Genome-wide DNA methylation patterns in monocytes derived from patients with primary Sjogren syndrome

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Abstract

Background: Epigenetics, especially DNA methylation, plays an important role in the pathogenesis of primary Sjogren syndrome (pSS). Our study aimed to reveal the role of DNA methylation in peripheral monocytes of pSS patients.

Methods: A total of 11 pSS patients and five age-matched healthy controls (HCs) were included in this study. Monocytes were isolated from peripheral blood mononuclear cells using magnetic microbeads. DNA methylation profiles were generated using Human Methylation 850K BeadChips.

Results: In monocytes from pSS patients, we identified 2819 differentially methylated positions (DMPs), comprising 1977 hypomethylated- and 842 hypermethylated-DMPs, corresponding to 1313 unique genes when compared with HCs. *IFI44L*, *MX1*, *PAARP9*, and *IFITM1*, which influence the interferon (IFN) signaling pathway, were among the genes hypomethylated in pSS. Functional analysis of genes with a minimum of two DMPs showed involvement in antigen binding, transcriptional regulation, cell adhesion, IFN-γ pathway, type I IFN pathway, antigen presentation, Epstein-Barr virus infection, human T-lymphotropic virus type 1 virus infection, and metabolic disease-related pathways. In addition, patients with higher serum IgG levels exhibited enrichment in Notch signaling and metabolic-related pathways. Upon comparing monocytes with salivary gland epithelial cells, an important overlap was observed in the cell cycle, cell senescence, and interleukin-17 signaling pathways. The differentially methylated genes were more enriched in the ribosome- and AMP-activated protein kinase signaling pathway in anti-Ro/SSA and anti-La/SSB autoantibodies double-positive patients.

Conclusion: Genome-wide DNA methylation profiling revealed significant differences in DNA methylation in monocytes isolated from patients with pSS.

Keywords: Sjogren syndrome; Monocyte; DNA methylation

Introduction

Primary Sjogren syndrome (pSS) is a chronic autoimmune disease, which primarily manifests as lymphocyte infiltration in the exocrine glands and is predominantly characterized by dryness of the mucosa, including the mouth and eyes. In addition to the lacrimal glands, salivary glands, and other exocrine glands, pSS can also cause damage to the kidney, liver, lungs, and other important organs.

Current evidence demonstrates that innate immunity plays an important role in pSS pathogenesis.[1] Monocytes/macrophages are one of the representatives of innate immune responses, and dysregulation of their actions might mediate autoimmunity. In salivary and lacrimal gland pathology, monocytes/macrophages are recruited to salivary gland tissue before lymphocytes, suggesting that macrophage infiltration is an early disease event that promotes further immune cell chemotaxis[2] and with disease progression in pSS patients, the number of macrophages increases in salivary tissue.[3] Thus, monocytes/macrophages may promote an inflammatory phenotype and also contributes to exocrine gland dysfunction in disease. Monocytes from pSS patients display a deficient

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clearance of apoptotic cells[4] or defective monocytes/ macrophages failing to clear apoptotic material may contribute to increased levels of antigens released from dying cells, therefore exaggerating disease severity.[5] Monocytes are also considered to be the main source of inflammatory cytokines. For example, type I interferon (IFN), which is responsible for the pleiotropic activation of immune cells and the elicitation of the IFN signature, is mainly produced by plasmacytoid dendritic cells (DCs) and monocytes.[6,7] In addition, monocytes secrete increasing levels of pro-inflammatory cytokines including interleukin and B cell-activating factor (BAFF), upon stimulation,[8] and reduce the levels of the nuclear factor kappa-B inhibitor.[9] Monocytes play a potential role in the pathogenesis of pSS. Thus, monocytes reflect the inflammatory state in pSS patients, and mature monocytes are proposed to contribute to salivary gland inflammation in pSS.

Previous investigations into pSS DNA methylation patterns have assessed different cell types, including naïve CD4+ T cells, CD19+ B cells, and salivary gland epithelial cells (SGECs). Results of these studies displayed significant changes and indicated that the IFN signature is detectable at the DNA methylation level and observed other signal pathways involved in the pathogenesis of pSS.[10-13] DNA methyltransferase 3A (DNMT3A) and the methylcytosine dioxygenase ten-eleven translocation (TET) 2 are essential for the *de novo* incorporation and oxidation/removal of methyl groups to cytosines. In monocyte, DNMT3A and TET are related to differentiation and activation during inflammatory responses.[14,15] In this regard, DNA methylation stands out as a major epigenetic mechanism, which potentially could reflect the influence of disease-associated inflammation in monocytes. Thus, DNA methylation in monocytes will likely provide new insights into pSS. A better knowledge of such processes could determine the detection of new therapeutic targets that are a major need for pSS.

However, DNA methylation patterns of monocytes are poorly explored when compared with other cell types. In this study, we performed a genome-wide DNA methylation study in peripheral blood monocytes obtained from pSS patients and healthy controls (HCs). In this regard, analysis of monocytes from patients with pSS might help to illustrate the pathogenesis of pSS.

Methods

Ethical approval

This study was approved by the Institutional Review Board of Peking Union Medical College Hospital. Written informed consent was obtained from each participating patient and HC.

Patients and controls

Peripheral blood samples were collected from 11 patients with pSS without treatment and five sex- and age-matched HCs. All patients underwent complete blood count, urinalysis, liver and renal function tests, erythrocyte sedimentation rate evaluations, C-reactive protein quantification, serum immunoglobulin level quantification, anti-nuclear antibody profiling, and salivary gland and ocular assessment among others. In addition, eight patients also underwent a tissue biopsy. Classification of pSS was based on the 2016 American College of Rheumatology and European League Against Rheumatism classification criteria for pSS.[16] EULAR Sjogren Syndrome Disease Activity Index (ESSDAI) is used to assess disease activity.[17]

Monocytes isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples obtained from patients using Ficoll-Paque density gradient centrifugation. Monocytes were separated from PBMCs by positive selection using CD14 microbeads (Miltenyi Biotec GmbH, Germany). The purity of monocytes was tested by flow cytometry and was observed to be over 95% in all the samples.

Illumina infinium human methylation 850K bead chip and data analysis

Genome-wide DNA methylation was assessed using the Illumina Infinium Human Methylation 850K BeadChip (Illumina Inc., San Diego, California, USA) according to the manufacturer's instructions. The array data were analyzed using the ChAMP package in R to derive the methylation level. The methylation status of all probes was denoted as the β value, which is the ratio of the methylated probe intensity to the overall probe intensity (sum of methylated and unmethylated probe intensities plus constant α, where $\alpha = 100$). CpG sites having $|\Delta\beta| \geq 0.20$ (in test *vs.* control) and adjusted P value ≤ 0.05 were considered as differentially methylated positions (DMPs). A CpG was considered hypermethylated if $\Delta\beta \geq 0.20$ or hypomethylated if $\Delta\beta \leq -0.20$. The average β values of promoters and CpG islands were compared between disease and normal monocytes. Promoters and CpG islands with $|\Delta\beta| \geq 0.20$ and adjusted P value ≤ 0.05 were considered for further analysis. All the laboratory examination data are listed in the article. The data meeting normal distribution are shown as mean (standard deviation [SD]) while the data that did not satisfy normal distribution are shown as median (interquartile range).

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

GO enrichment analysis of differentially DMP target genes were implemented by the GOseq R package, with the gene length bias being corrected. GO terms with corrected P value < 0.05 were considered significantly enriched by differentially expressed genes. KEGG was a database resource for understanding high-level functions and utilities of the biological system, and KOBAS software was used to test the statistical enrichment of differential DMP target genes in KEGG pathways.

Results

We investigated the difference in methylation levels in monocytes derived from pSS patients and HCs. Patient demographic and clinical characteristics are shown in

Table 1: Characteristic of patients with pSS and control individuals.

Variables	pSS patients (n = 11)	HCs (n = 5)
Female, n	10	4
Age at onset (years), mean (SD)	45.3 (13.7)	42.8 (10.1)
Fatigue, n	2	0
Oral dryness, n	11	0
Ocular dryness, n	8	0
Overall dryness, n	8	0
Joint pain, n	6	0
Hyperglobulinemia, n	8	0
Parotid glands, n	3	0
Cutaneous, n	2	0
Pulmonary, n	4	0
Autoantibody frequency, n		
ANAs	11	0
Anti-SSA antibodies	11	–
Anti-SSB antibodies	9	–
Serological features, median (IQR)		
IgG (g/L)	23.05 (16.20–32.34)	–
IgM (g/L)	1.33 (0.55–1.91)	–
IgA (g/L)	4.20 (3.10–4.70)	–
ESR (mm/h)	49.50 (18.25–66.00)	–
CRP (mg/L)	2.14 (1.23–9.31)	–
RF (IU/mL)	83.25 (39.90–351.58)	–
C3 (g/L)	1.06 (0.90–1.16)	–
C4 (g/L)	0.19 (0.14–0.20)	–
Biopsy, n	8	0
ESSDAI, mean (SD)	2.64 (1.15)	0

ANAs: Anti-nuclear antibodies; CRP: C-reactive protein; ESSDAI: EULAR Sjogren Syndrome Disease Activity Index; ESR: Erythrocyte sedimentation rate; HCs: Healthy controls; IQR: Interquartile range; pSS: Primary Sjogren syndrome; SD: Standard deviation.

Table 1. At the time of diagnosis, their mean age was 45.3 ± 13.7 years and ten patients were women. All patients presented with oral dryness, eight patients had ocular dryness, six suffered from joint pain, three had parotid glandular swelling, two reported fatigue, and two presented with urticarial vasculitis. In addition, four patients developed interstitial lung disease, which was confirmed using high-resolution computed tomography. All the patients were positive for anti-SSA antibodies, and nine had anti-SSB antibodies, and the mean (SD) ESSDAI was 2.64 (1.15).

Methylation status of monocytes from patients with pSS

We identified 2819 (1977 hypomethylated and 842 hypermethylated) DMPs [Supplementary Table 1, http://links.lww.com/CM9/A515]. The hierarchical clustering analysis of these DMPs is shown in Figure 1A (Gene Expression Omnibus database: GSE146116). DMPs were distributed across 1313 genes in monocytes from patients with pSS, including 370 (28.18%) genes with only hypermethylated CpG sites, 883 (67.25%) genes with only hypomethylated CpG sites, and 60 (4.57%) genes displaying a mixed methylation pattern [Figure 1B]. A total of 460 genes showed differential methylation in the gene start/promoter regions corresponding to 129 hypermethy-

lated (28%), 299 hypomethylated (65%), and 32 genes (7%) with a mixed methylation pattern [Figure 1B]. Overall, in circulating monocytes from pSS patients, differences in DNA methylation appear to present predominantly as hypomethylation.

An average difference in β values of > 0.6 between cases and controls was identified in 25 of the 2819 DMPs, of which 16 were hypomethylated [Supplementary Table 2, http://links.lww.com/CM9/A516]. These 16 DMPs were annotated to 11 genes [Figure 1C]. Among the 11 hypomethylated genes [Supplementary Table 3, http://links.lww.com/CM9/A517], *IFI44L* showed the most distinct DMPs, which are located in the 5′-untranslated regions. We also observed hypomethylation of *MX1*, *PARP9*, *DTX3L*, *EPSTI1*, and *IFITM1*, which influence the IFN signaling pathway, in pSS monocytes. The DMP-associated genes and potential signaling pathways are shown in Figure 1D. To correlate DNA methylation with disease activity, we analyzed the methylation status of these DMPs with ESSDAI, but we could not find any significant correlation between them (data not shown).

Pathway analysis

To identify pathways possibly influenced by the differential methylation in monocytes, we performed a GO analysis using genes with a minimum of two DMPs. Analysis of gene function has shown that most genes are related to antigen binding and transcriptional regulation, including RNA polymerase II, RNA polymerase II distal enhancer sequence binding, and so on. The biological processes included cell adhesion pathways, IFN-γ pathway, type I IFN pathway, and antigen presentation [Figure 2A]. The results of a KEGG analysis showed that the differential genes were involved in antigen presentation ($P = 1.57E-05$), cell adhesion ($P = 2.14E-05$), Epstein-Barr virus infection ($P = 1.50E-05$), and HTLV-1 virus infection ($P = 8.52E-04$). Metabolic disease-related pathways such as type 1 diabetes ($P = 4.28E-07$), immune system diseases, including allograft rejection ($P = 2.45E-06$), graft *vs.* host disease ($P = 4.12E-06$), autoimmune thyroid disease ($P = 1.73E-05$), and related pathways [Figure 2B] were also included.

Comparison of differential methylation in monocytes and salivary glands

DMPs of monocytes from peripheral blood were compared with publicly available data from SGECs.[11] As presented in Figure 3A, 20 DMPs corresponding to 12 genes were found to overlap in pSS monocytes and SGECs, namely *PTPRN2, TNK1, WDR8, TSPAN9, VIPR2, OBSCN, KCNT1, ZNF703, NEURL3, LMX1B, LOC146336,* and *FTSJD2.* Compared to the common functional characteristics of genes with aberrant DNA methylation, the potential signaling pathways of the 12 genes included cell cycle, cellular senescence, and the IL-17 signal pathway [Figure 3B and Supplementary Table 4, http://links.lww.com/CM9/A518].

Association of DNA methylation with serological features

To identify the link between DNA methylation and serological features, we first analyzed the methylation status of monocytes from patients with pSS who were both
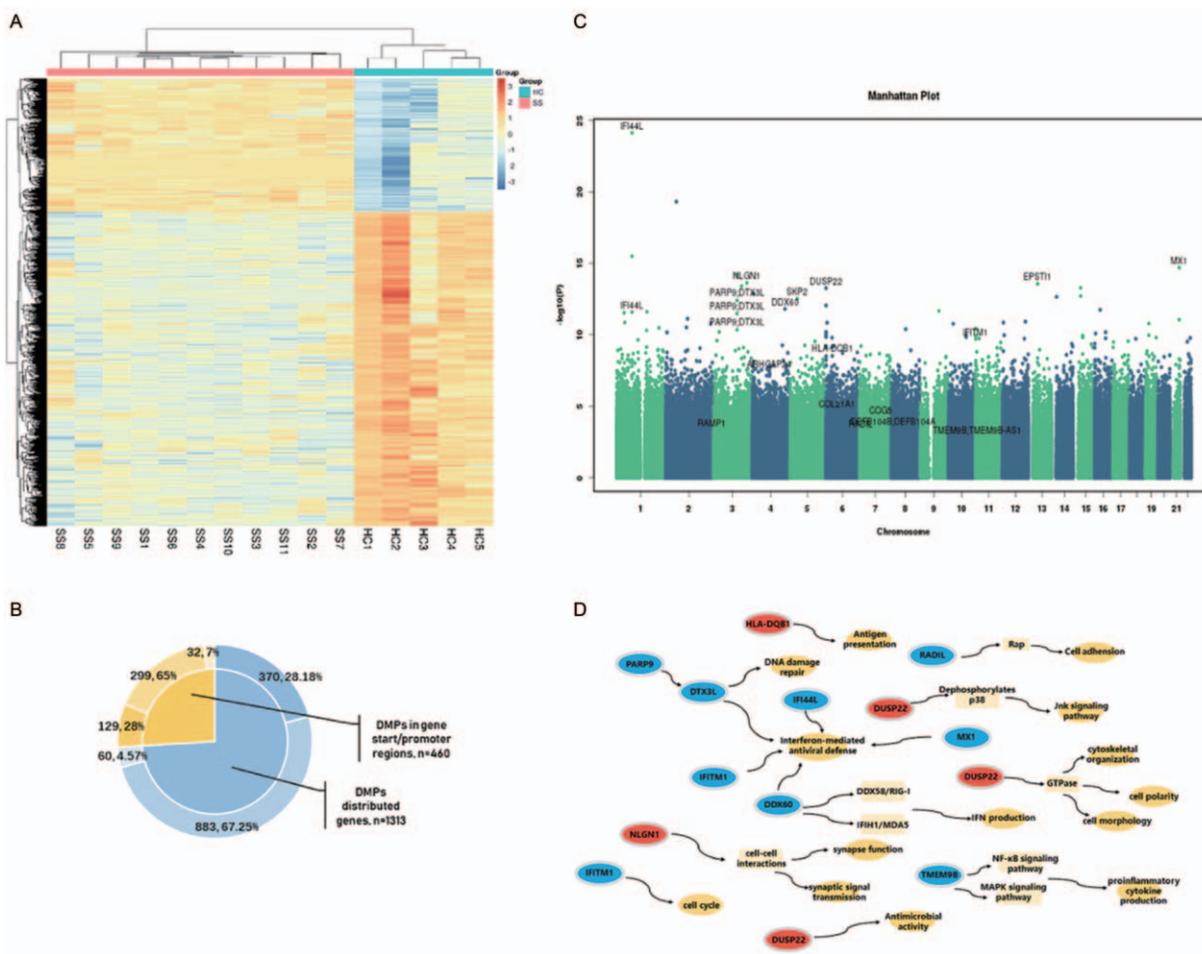
Figure 1: Methylation of monocytes of pSS patients. (A) Heat maps for the hierarchical clustering of DMPs in monocytes of pSS patients. (B) Statistical analysis of the patterns of the DMPs in pSS monocytes; blue stands for the pattern proportion of the DMPs distributed genes, while yellow stands for the pattern proportion of the DMPs in gene start/promoter regions. (C) Manhattan map of the DMPs in pSS monocytes (DMPs with an average difference in β values > 0.6 were screened out). (D) Potential gene signaling pathways or gene functions for the DMPs with β values > 0.6. The blue and red nodes represent the DMPs annotated genes, the red nodes indicate the hypermethylated DMPs annotated genes, blue nodes indicate the hypomethylated DMPs annotated genes, and the shades of the color represent the degree of methylation of DMPs. The yellow nodes represent the relevant pathways or gene functions. DMPs: Differentially methylated position; pSS: Primary Sjogren syndrome.
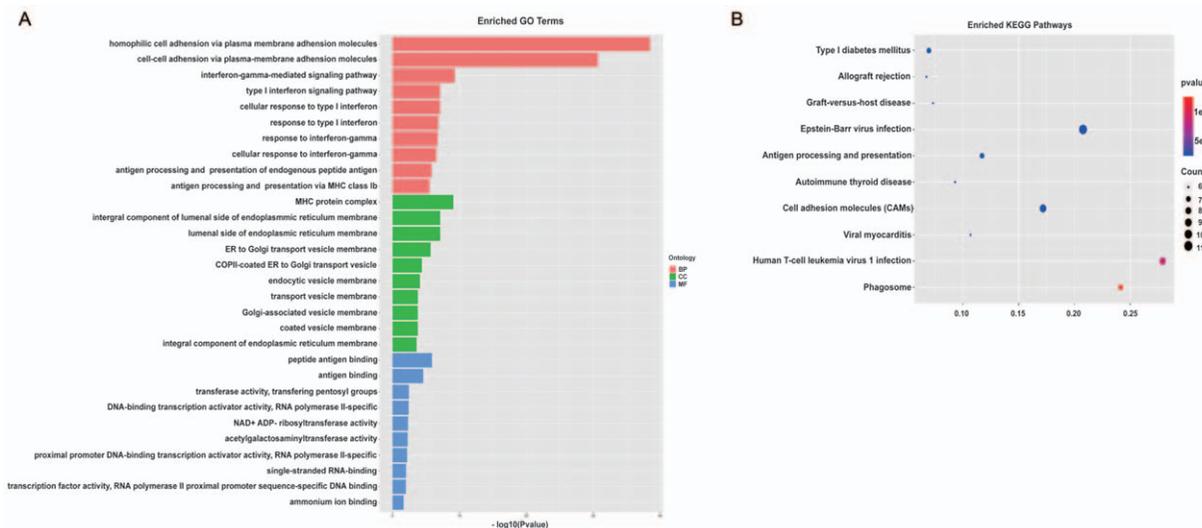
Figure 2: GO and KEGG analysis of the DMPs in pSS monocytes. (A) GO analysis for DMPs in pSS monocytes. (B) KEGG analysis results for the DMPs. BP: Biological process; CC: Cellular component; DMPs: differentially methylated position; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecule function; pSS: Primary Sjogren syndrome.
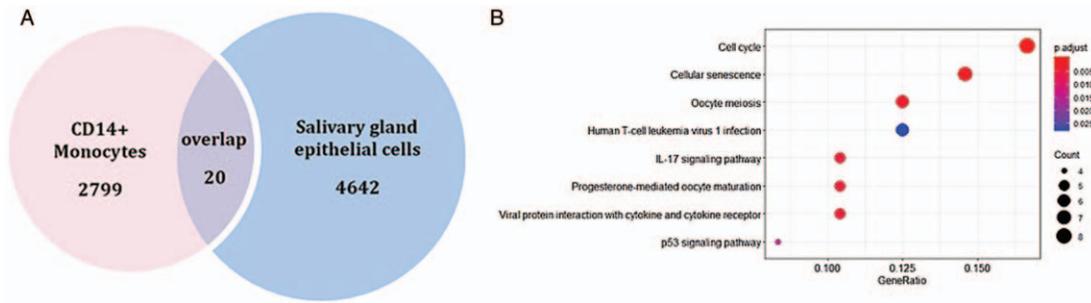
Figure 3: The overlapped DMPs in pSS CD14+ monocytes and SGECs. (A) Venn diagram presenting the overlapped DMPs found in pSS CD14+ monocytes and pSS salivary gland epithelial cells. (B) The KEGG analysis results for the 20 overlapping genes. DMPs: Differentially methylated position; KEGG: Kyoto Encyclopedia of Genes and Genomes; pSS: Primary Sjogren syndrome; SGECs: Salivary gland epithelial cells.
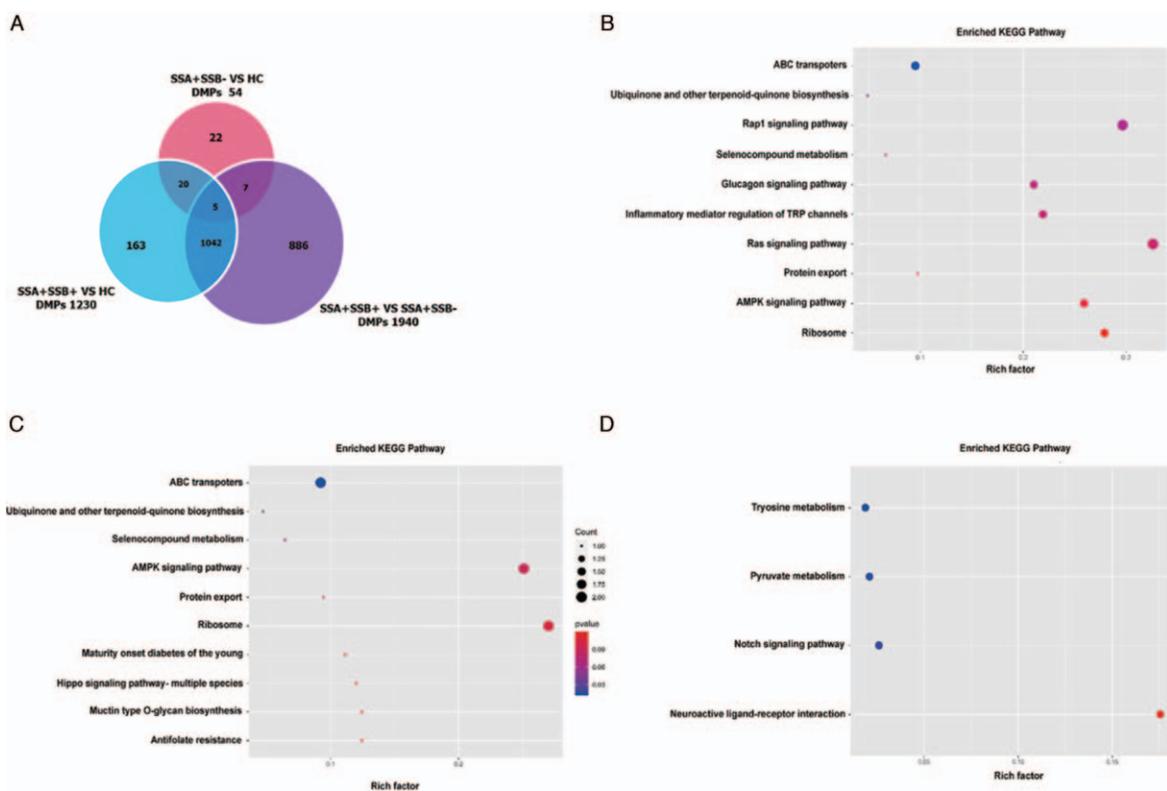
Figure 4: The relationship between DMPs in pSS monocytes and patients' clinical features. (A) Comparison of DMPs for the DP patients *vs.* HCs and SP patients *vs.* HCs. (B) KEGG analysis for the DMPs between DP patients and HCs. (C) KEGG analysis for DMPs between DP and SP patients. (D) KEGG analysis of DMPs between the pSS patients with hyperglobulinemia (IgG >18 g/L) and pSS patients without hyperglobulinemia (DP: anti-SSA+/anti-SSB+; SP: anti-SSA+/anti-SSB−; HCs). DMPs: Differentially methylated position; DP: Double-positive; HCs: Healthy controls; KEGG: Kyoto Encyclopedia of Genes and Genomes; pSS: Primary Sjogren syndrome; SP: Single-positive.

anti-SSA antibody and anti-SSB antibody positive (anti-SSA+/anti-SSB+, double-positive [DP]), or anti-SSA antibody positive and anti-SSB antibody negative (anti-SSA +/anti-SSB−, single-positive [SP]) compared with the control cohort [Figure 4A]. Compared with HCs, DP pSS patients had 1230 DMPs annotated to 984 genes, with 113 showing differential methylation in the gene start/promoter regions. Among the SP patients, only 54 DMPs were annotated to 27 genes, nine of which showed a significant difference compared with the HCs in the gene start/promoter regions. The number of DMPs in patients who were SP was lower than in patients who were DP. We

subsequently used GO and KEGG analysis to enrich the functions and the potential signaling pathways of the DMPs (DP *vs.* HCs, SP *vs.* HCs, and DP *vs.* SP). The DMPs between DP patients and HCs were enriched in the Ras signaling pathway, ribosome pathway, Rap1 signaling pathway, AMPK signaling pathway, and so on [Figure 4B], while the distinct DMPs between SP patients and HCs were only enriched in the Notch signaling pathway [Supplementary Figures 1 and 2, http://links.lww.com/CM9/A519]. Upon comparing the DP and SP patients, we found that the differentially methylated genes were more enriched in the ribosome pathway, AMPK signaling pathway, and so on

[Figure 4C]. These signaling pathways were potentially correlated more strongly in the DP patients.

To identify the potential relationship between serum IgG level and DNA methylation patterns, we compared the DNA methylation in patients with high IgG levels (IgG ≥18 g/L) and patients without high IgG level (IgG <18 g/L). Compared with the non-high IgG group, the high-IgG group had 89 DMPs, including 21 hypermethylated DMPs and 68 hypomethylated DMPs, annotated to 49 genes, of which 22 were displayed in the gene start site/promoter region. Through functional analysis, we found that the neuroactive ligand-receptor interaction pathway, Notch signaling pathway, pyruvate metabolism pathway, and tyrosine metabolism pathway were highly enriched in patients with higher serum IgG levels [Figure 4D].

Discussion

In this study, we have performed a comprehensive analysis of DNA methylation changes in monocytes of pSS patients. Our analysis has identified that circulating monocytes in pSS had more hypomethylation sites than in HCs, which is consistent with previous studies in B cells, T cells, and salivary glands.[10-12] Increased hypomethylation indicated that more genes were abnormally activated in the monocytes of pSS patients. Combining the above results, we showed that the DNA methylation pattern of various cells in pSS patients is modified and the expression of some genes may change due to methylation. The type I IFN signal has been proposed over the last decade as a central contributor in pSS and acted as the link between innate and adaptive immune responses in pSS.[18] In this study, the top DMPs annotated genes are IFN-related genes including *IFI44L*, *MX1*, *EPSTI1*, *PAPR9/DTX3L*, *IFITM1*, and so on. They were found to be hypomethylated in the monocytes of pSS patients, which is in accordance with other epigenome-wide association studies conducted in CD4⁺ T cells, CD19⁺ B cells, and SGECs.[13] Combined with previous research, we found that the global hypomethylation of IFN-inducible genes is well-described and confirmed in different immune cell types of patients with pSS. In systemic lupus erythematous and rheumatoid arthritis, *IFI44L* methylation level can be used as a diagnostic marker and associated with clinical features and treatment outcomes.[19,20] Brkic *et al*[21] also reported that there was a systemic upregulation of IFN type I inducible genes like *IFI44L, IFI44, IFIT3, LY6E,* and *MX1* in monocytes of pSS patients, and this correlated with high disease activity, higher serological IgG titers, and BAFF gene expression. Zhao *et al*[19] also found that DNA hypomethylation of *IFI44L* is not directly induced by type I IFN.[19] So, we think the DNA methylation in IFN-related genes may be an effective tool for the diagnosis of pSS in the peripheral blood. In addition, our data confirmed the importance of DNA methylation on the IFN signaling pathway and assumed that such hypomethylated trend could cause enhanced expression and abnormal activation of the correlated genes, which might give rise to the activated type I IFN response in monocytes of pSS patients. Adaptive immune was considered as the hallmarks of pSS for a long time. However, in our study, we also emphasize the participation of monocytes, a part of the innate immune. A better knowledge of abnormal cell subsets could determine the detection of new therapeutic targets.

GO analysis elucidated that the biological functions of the annotated genes were mainly enriched in cell adhesion, antigen presentation, and IFN-related pathways. These findings confirmed that diverse abnormally activated monocyte functions were involved in the pathogenesis of pSS. Previous studies with mouse models have confirmed that antigen-presenting cells (APCs), including macrophages and DCs, could infiltrate the salivary glands of pSS patients and promote the development of pSS.[2] Abnormalities in antigen presentation related pathways might be related to pSS autoantibody production. While the function of antigen presentation in pSS, and the role of monocytes as APCs remains to be clarified. KEGG analysis showed that genes enriched in the metabolic-related pathway, especially patients with higher serum IgG levels, including pyruvate metabolism pathway and tyrosine metabolism pathway. Metabolic reprogramming supports cell activation and promotes metabolic pathways to match the needs of specific cell functions. Monocytes/macrophages are capable of reprogramming their metabolism to acquire pro-inflammatory M1 or anti-inflammatory M2 phenotypes.[22] Therefore, the changes in methylation patterns of metabolism-related genes may influence IgG production by affecting monocyte differentiation. However, the exact roles of metabolic abnormalities in monocytes and IgG production in SS are not clear. In addition, the Notch signaling pathway was enriched in patients with elevated serum IgG. Notch-related genes were reported to be highly expressed in CD14⁺ monocytes in rheumatoid arthritis and the hyperactivity of the Notch signaling pathway in monocytes could augment the macrophage differentiation and promote cytokine production.[23,24] In Murphy Roths Large/lymphoproliferation (MRL/lpr) mice, inhibiting the Notch pathway effectively reduces the serum total IgG level and autoimmune markers.[25] Abnormally activation of Notch genes like *DTX3L via* hypomethylation might have a similar role in the pathogenesis of pSS, especially pSS with elevated serum IgG levels; this still needs to be investigated experimentally. However, when we evaluated the disease activity of the patients in our cohort, we did not find any significant correlation between ESSDAI and DNA methylation (data not shown). The presence of anti-SSB antibody has been associated with recurrent parotid gland enlargement and a higher proportion of extra-glandular manifestations and lymphoma. However, little is known about the functional characteristics and mechanisms of anti-SSB antibody specific monocytes in pSS patients. In our study, the comparison of patients with DP and SP showed that the former had more DNA methylation sites, which means that the signaling pathways involved in DP patients are more extensive and revealed that immune response and inflammatory response were involved in the production of autoantibodies based on the functional analysis. However, more research is needed to clarify their possible role in pSS pathogenesis, especially for a different type of pSS.

Furthermore, we found the overlap in DNA methylation patterns between monocytes and SGECs, suggesting misregulation of similar pathways between these two cellular subsets. As represented in Figure 3A and Supplementary Table 3, http://links.lww.com/CM9/A517, 20 differentially

methylated genes enriched in the cell cycle, cell senescence, IL-17 signaling pathway, and so on.

We conclude that our results identified aberrant methylation of monocytes for the first time from pSS patients. These data indicate that abnormal DNA methylation exists in pSS monocytes and emphasized the potential role of DNA methylation changes in the pathogenesis of pSS and indicated differential methylation of IFN-related genes, and the genes involved in the Notch signaling pathway and the antigen processing/presentation pathways, in addition to other key genes and pathways involved in the pathogenesis of pSS. Future studies to replicate and determine the functional consequences of the observed methylation changes on pSS pathophysiology should be warranted.

Conflicts of interest

None.

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