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, SS 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 T1 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
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×CGGG repeat of the **IRF5** promoter contains two Sp1 binding sites, whereas the 4×CGGG 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. Abrupt 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** mRNA 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 streptomycin 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-cm2 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% CO2. 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 phytomagemagglutinin (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 RNasy Mini kit (Qiagen, Courtaboeuf, France). DNA 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 AGG TT-3′; IRF5 ATG region, (forward) 5′-GTG TAG GTT TAG ATT GGG TTT-3′, (reverse) 5′-Biotin-CTT AAA TCA CTA AAC TCC CC-3′, (sequencing primer 1) 5′-TTCC GGC TTT TTT AGG TA-3′, (sequencing primer 2) 5′-GGG ATG ATT GGA GTA-3′; (sequencing primer 3) 5′-GGG GGG GTG TTT ATA GTA-3′ and (sequencing primer 4) 5′-GTT TGG 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...
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 /H9262 M) 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 TH1 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,
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 hyper-methylation 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 micro-RNAs in patients with systemic lupus erythematosus [29]. Interestingly, transfection of miR-146a in patients with systemic lupus erythematosus demonstrated increased 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


