J, Ainsworth JR. Estimating the prevalence among Caucasian women of primary Sjogren's syndrome in two general practices in Birmingham, UK. *Scand J Rheumatol* 2004; 33: 39-43.
3. Chused TM, Kassan SS, Opelz G, Moutsopoulos HM, Terasaki PI. Sjogren's syndrome association with HLA-Dw3. *N Engl J Med* 1977; 296: 895-7.
4. Kang HI, Fei HM, Saito I, *et al*. Comparison of HLA class II genes in Caucasoid, Chinese, and Japanese patients with primary Sjogren's syndrome. *J Immunol* 1993; 150: 3615-23.
5. Gottenberg JE, Cagnard N, Lucchesi C, *et al*. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. *Proc Natl Acad Sci U S A* 2006; 103: 2770-5.
6. Hjelmervik TO, Petersen K, Jonassen I, Jonsson R, Bolstad AI. Gene expression profiling of minor salivary glands clearly distinguishes primary Sjogren's syndrome patients from healthy control subjects. *Arthritis Rheum* 2005; 52: 1534-44.
7. Miceli-Richard C, Comets E, Loiseau P, Puechal X, Hachulla E, Mariette X. Association of an IRF5 gene functional polymorphism with Sjogren's syndrome. *Arthritis Rheum* 2007; 56: 3989-94.
8. Miceli-Richard C, Gestermann N, Ittah M, *et al*. The CGGGG insertion/deletion polymorphism of the IRF5 promoter is a strong risk factor for primary Sjogren's syndrome. *Arthritis Rheum* 2009; 60: 1991-7.
9. Barnes BJ, Moore PA, Pitha PM. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *J Biol Chem* 2001; 276: 23382-90.
10. Nordmark G, Kristjansdottir G, Theander E, *et al*. Additive effects of the major risk alleles of IRF5 and STAT4 in primary Sjogren's syndrome. *Genes Immun* 2009; 10: 68-76.
11. Dideberg V, Kristjansdottir G, Milani L, *et al*. An insertion-deletion polymorphism in the interferon regulatory Factor 5 (IRF5) gene confers risk of inflammatory bowel diseases. *Hum Mol Genet* 2007; 16: 3008-16.
12. Sigurdsson S, Goring HH, Kristjansdottir G, *et al*. Comprehensive evaluation of the genetic variants of interferon regulatory factor 5 (IRF5) reveals a novel 5 bp length polymorphism as strong risk factor for systemic lupus erythematosus. *Hum Mol Genet* 2008; 17: 872-81.
13. Lu Q, Wu A, Tesmer L, Ray D, Yousif N, Richardson B. Demethylation of CD40LG on the inactive X in T cells from women with lupus. *J Immunol* 2007; 179: 6352-8.
14. Lu Q, Wu A, Richardson BC. Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. *J Immunol* 2005; 174: 6212-9.
15. Shin SH, Kim BH, Jang JJ, Suh KS, Kang GH. Identification of novel methylation markers in hepatocellular carcinoma using a methylation array. *J Korean Med Sci* 2010; 25: 1152-9.
16. Lu R, Au WC, Yeow WS, Hageman N, Pitha PM. Regulation of the promoter activity of interferon regulatory factor-7 gene. Activation by interferon and silencing by hypermethylation. *J Biol Chem* 2000; 275: 31805-12.
17. Vitali C, Bombardieri S, Jonsson R, *et al*. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; 61: 554-8.
18. Dimitriou ID, Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Establishment of a convenient system for the long-term culture and study of non-neoplastic human salivary gland epithelial cells. *Eur J Oral Sci* 2002; 110: 21-30.
19. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. *Nat Protoc* 2007; 2: 2265-75.
20. Savitsky DA, Yanai H, Tamura T, Taniguchi T, Honda K. Contribution of IRF5 in B cells to the development of murine SLE-like disease through its transcriptional control of the IgG2a locus. *Proc Natl Acad Sci U S A* 2010; 107: 10154-9.
21. Lien C, Fang CM, Huso D, Livak F, Lu R, Pitha PM. Critical role of IRF-5 in regulation of B-cell differentiation. *Proc Natl Acad Sci U S A* 2010; 107: 4664-8.
22. Yasuda K, Richez C, Maciaszek JW, *et al*. Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J Immunol* 2007; 178: 6876-85.
23. Krausgruber T, Blazek K, Smallie T, *et al*. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* 2011; 12: 231-8.

24. Jarvinen TM, Hellquist A, Koskenmies S, *et al*. Tyrosine kinase 2 and interferon regulatory factor 5 polymorphisms are associated with discoid and subacute cutaneous lupus erythematosus. *Exp Dermatol* 2010; 19: 123-31.

25. Kristjansdottir G, Sandling JK, Bonetti A, *et al*. Interferon regulatory factor 5 (IRF5) gene variants are associated with multiple sclerosis in three distinct populations. *J Med Genet* 2008; 45: 362-9.

26. Sigurdsson S, Padyukov L, Kurreeman FA, *et al*. Association of a haplotype in the promoter region of the interferon regulatory factor 5 gene with rheumatoid arthritis. *Arthritis Rheum* 2007; 56: 2202-10.

27. Balasa A, Gathungu G, Kisfali P, *et al*. Assessment of DNA methylation at the interferon regulatory factor 5 (IRF5) promoter region in inflammatory bowel diseases. *Int J Colorectal Dis* 2010; 25: 553-6.

28. Stagakis E, Bertsias G, Verginis P, *et al*. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. *Ann Rheum Dis* 2011; 70: 1496-506.

29. Tang Y, Luo X, Cui H, *et al*. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum* 2009; 60: 1065-75.

30. Jeffries MA, Dozmorov M, Tang Y, Merrill JT, Wren JD, Sawalha AH. Genome-wide DNA methylation patterns in CD4+ T cells from patients with systemic lupus erythematosus. *Epigenetics* 2011; 6: 593-601.