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ORIGINAL RESEARCH

Identification of Epigenetic Alteration of the IFI44L Gene in B Cells of Sjogren's Syndrome as a Clinical Biomarker and Molecular Significance

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Objective: Type 1 interferon (IFN-I)-related genes play a critical role in Sjögren's syndrome (SS). However, study on its role and impact in peripheral blood B cells of SS is limited. This study investigated gene expression and epigenetic changes in IFI44L, analyzing its correlation with disease activity and clinical indicators.

Methods: Differentially expressed genes (DEGs) were identified from the GSE199868 dataset, while IFN-I-related genes were collected from GeneCards. Intersection analysis revealed IFN-I-related DEGs in SS. ClueGO, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, PPI network construction, and hub gene identification were conducted, with hub genes validated in GSE135809. Following this, we recruited 30 SS patients as the disease group and 11 healthy individuals as the control group. Clinical information and peripheral blood samples were collected from all participants. After isolating B cells from the peripheral blood, we used quantitative real-time polymerase chain reaction (RT-qPCR) and pyrosequencing to examine the mRNA expression and DNA methylation status of the IFI44L gene. Further analyses were conducted in conjunction with clinical indicators.

Results: GSE199868 analysis revealed 125 upregulated and 16 downregulated DEGs. Among 2794 IFN-I-related genes from GeneCards, 26 DEGs overlapped, enriched in viral genome replication and IFN-I pathways. 14 hub genes were identified, with 7 genes confirmed in GSE135809: ISG15, IFIT1, OASL, IFI6, RSAD2, IFI44L, and USP18. IFI44L mRNA expression was significantly higher in SS B cells ($P < 0.05$), while its DNA methylation status was significantly lower ($P < 0.05$). IFI44L expression correlated positively with ESSDAI, ESSPRI, and IgG levels, while methylation correlated negatively with ESSDAI and ESSPRI ($P < 0.05$). ROC analysis revealed that the IFI44L gene had high diagnostic value (AUC = 0.8515).

Conclusion: The study highlights the clinical relevance of IFI44L mRNA expression and DNA hypomethylation in SS, underscoring its potential as a biomarker for disease activity and progression.

Keywords: Sjögren's syndrome, IFI44L, DNA methylation, B cell, bioinformatics

Introduction

In 1933, Henrik Sjögren described a disease marked by dysfunction of salivary and lacrimal glands, leading to xerostomia (dry mouth) and xerophthalmia (dry eyes). This condition, later named Sjögren's syndrome (SS), is now recognized as a systemic autoimmune disease with characteristic lymphocytic infiltration of exocrine glands. While the primary symptoms include dry eyes and mouth, SS can also affect vital organs such as the kidneys, liver, and lungs.[1] The systemic manifestations of SS are diverse, ranging from fatigue and joint pain to severe complications like interstitial lung disease, renal tubular acidosis, and neuropathies. SS often overlaps with other autoimmune disorders, such as

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primary biliary cholangitis, sharing features like exocrine gland involvement and autoantibody seropositivity, including anti-SSA and anti-SSB antibodies.[2] This clinical complexity underscores the need for novel biomarkers to improve diagnosis and disease stratification.

Hypergammaglobulinemia, particularly elevated IgG levels, is a hallmark of SS, highlighting the crucial role of B cells in its pathogenesis.[3] The development and progression of SS are closely associated with the production of various autoantibodies by autoreactive B cells and plasma cells, including anti-nuclear antibody (ANA) as well as anti-SSA and anti-SSB antibodies.[4,5] Recent studies have uncovered additional roles of B cells in the pathogenesis of SS, such as cytokine production[6] and antigen presentation.[7] Related research has elaborated on the functional diversity of B cell subsets in both immune and autoimmune pathogenesis.[8] These findings collectively underscore the significance of B cell hyperactivation in the pathogenesis of SS. With rapid advancements in bioinformatics and microarray sequencing technologies, numerous biomarkers with specific expression patterns resulting from DNA methylation alterations have been discovered. Epigenetic changes are crucial in the pathogenesis of autoimmune diseases, offering valuable insights into the mechanisms and pathophysiology of SS. Studies indicate that inflammation in the salivary glands of SS patients is regulated by DNA methyltransferases and results from dynamic changes in DNA methylation.[9] Genome-wide analyses have identified more pronounced differentially methylated sites in B cells from SS patients, particularly hypomethylation of genes related to the type I interferon (IFN-I) pathway.[10] This underscores the importance of investigating the DNA methylation status of IFN-I pathway-related factors in the B cells of SS patients.

Interferon-Induced Protein 44-Like (IFI44L) is a key stimulatory gene within the IFN-I pathway, and serves as a feedback regulator of IFN function.[11] Currently, IFI44L expression has been found to be upregulated in patients with SS and significantly increased following stimulation with IFN-α and IFN-β.[12] In systemic lupus erythematosus and rheumatoid arthritis, the methylation level of IFI44L has been identified as a potential diagnostic marker, correlating with clinical features and treatment outcomes.[13,14] Despite these findings, research on the methylation status of IFI44L in SS is limited, particularly in B cells, which play a central role in the disease. Investigating whether hypomethylation of IFI44L in SS B cells contributes to its overexpression and the subsequent activation of the IFN-I pathway is critical for understanding the disease's underlying mechanisms.

This study aims to comprehensively analyze the DNA methylation status of IFI44L in SS patients and its potential impact on gene expression and immune activation. Through mining publicly available Gene Expression Omnibus (GEO) datasets, conducting genome-wide DNA methylation profiling, and performing Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, we sought to uncover epigenetic patterns associated with SS. Furthermore, experimental validation was carried out to confirm the findings, and clinical indicators were integrated to explore correlations between IFI44L methylation, gene expression, and disease activity. These insights may facilitate the identification of novel diagnostic markers and therapeutic targets for SS. Figure 1 provides an overview of the study design.

Methods

Exploration of Microarray Data and IFN-I-Related Genes

The microarray dataset GSE199868 was retrieved from the GEO (https://www.ncbi.nlm.nih.gov/). This dataset comprises human peripheral blood B lymphocytes from 14 patients with SS and 13 healthy individuals. IFN-I-related genes were downloaded from GeneCards (https://www.genecards.org/).

Acquisition of IFN-I-Related Differentially Expressed Genes (DEGs)

The transcriptome database was analyzed using the Easy Visualization and Inference Toolbox for Transcriptomic Analysis (eVITTA6)[15] for DEGs identification. A threshold of $P<0.05$ and $\log2|FC|\geq1$ was applied for screening DEGs in the GSE199868 dataset. To identify IFN-I-related DEGs in SS, the online tool Venn (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to overlap the IFN-I-related genes from GeneCards with the identified DEGs from GSE199868. Finally, the online tool Xiantao Academic (https://www.xiantaozi.com/) was utilized for visualizing the volcano plot of the identified IFN-I-related DEGs in SS.

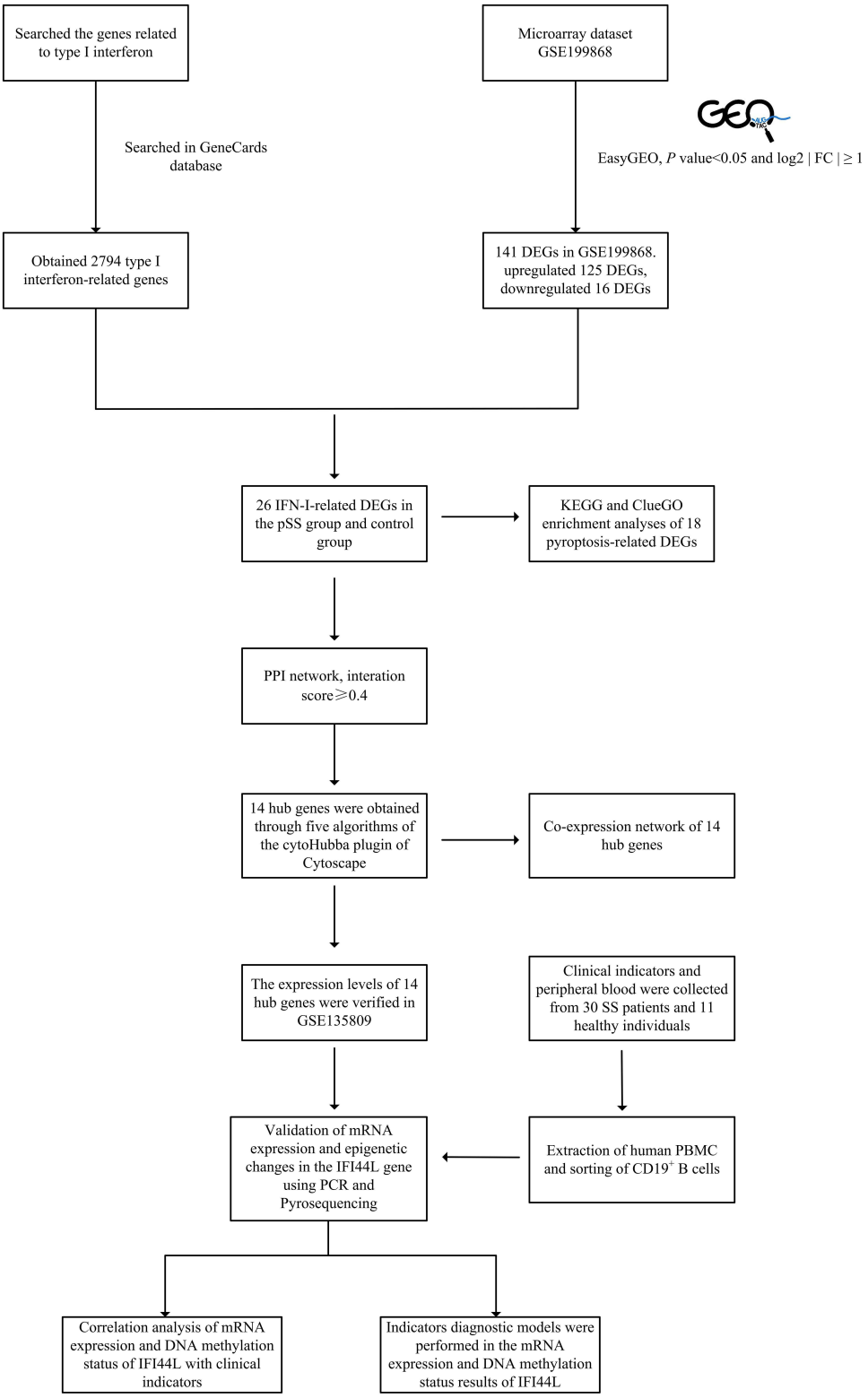

Figure 1 Flowchart of the study.

Protein-Protein Interaction (PPI) and Enrichment Analysis

PPI analysis of IFN-I-related DEGs in SS was conducted using STRING (http://www.string-db.org).[16] The results were imported into Cytoscape (http://www.cytoscape.org)[17] for visualization. GO enrichment analysis was performed using ClueGO, while KEGG enrichment analysis was conducted through the DAVID database (https://david.ncifcrf.gov/).

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A free online platform Wei Sheng Xin (https://www.bioinformatics.com.cn) was utilized for visualizing the results. $P<0.05$ was considered statistically significant.

Analysis of Hub Genes

Hub genes were identified using the CytoHubba plugin in Cytoscape. The top 15 hub genes were determined using five algorithms: Maximum Clique Centrality (MCC), Maximum Neighborhood Component (MNC), Degree, Closeness, and Edge Percolated Component (EPC). The genes identified by these algorithms were intersected to obtain the final set of critical genes. Subsequently, a co-expression network of these hub genes was constructed using GeneMANIA (http://www.Genemania.org/).[18]

Validation of the Expression of Hub Genes

The mRNA expression of the identified hub genes was validated in the GSE135809 dataset. Comparisons between groups were performed using *t*-test. *P*-value<0.05 was considered significant.

Subject Information

A total of 30 patients diagnosed with SS were recruited from the Second Affiliated Hospital of Zhejiang Chinese Medical University between May 2023 and March 2024. Diagnoses were made according to the 2016 ACR/EULAR classification criteria for SS.[19] Additionally, 11 healthy controls were enrolled from individuals undergoing routine health examinations at the same hospital during the same period. The exclusion criteria for SS patients included: (1) individuals with severe primary diseases of the cardiovascular system, liver, kidneys, brain, or hematopoietic system, or with a history of mental illness; (2) individuals with other autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis, acute or chronic infectious diseases, or malignant tumors; and (3) pregnant women. This study was conducted in full compliance with clinical trial laws and regulations as outlined in the Helsinki Declaration. The use of patient information and peripheral blood samples was approved by the Ethics Committee of The Second Affiliated Hospital, Zhejiang Chinese Medical University (Ethics Approval No. 2023-KL-003-A01). All participants were fully informed of the content of the Informed Consent Form prior to the commencement of the study and voluntarily provided their consent for participation by signing the form.

Collection of Clinical Indicators

Clinical indicators were collected from 30 patients with SS, including the EULAR Sjögren's syndrome disease activity index (ESSDAI),[20] EULAR Sjögren's syndrome patient reported index (ESSPRI),[20] fatigue severity scale (FSS), erythrocyte sedimentation rate (ESR), ANA, anti-SSA antibody, anti-SSB antibody, and IgG levels.

Isolation of Human $CD19^+$ B Cells

A total of 2.5 mL of fresh EDTA-anticoagulated peripheral blood was collected. Lymphocyte separation medium was used to isolate peripheral blood mononuclear cells (PBMCs) from the blood sample. After isolation, the cells were resuspended in 1 mL of PBS containing 0.1% BSA. 10 μL of the suspension was pipetted onto a hemocytometer for cell counting, and the cell concentration was adjusted to 1×10^7 cells/mL under a microscope. Next, buffer 1 (BSA: PBS = 0.1g/100mL) and buffer 2 (FCS: 1640 = 1:99) were prepared. A $CD19^+$ B cell positive selection kit was utilized for magnetic bead separation. The magnetic beads were washed with buffer 1, and 25 μL of the bead mixture was added per 1 mL of the cell suspension (1×10^7 cells/mL) into a centrifuge tube. The tube was incubated at 4°C for 15 minutes. After incubation, the tube was placed in a magnetic field to remove the supernatant, and then washed with 1 mL of buffer 1 by pipetting 2–3 times before returning it to the magnetic field. The $CD19^+$ B cells bound to the magnetic beads were washed, and the cell pellet was resuspended in 100 μL of buffer 2, followed by the addition of 10 μL of release reagent and incubation at room temperature for 45 minutes. After incubation, the tube was placed back into the magnetic field, and the supernatant containing the released cells was transferred to a new centrifuge tube. An appropriate amount of buffer 2 was added for washing, and the sample was centrifuged at 1500 rpm for 5 minutes at 4°C to remove the release reagent. The resulting pellet contained the $CD19^+$ B cells, which were labelled appropriately and stored at −80°C for future use.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) Assay

For RNA extraction, total RNA was isolated from $CD19^+$ B cells (1×10^7 cells) using the Total RNA Purification Kit (Thermo Fisher, 15596026) according to the manufacturer's instructions. To obtain cDNA, reverse transcription was performed using PrimeScript RT Master Mix (TaKaRa, RR036A). For RT-qPCR, the CFX96 Real-Time PCR Detection System and SYBR Green Premix were applied, with GAPDH serving as the internal control to normalize gene expression levels. The specific PCR primers and genes examined are listed in Table 1. The PCR conditions included an initial denaturation at 95°C for 30 seconds, followed by 50 cycles of denaturation at 95°C for 10 seconds and annealing at 60°C for 30 seconds. Gene expression was normalized using the $2^{-\Delta\Delta Ct}$ method and expressed as the ratio of target gene to GAPDH.

Pyrosequencing

Genomic DNA was first extracted using a standard phenol/chloroform extraction followed by ethanol precipitation. Eight micrograms (8 μg) of DNA were added to 192 μL of 0.05 mm NaOH and incubated at 37°C for 30 minutes. The resulting solution was then divided equally into two portions. One portion was subjected to oxidation using 4 μL of KRuO4 (15 mm in 0.05 mm NaOH, Alpha Aesar, MA, USA) on ice for 1 hour, followed by purification using a MicroSpin S-400 hR column (Roche, CA, USA). Two micrograms (2 μg) of the purified DNA were denatured in 0.3 mm NaOH at 50°C for 20 minutes. The denatured DNA was diluted in 500 μL of freshly prepared 10 mm hydroquinone and 3 M sodium bisulfite, and incubated at 70°C for 3 hours to convert unmethylated cytosines to uracils. The second portion of DNA underwent a mock oxidation treatment without KRuO4, with all other procedures remaining the same. To identify the CpG islands within the gene promoter regions, gene information from PubMed and other bioinformatics tools were utilized. PCR primers and sequencing primers were designed using the PyroMark Assay Design software (v 2.0), with their sequences provided in Table 2. Target fragments were amplified using a 50 μL PCR reaction mixture containing components from the Qiagen PyroMark PCR kit (PCR Buffer 2.5 μL, dNTPs 2 μL, forward and reverse primers each 0.5 μL, Taq DNA polymerase 0.2 μL, and cDNA template 1 μL). Five microliters (5 μL) of the PCR products were subjected to 1.5% agarose gel electrophoresis to confirm the presence of clear bands of the expected size. Subsequently, 40 μL of PCR product was mixed with 36 μL of binding buffer and 4 μL of streptavidin-coated agarose beads. The beads were then transferred to a 96-well plate containing annealing buffer, heated to 80°C for 2 minutes for denaturation, and then cooled to room temperature. The methylation status of each CpG site was analyzed using the PyroMark Q48 sequencing instrument. The resulting pyrograms were processed using the PSQ48MA software (v 2.0.6) to covert the pyrograms into peak heights and calculate the methylation ratio (C/T) at each base position. Finally, the Pyro Q-CpG software integrated within the pyrosequencing instrument automatically analyzed the methylation status of each CpG site.

Table 1 PCR Primer Sequence

Gene	Primer Sequence
IFI44L	F: 5'- CTAGTGTTCATGGAGGTAGCAT-3'
	R:5'- TGGCTCTGTAGAACTTTCATGT-3'
GAPDH	F: AGATCCCTCCAAAATCAAGTGG
	R: GGCAGAGATGATGACCCTTTT

Table 2 Sequence Information of Pyrophosphate Sequencing Primer

Primer name	Sequence (5'to3')	5' Modification
2.cg17980508-F (77bp)	ATTTTAAGAAATAGGGATAGTTATAGTGTT	5'-Biotin
2.cg17980508-R (Q48)	ACTACATACCTTTTCCTAATTTCTCC	
2.cg17980508-S	CTTTTCCTAATTTCTCCTCAA	

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Construction of the Receiver Operating Characteristic (ROC) Model

ROC curves were constructed to evaluate the predictive values of the mRNA and DNA methylation expression of IFI44L for SS by comparing the two groups. The ROC curves were numerically integrated to determine the plot area under the curve (AUC).

Statistical Analysis

Numerical results were presented as means ± standard deviations (SD). Differences between the two groups were analyzed using an unpaired *t*-test with Welch's correction. All count data were expressed in frequency (percentage) and analyzed using the Chi-square test or the Fisher's exact test. All analyses were performed using GraphPad Prism (v 8.0.2) and the statistical software SPSS (v 22.0). $P<0.05$ was considered statistically significant.

Results

Identification of IFN-I-Related DEGs in SS

Based on the established criteria, we screened out 125 upregulated and 16 downregulated DEGs from the GSE199868 dataset (Figure 2A). Additionally, we identified 2,794 genes with an IFN-I correlation score exceeding 10. By intersecting the DEGs with the IFN-I-related genes, we obtained a total of 26 IFN-I-related DEGs (Figure 2B).

PPI Analysis and Enrichment Analysis

The PPI analysis revealed that genes, including ISG15, CDK1, IFI44L, IFIT1, RSAD2, and IFI6 among the IFN-I-related DEGs, demonstrated strong interaction patterns, with their colors ranging from yellow to red, indicating an increasing Degree value (Figure 3A). The ClueGO results showed significant enrichment of these 26 IFN-I-related DEGs in signaling pathways such as viral genome replication and cellular response to IFN-I (Figure 3B). Additionally, the KEGG enrichment analysis indicated that these IFN-I-related DEGs were enriched in pathways related to asthma, as well as Th1, Th2, and Th17 cell differentiation (Figure 3C).

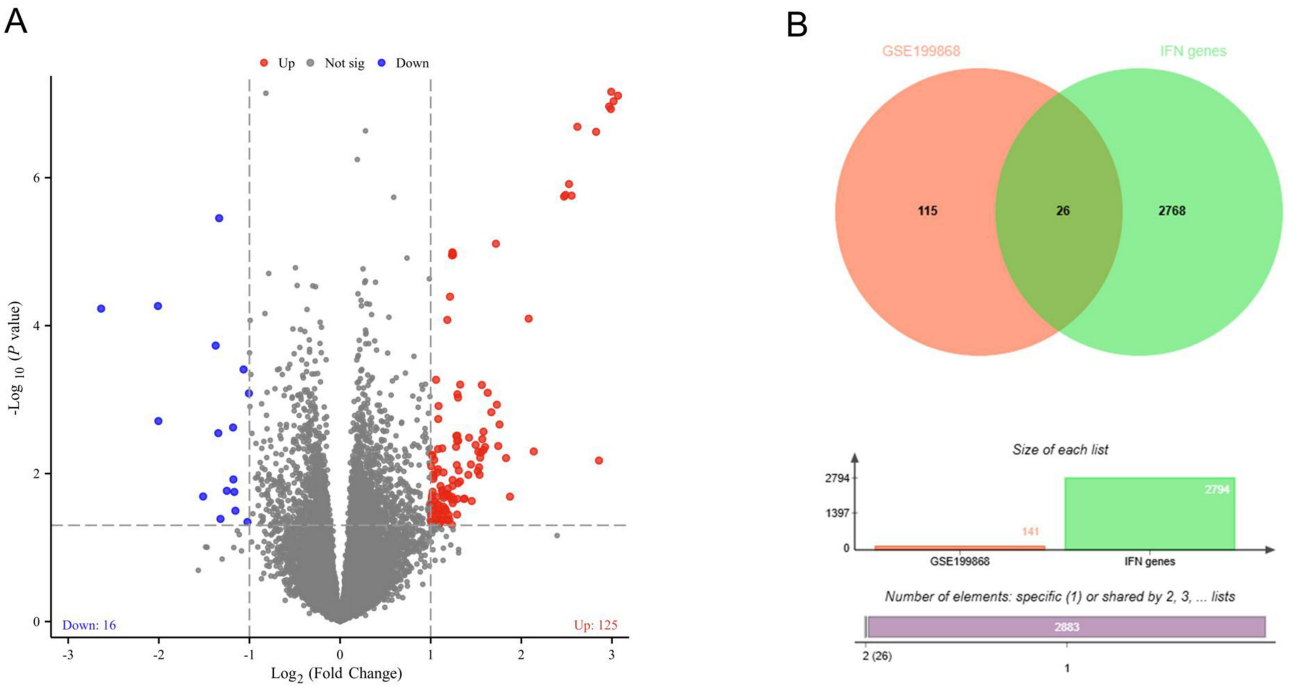

Figure 2 Identification of IFN-I-related DEGs. (**A**) Volcano map of GSE199868. (**B**) Venn diagram showing the intersection between IFN-I-related genes and DEGs.

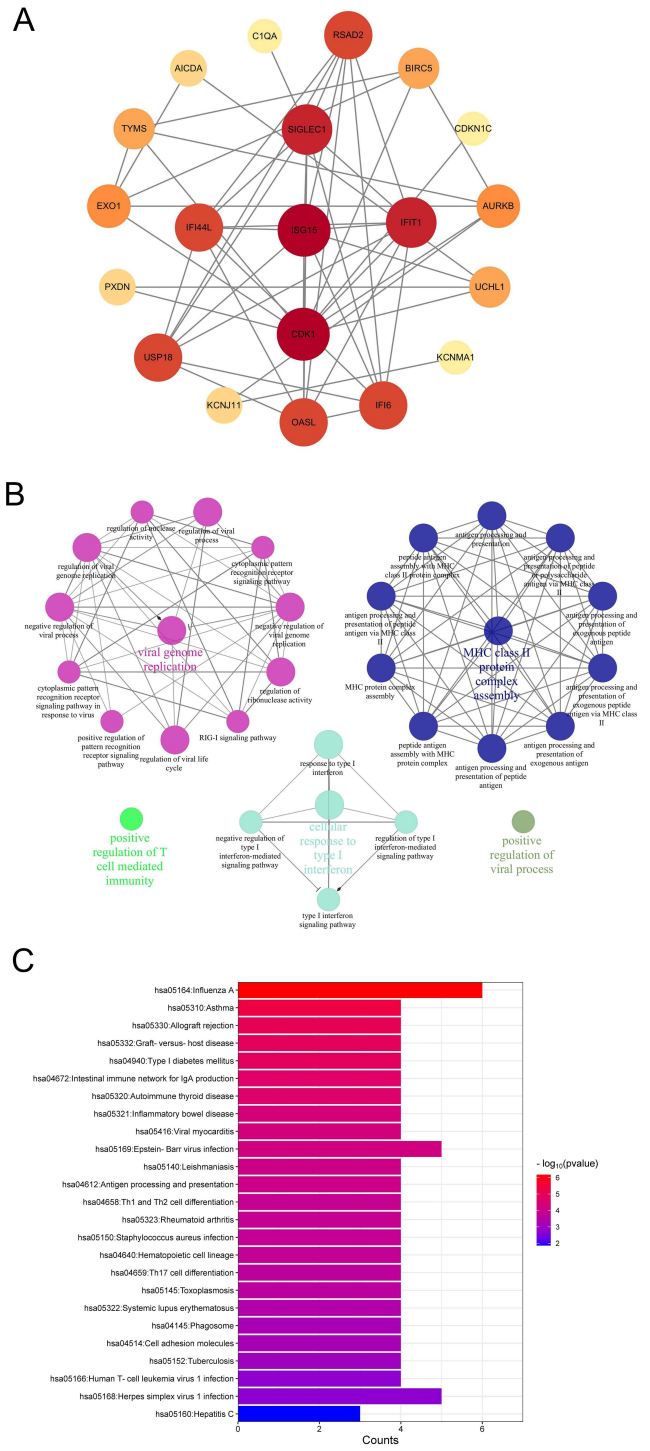

Figure 3 Biological functions of IFN-I-related DEGs. (**A**) PPI network of IFN-I-related DEGs, with a gradual change in color from yellow to red indicating the increasing degree values. (**B**) ClueGO enrichment analysis of IFN-I-related DEGs. (**C**) KEGG enrichment analysis of IFN-I-related DEGs.

Hub Gene Selection and Analysis

To further identify hub genes among the IFN-I-related genes in SS, we employed five algorithms: MCC, MNC, Degree, Closeness, and EPC, to select the top 15 hub genes. By intersecting the results from these five algorithms, we ultimately identified 14 IFN-I-related hub genes: ISG15, IFIT1, SIGLEC1, OASL, IFI6, RSAD2, IFI44L, USP18, CDK1, AURKB, EXO1, BIRC5, TYMS, and UCHL1 (Figure 4A and Table 3). Then, we constructed a gene interaction network centered on

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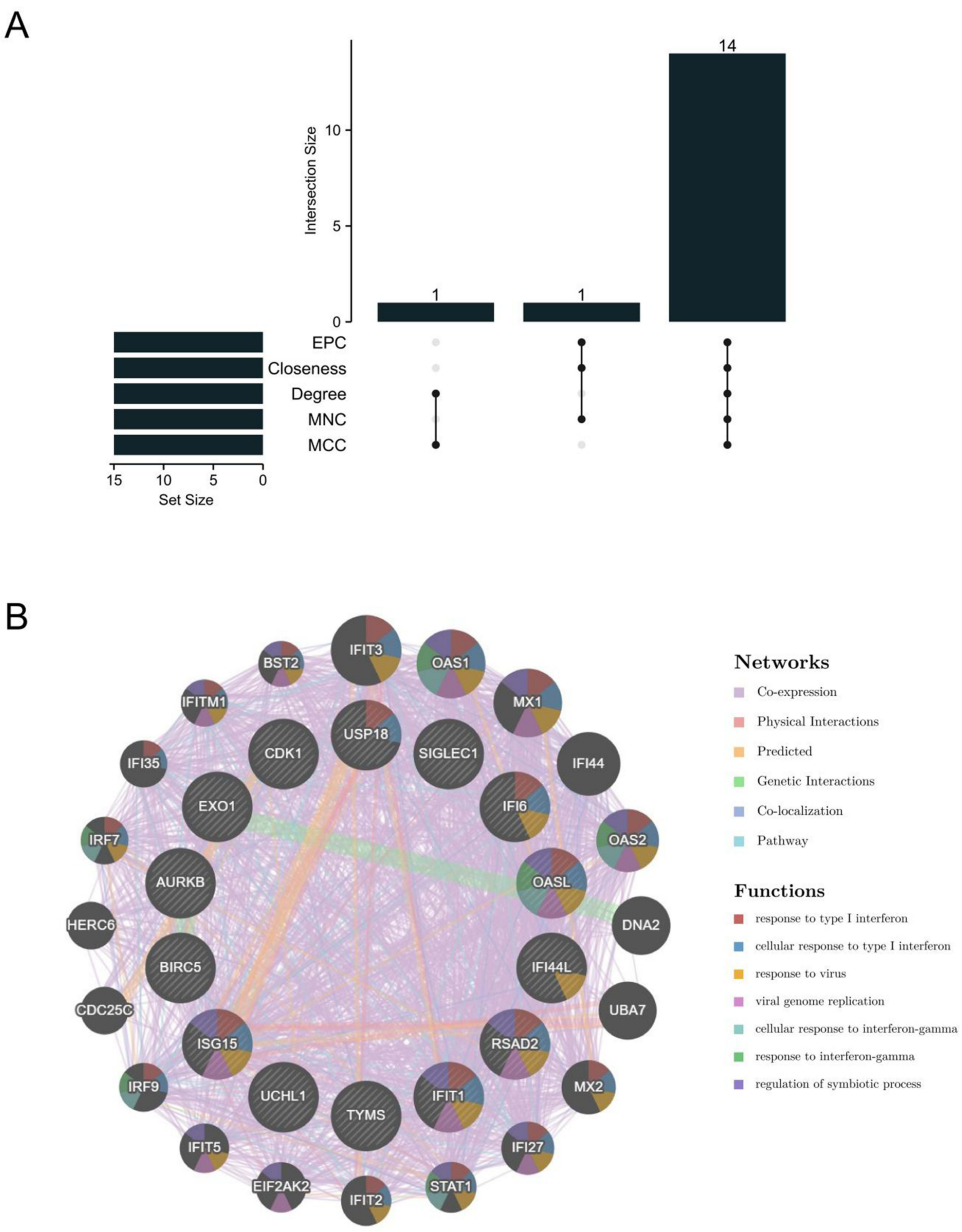

Figure 4 Co-expression network of IFN-I-related hub genes. (**A**) Upset diagram showing the 14 overlapping hub genes identified by five PPI algorithms. (**B**) Interaction network of the 14 hub genes demonstrated through GeneMANIA.

these 14 hub genes. The analysis indicated that IFIT1, IFI44L, OASL, and IFI6 exhibited strong interactions, with functions related to response to IFN-I, cellular response to IFN-I, response to virus, and viral genome replication (Figure 4B).

Validation of the Expression of Hub Genes

To further validate the differential expression of hub genes between groups, we performed inter-group gene expression analysis using the GSE135809 validation set (Figure 5). While SIGLEC1, CDK1, AURKB, EXO1, BIRC5, TYMS, and UCHL1 showed no significant differences between the groups ($P>0.05$), ISG15, IFIT1, OASL, IFI6, RSAD2, IFI44L, and USP18 were significantly overexpressed in the SS group ($P<0.05$).

Table 3 The Top 15 Hub Genes Rank in CytoHubba

MCC	MNC	Degree	Closeness	EPC
ISG15	ISG15	CDK1	CDK1	ISG15
IFIT1	IFIT1	ISG15	ISG15	IFIT1
SIGLEC1	OASL	IFIT1	IFIT1	IFI44L
OASL	IFI6	SIGLEC1	SIGLEC1	USP18
IFI6	RSAD2	OASL	OASL	RSAD2
RSAD2	SIGLEC1	IFI6	IFI6	SIGLEC1
IFI44L	IFI44L	RSAD2	RSAD2	IFI6
USP18	USP18	IFI44L	IFI44L	OASL
CDK1	CDK1	USP18	USP18	CDK1
AURKB	AURKB	AURKB	UCHL1	EXO1
EXO1	BIRC5	EXO1	EXO1	TYMS
BIRC5	TYMS	UCHL1	AURKB	UCHL1
TYMS	EXO1	BIRC5	BIRC5	BIRC5
UCHL1	UCHL1	TYMS	TYMS	AURKB
AICDA	PXDN	AICDA	PXDN	PXDN

Baseline Comparison Between Groups

Baseline information, including clinical information, disease-related scales, and laboratory indicators, was collected from 30 patients and 11 healthy individuals. Detailed data for each item are presented in Table 4. Comprehensive patient data can be found in Supplementary Table 1.

PCR and Pyrosequencing Results

Based on the analytical results and literature review, the IFI44L gene was ultimately selected for validation among the identified hub genes. $CD19^+$ B cells were extracted from 30 patients with SS and 11 healthy individuals. Through verification by RT-qPCR, a significant increase was revealed in the mRNA expression of IFI44L in $CD19^+$ B cells from SS patients compared to healthy individuals (Figure 6A). Additionally, by subjecting $CD19^+$ B cells to pyrosequencing, a significant decrease was observed in the DNA methylation status in $CD19^+$ B cells from SS patients relative to healthy individuals (Figure 6B). The mRNA expression of the IFI44L gene exhibited a significant negative correlation with its DNA methylation status ($P<0.05$), suggesting the regulation of mRNA expressionthe of IFI44L by its the DNA methylation status (Figure 6C).

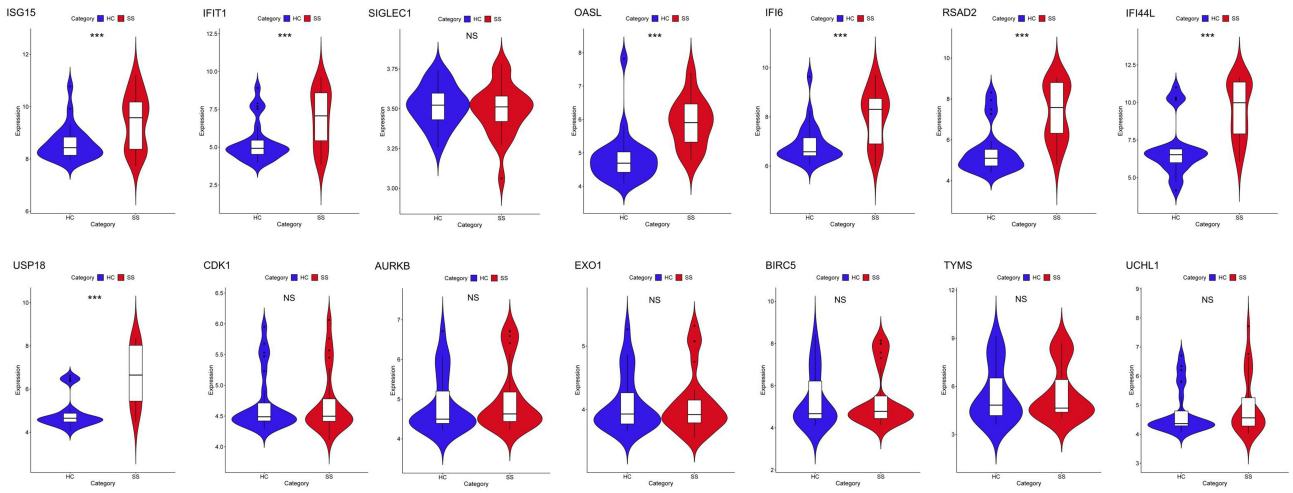

Figure 5 The expression of hub genes in GSE135809. ***$P < 0.001$ indicates a statistically significant difference, while NS (not significant) represents $P > 0.05$, indicating no statistical significance.

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Table 4 Patients Basis Information

Type	SS	HC	*P*-value
Age (years)	51.40±12.02	42.55±18.92	0.083
Gender (Female, %)	30(100.00%)	10(90.9%)	0.112
FS+ (%)	30(100.00%)	NA	NA
ANA + (%)	30(100.00%)	NA	NA
Anti-SSA+ (%)	23(76.67%)	NA	NA
Anti-Ro52+ (%)	17(56.67%)	NA	NA
Anti-SSB+ (%)	9(30.00%)	NA	NA
ESSDAI	5.47±3.76	NA	NA
ESSPRI	4.48±1.93	NA	NA
FSS	26.70±18.24	NA	NA
ESR	28.87±16.60	NA	NA
CRP	2.36±2.63	1.09±1.02	0.160
IgG	16.18±3.74	NA	NA

Note: NA indicates not applicable.
Abbreviations: FS+, Focus Score ≥1; ANA+, Anti Nuclear Antibody Positive.

Association Between Epigenetic Regulation of IFI44L and SS Disease Scores and Clinical Indicators

The mRNA expression of IFI44L was significantly positively correlated with ESSDAI, ESSPRI, and IgG levels ($P<0.05$), but did not correlate significantly with ESR and FSS ($P>0.05$) (Figure 7A–E). Conversely, the DNA methylation status of IFI44L was negatively correlated with ESSDAI and ESSPRI ($P<0.05$), with no significant correlations observed with ESR, FSS, or IgG levels ($P>0.05$) (Figure 7F–J).

Combined ROC Analysis

ROC analysis was conducted to evaluate the sensitivity and specificity of mRNA expression and DNA methylation status of the IFI44L gene as potential diagnostic biomarkers for SS. The AUC for mRNA expression was 0.8333 ($P=0.0012$) (Figure 8A), while the AUC for DNA methylation was 0.7939 ($P=0.0043$) (Figure 8B). To enhance diagnostic accuracy, we integrated the mRNA expression and DNA methylation status of IFI44L to develop a multi-marker diagnostic model through ROC regression analysis. This model could effectively predict the diagnosis of SS (AUC=0.8515, $P=0.0006$) (Figure 8C).

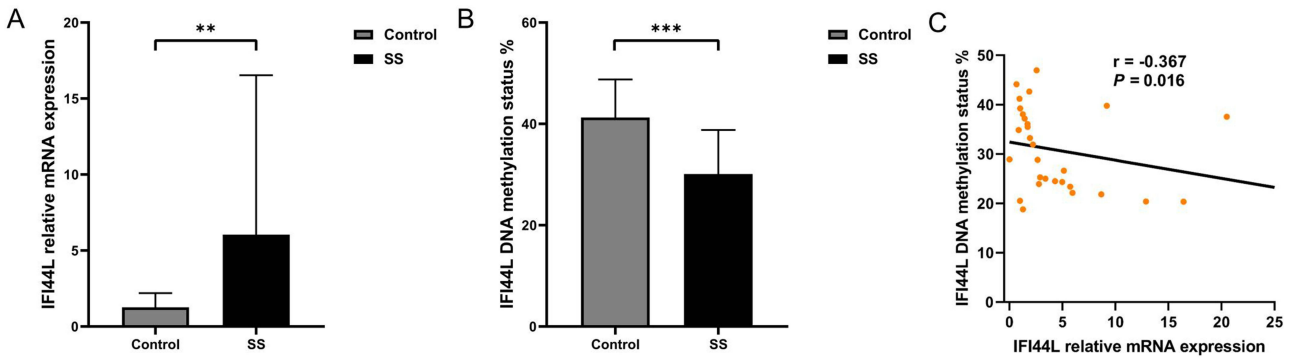

Figure 6 PCR and pyrosequencing analysis of the IFI44L gene in B cells from 30 SS patients and 11 healthy individuals. (**A**) mRNA expression levels of IFI44L in both groups **$P<0.01$. (**B**) DNA methylation status of IFI44L in both groups ***$P< 0.001$. (**C**) Correlation analysis of DNA methylation status and mRNA expression of IFI44L.

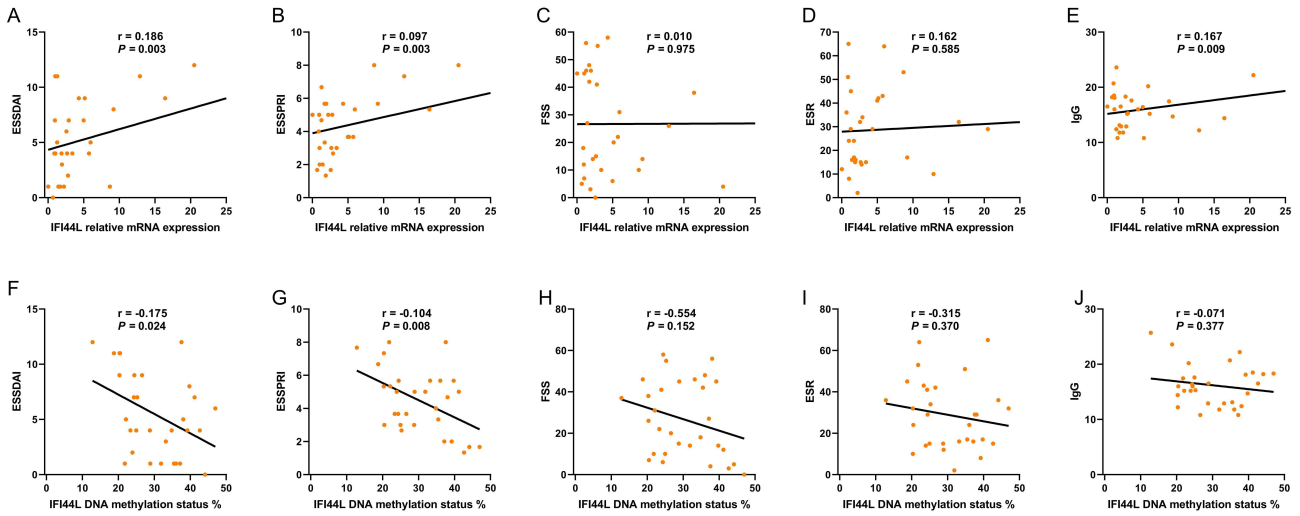

Figure 7 Correlation of IFI44L expression levels with Disease Assessment Scale scores and laboratory indicators. (**A**) ESSDAI and mRNA expression. (**B**) ESSPRI and mRNA expression. (**C**) FSS and mRNA expression. (**D**) ESR and mRNA expression. (**E**) IgG and mRNA expression. (**F**) ESSDAI and DNA methylation status. (**G**) ESSPRI and DNA methylation status. (**H**) FSS and DNA methylation status. (**I**) ESR and DNA methylation status. (**J**) IgG and DNA methylation status.

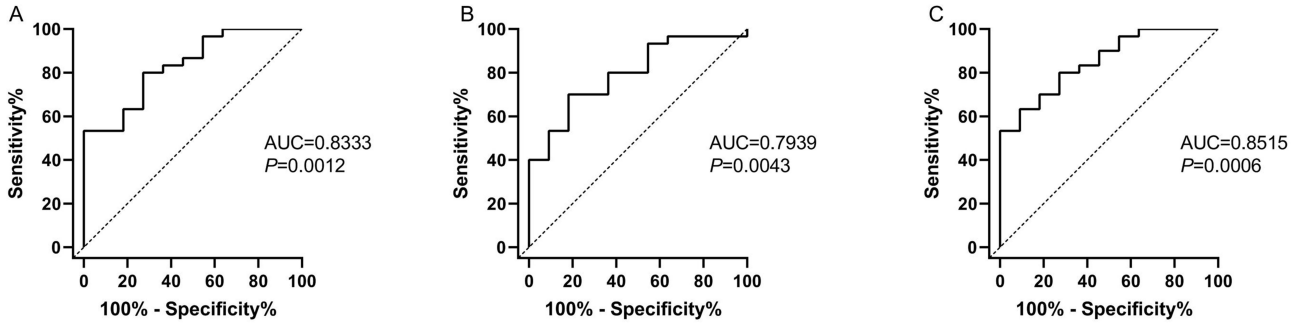

Figure 8 ROC curves analysis of PCR and pyrosequencing results in SS patients. (**A**) ROC analysis of mRNA expression levels. (**B**) ROC analysis of DNA methylation status. (**C**) Combined ROC curve analysis of mRNA expression levels and DNA methylation status.

Discussion

Over the past decade, IFN-I signaling has been recognized as a central contributor to SS, serving as a critical link between innate and adaptive immune responses in SS.[21] Clinical features of SS correlate strongly with the expression of IFN-I-related genes, and disease activity in SS is significantly increased in IFN-I-positive patients.[22,23] IFN-I mediates B-cell infiltration of lymphocytes into salivary glands, activates B cells to induce the expression of major histocompatibility complex (MHC) and costimulatory molecules on salivary gland epithelial cells, and stimulates the production of autoantibodies, thereby promoting the apoptosis of salivary gland epithelial cells and leading to the development of salivary gland inflammation.[24] Additionally, B-cell activating factor (BAFF), a key cytokine involved in the survival of circulating B cells, has been reported to show a significant correlation with higher levels of BAFF and BAFF mRNA expression in monocytes of SS patients and the presence of IFN-I scores. IFN-I induces the expression of BAFF through the IRF family, participating in B-cell activation.[25] Additionally, IFN-I induces the expression of chemokines such as C-X-C motif chemokine ligand 13 (CXCL13), recruiting C-X-C chemokine receptor type 5 $(CXCR5)^{+}CD19^{+}$ B cells to accumulate in affected glands.[26] A study on RNA-sequencing (RNA-seq) found that genes upregulated in B cells of SS patients are mostly related to the IFN-I pathway, including IFI44L, IFI44, interferon-induced gene-1 (IFIT1), IFITM1, IFIT3, IFN regulatory factor 7 (IRF7), interferon-stimulated gene 15 (ISG15).[27] Therefore, the overexpression of IFN-I signature genes in B cells is of great significance in the pathogenesis and progression of SS.

In recent years, alterations in DNA epigenetics have emerged as one of the key mechanisms for understanding how genetic predispositions and environmental variables interact to contribute to chronic autoimmune diseases. Notably, global

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hypomethylation of IFN-I-induced genes has been well-documented and confirmed in various immune cell types in patients with SS. The DNA methyltransferases (DNMTs)-mediated DNA methylation process in SS regulates B-cell activation-related genes, such as CD86, POU domain class 2 transcription factor 2 (POU2F2), nuclear factor of activated T cells c1 (NFATc1), and Nfatc2, thereby limiting B-cell activation and modulating plasma cell differentiation.[28] Additionally, hypomethylation of the CD70 promoter region has been observed in SS patients, which correlates with excessive B-cell activation.[29] It is evident that the DNA methylation is involved in the production of autoantibodies in SS by affecting B-cell differentiation and activation, ultimately leading to the destruction of lacrimal and salivary glands and contributing to the onset and progression of SS. Studies have identified methylation alterations of various immune genes in $CD4^+$ T cells, $CD19^+$ B cells, whole blood, and salivary gland tissues of patients with SS,[30–32] with these hypomethylation differences being more pronounced in B cells.[10,33] Genome-wide DNA methylation studies have revealed that IFN-I-regulated genes are hypomethylated in $CD19^+$ B cells of SS patients. Through evaluations[10,34] of the methylation status of $CD19^+$ B cells, the differential sites were found to be mainly IFN-I-related genes, such as myxovirus resistance 1 (MX1), IFI44L, IRFs, poly polymerase family member 9 (PARP9), and IFITM1, as compared to the controls. Notably, the number of these differentially methylated gene loci is higher in B cells than in T cells, and the differential sites correlate with disease activity and autoantibody positivity. A study conducted by IMGENBERG et al[34] indicated that genes related to the IFN-I pathway are hypomethylated in SS B cells and correlates with an increase in B-cell numbers. The methylation changes of IFN-I signature genes in B cells were positively correlated with disease activity, further emphasizing the central role of B cells and the relevance of DNA methylation alterations in the pathogenesis of SS. Thus, DNA methylation changes in IFN-I-related genes may serve as effective biomarkers for assessing disease activity and clinical prognosis in SS.

IFI44L is an IFN-I-stimulated gene that functions as a feedback regulator to modulate and influence the function of IFNs. A genome-wide DNA methylation analysis revealed that the DNA methylation status of the IFI44L gene is significantly lower in isolated monocytes from SS patients compared to healthy individuals.[31] In other autoimmune diseases, such as mixed connective tissue disease, genetic risk is increased due to DNA methylation changes in genes like IRF7 and IFI44, as well as in the HLA region.[35] These studies suggest that the high expression of IFI44L is regulated by its DNA methylation status and may play a critical role in the immune response mechanisms of the IFN-I pathway in SS. Despite numerous studies highlighting the role of the IFI44L gene in SS, the relationship between its mRNA expression and DNA methylation status in B cells has not been fully elucidated. To address this, we carried out bioinformatics analyses to identify IFI44L as a key IFN-I-related gene in B cells of SS patients and employed RT-qPCR and second-generation high-throughput pyrosequencing to detect the mRNA expression and DNA methylation status of IFI44L in B cells, respectively. The results revealed that the mRNA levels of IFI44L were significantly higher in the SS group compared to healthy individuals, while the DNA methylation status was significantly lower. Moreover, a significant negative correlation was observed between the mRNA expression and DNA methylation status of IFI44L, confirming hypomethylation of the IFI44L gene in B cells of SS patients. This hypomethylation affects the mRNA expression of IFI44L in B cells and, to some extent, indicates an overactivated state of the IFN-I system in SS patients.

By integrating the clinical data and laboratory indicators of 30 SS patients and conducting Pearson correlation analysis, we found that the mRNA expression of IFI44L is significantly positively correlated with ESSDAI, ESSPRI, and IgG levels, while the DNA methylation status of IFI44L is significantly negatively correlated with ESSDAI and ESSPRI. These findings suggest that the mRNA expression regulated by the DNA methylation status of the IFI44L gene can partially reflect the disease activity and subjective symptoms of SS patients. Thus, assessing the disease activity of SS through the DNA methylation status of IFN-I signature genes may emerge as a more precise diagnostic method. However, our study did not find significant correlations between the methylation status of IFI44L and IgG, ESR, or FSS. This may be attributed to the presence of some SS patients with normal and relatively low ESR and IgG levels, who exhibit overall low disease activity, minimal organ damage, and low fatigue scores, while a small subset of patients display higher DNA methylation status. To enhance the robustness of our findings, future studies should consider expanding the sample size to include more SS patients with moderate to high disease activity and more pronounced fatigue symptoms. Additionally, increasing the number of experimental validations of IFN-I signature genes could help more accurately characterize the overall DNA methylation expression profile of IFN-I-related genes.

Conclusion

In conclusion, this study establishes that the high expression of the IFI44L gene in SS is regulated by its DNA methylation status, and the expression status of IFI44L significantly correlates with disease activity scores and clinical indicators of SS patients. Thus, the DNA methylation status of the IFN-I signature gene IFI44L may serve as a valuable marker to assess the disease activity of SS, providing novel insights for the precise diagnosis and treatment of SS.

Data Sharing Statement

The datasets analysed in the current study were retrieved from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), with accession numbers: GSE199868 and GSE135809, and additional data are available from the corresponding author upon reasonable request. All methods were carried out in accordance with relevant guidelines and regulations.

Institutional Review Board Statement

This study was performed in accordance with the Declaration of Helsinki. Approval was obtained from the Institutional Ethics Committee of The Second Affiliated Hospital, Zhejiang Chinese Medical University (No. 2023-KL-003-A01).

Informed Consent Statement

Participants provided written consent to the storage of their samples for future research purposes.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests related to this work.

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