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RESEARCH ARTICLE

Aberrant DNA methylation of the promoters of JAK2 and SOCS3 in juvenile systemic lupus erythematosus

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ABSTRACT. Cytokine dysregulation is one of the important hallmarks of systemic lupus erythematosus (SLE) in both pediatric and adult patients. Owing to the substantial role of Janus kinase (JAK) and suppressor of cytokine signaling (SOCS) in cytokine signaling, we compared the methylation status of the promoter of JAK2 and SOCS3 between patients with JSLE and healthy controls. *Methods:* Peripheral blood samples were obtained from patients with JSLE and healthy controls. The promoter methylation was assessed by using the bisulfite conversion system and real-time quantitative multiplex methylation-specific PCR (QM-MSP). *Results:* The methylation assessments were performed on the blood samples of 25 patients with JSLE and 24 healthy controls. The promoter of JAK2 was significantly hypomethylated in patients with JSLE compared to healthy controls. The median relative unmethylation of the promoter of JAK2 was higher in the JSLE group compared to the control group [0.44 (0.32, 0.59) vs. 0.18 (0.12, 0.86), respectively; P-value 0.026]. The promoter of SOCS3 was significantly hypermethylated in patients with JSLE compared to the controls. The median relative unmethylation of the promoter of SOCS3 was lower in the JSLE group compared to the control group [0.52 (0.10, 1.41) vs 1.18 (0.39, 2.19), respectively ; P-value 0.032]. *Conclusion:* According to the results of our study, hypomethylation of the promoter of JAK2 and hypermethylation of the promoter of SOCS3 associate with JSLE. These alterations are possible mechanisms for activation of the JAK2 and suppression of the SOCS3 gene, respectively.

Key words: childhood-onset SLE, cytokine, JAK2, SOCS3, promoter, methylation, epigenetic, autoimmunity, signaling

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that leads to multi-organ damage due to the activation of autoreactive T cells and B cells and increased production of autoantibodies [1]. The manifestations and clinical course of SLE begin before the age of 18 in roughly 20% of patients and it is known as juvenile SLE (JSLE) [2]. JSLE has some similarities with adult-onset SLE; however, JSLE has been shown with more severity and mortality, which necessitate a special attention to its pathogenesis [3, 4].

Various cytokines and inflammatory mediators contribute to the autoimmunity in SLE [5]. Interleukin-6 (IL-6) is one of the most important mediators that have a role in the differentiation of autoreactive B cells and the production of autoantibodies [6]. IL-12 family members IL-12 and IL-23 contribute to pathogenesis of SLE through induction of a T helper 1 (Th1) response and activation of Th17/IL-17 axis, respectively [7, 8]. Interferon gamma (IFN-γ) contributes to pathogenesis of SLE by induction of Th1, chemokines, type I IFNs, and B lymphocyte stimulator (BLyS), which is a central player in B cell proliferation, differentiation, and survival [9-11]. IFN-α, a type I IFN family member, also has an essential role in etiology and pathogenesis of SLE. The development of a SLE-like disease upon the administration of IFN-α has been reported in some studies [12, 13]. IL-21 is another cytokine contributing to pathogenesis of SLE through the induction of autoreactive B cells, T follicular helper cell, and Th17/ IL-17 axis [14-17].

The cytokine dysregulation is caused by alterations in the expression level of cytokines [6, 16, 18-20] and their shared downstream pathways that simultaneously mediate the function of multiple cytokines [8, 21-24]. These cytokines bind to their receptors whose intracellular

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domains activate the non-receptor Janus kinase (JAK) and subsequently cause the phosphorylation of signal transducer and activator of transcription (STAT) family members. The phosphorylated STAT translocates into the nucleus to regulate transcription of genes through binding to DNA [25]. JAK2 is a member of Janus kinases family that connects to the receptors of several inflammatory cytokines, including IL-6, IL-12, and IFN-γ, and shows an increased expression in SLE [21, 22, 26, 27]. The activity of IFN-γ and other cytokines, such as IL-6, IL-21, CXCL2, and BLyS, is relayed through the JAK2/STAT signaling pathway [27]. The JAK-STAT signaling pathway is regulated through different mechanisms and members of the suppressor of cytokine signaling (SOCS) family are one of the most important modulators of this pathway [28]. SOCS3 is one of these proteins that could inhibit both JAK2 and cytokine receptors. This characteristic underlies its central role and specificity in inhibition of cytokine activity [29, 30]. Therefore, alterations of JAK2 and the regulator of cytokines, SOCS3, are of great importance in SLE.
Studies showed that both genetic and epigenetic alterations account for cytokine dysregulation [31-33]. Recently, assessment of epigenetic alterations, particularly DNA methylation of the genes responsible for autoimmunity, has gained more attention in adult-onset SLE [34-36]. Owing to the facts that cytokine dysregulation has also been observed in JSLE [37-39] and DNA methylation plays an important role in pathogenesis of JSLE [40, 41], we conducted this study to understand whether methylation status of the promoters of JAK2 and SOCS3 is altered in JSLE.

METHODS

Study Design

To evaluate the differences of DNA methylation of the promoters of JAK2 and SOCS3 between patients with JSLE and healthy controls, a case-control study was designed. Following designing the protocol and receiving an ethical approval from the National Institute for Medical Research Development (NIMAD), the study was conducted at the Research Center for Immunodeficiencies (RCID), Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran.

Patients and Healthy Participants

Patients under 18 years old who had a confirmed diagnosis of JSLE according to the Systemic Lupus International Collaborating Clinics (SLICC) criteria [42] and were referred to the rheumatology clinic of Children's Medical Center were enrolled. Healthy controls without any clinical manifestations of autoimmunity, inflammatory disease, or abnormal laboratory tests were also included. After taking informed consent, peripheral blood samples were obtained.

Genomic DNA Extraction

Extraction of the genomic DNA from the blood samples was performed by using the High Pure PCR Template Preparation Kit (Roche) based on the instructions provided by the company. The quality of the extracted genomic DNA was assessed by the measurement of optical density (260/280 ratio) and the DNA samples were kept at –20 °C.

Bisulfite Conversion

The bisulfite conversion of the extracted DNA was performed by using the BislFast DNA Modification Kit (TOYOBO, Co., Ltd., Osaka, Japan) based on the instructions provided by the company. Treatment with sodium bisulfite is the step in which the unmethylated cytosine residues are converted to uracil but the 5-methylcytosines (5mC) are not changed. During PCR amplification, the uracil residues are converted to thymine as well.

Methylation Evaluation

The methylation status of CpG islands around the promoters of JAK2 and SOCS3 was evaluated by application of the real-time quantitative multiplex methylation-specific PCR (QM-MSP) method [43] and a SYBR green dye-based DNA methylation assay.
During the process, two sequential steps of PCR, that is, conventional and real-time PCR, were necessary to perform the highly sensitive and specific MethySYBR procedure. In the first or multiplex step, a conventional PCR was performed to amplify the region of interest. The amplification was performed by designing the external primers. The external forward and reverse primers for the promoters of JAK2 and SCOS3 that were applied to the study are given in *table 1*. A volume of 25 μL consisting of 1 μL of converted genomic DNA along with 24 μL of other reaction materials was prepared to perform the conventional PCR. The reaction was undertaken at the following cycles and times: one cycle at 95°C for 5 min; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and finally the extension at 72°C for 5 min.
In the second or quantification step, the real-time PCR was performed on the specific methylated target obtained from the first step. Nested real-time PCR was performed by using the methylation-independent (external) and methylation-specific (internal) primer sets for the promoters of JAK2 and SCOS3 (*table 1*).

Table 1
Primers. The external (bisulfite-specific) primers were used for the conventional PCR and the external and internal (methylation-specific) primers were used for the nested real-time PCR.

Primer name	Primer sequence (5' → 3')
JAK2	EXF: TAAGGTGGTTGATGGGAGTTAG EXR: TAACTCACCCTAACTAACTCCCC INF: CGGGTTTGTTGTATTCGG INR: AAACCGAACTACCTCCGC
SOCS3	EXF: GTAGGGAGGTGACGAGGTAG EXR: ACAAAATAACCCCGAACAACC INF: GGAGATTTTAGGTTTTCGGA INR: CCCGAAACTACCTAAACGCC

EXF: external forward primer; EXR: external reverse primer; INF: internal forward primer; INR: internal reverse primer.

The UCSC database was utilized for defining the methylation profile of the CpG islands of promoters. The MethBlast tool was also utilized for prediction of CpG islands and primer blasting. In volume of 10 µL reaction well that contained 0.25 µL of each of the methylated primers, 1 µL of bisulfite-treated DNA, 5 µL SYBR® Green Master Mix, and 3.5 µL DDW, the real-time PCR amplification was performed. Cycling conditions were performed by using a 7500 real-time PCR system as follows: one cycle at 95°C for 1 min; 30 cycles at 94°C for 30 s, at 60°C for 1 min, at 72°C for 30 s; and finally the extension at 72°C for 5 min.

As a positive control, a fully converted methylated human DNA, that is, a 100% methylated reference, was applied in each run. It was utilized as a reference to calculate the relative unmethylation of DNA samples. No negative controls were included.

The $2^{-\Delta\Delta Ct}$ method, which has been previously described by other groups [44, 45], was utilized to calculate the ratio of unmethylated DNA. To use the method, cycle threshold (CT) values were obtained from the exponential phase of the product of bisulfite-specific primer (BSP) and methylation-specific primer (MSP). The formula of this method is as follows:

$$Unmethylated\ DNA\ level = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta C_{t\,sample} - \Delta C_{t\,reference}$$

$$\Delta C_{t\,sample} = C_{t\,MSP} - \Delta C_{t\,BSP}$$

$$\Delta C_{t\,reference} = C_{t\,MSP} - C_{t\,BSP}$$

Statistical analysis

The statistical analysis was undertaken by using the IBM SPSS Statistics V21.0 and GraphPad Prism V8. To compare the relative DNA unmethylation of the promoters between patients and controls, the Mann–Whitney *U* test was applied. To calculate odds ratio (OR), the Fisher exact test was used. The tests were two sided, with the significance level of 0.05.

RESULTS

Twenty-five patients with JSLE and 24 healthy controls were enrolled in the study. Demographic characteristics of the patients are summarized in *table 2*.

Table 2
Demographic characteristics of 25 patients with JSLE.

Characteristic	Value
Age	
Median	8
Range	5-16
Sex	
Male	7
Female	18
SLEDAI Score (available for 15 patients)	
Median	18
Range	13-29

SLEDAI: SLE Disease Activity Index.

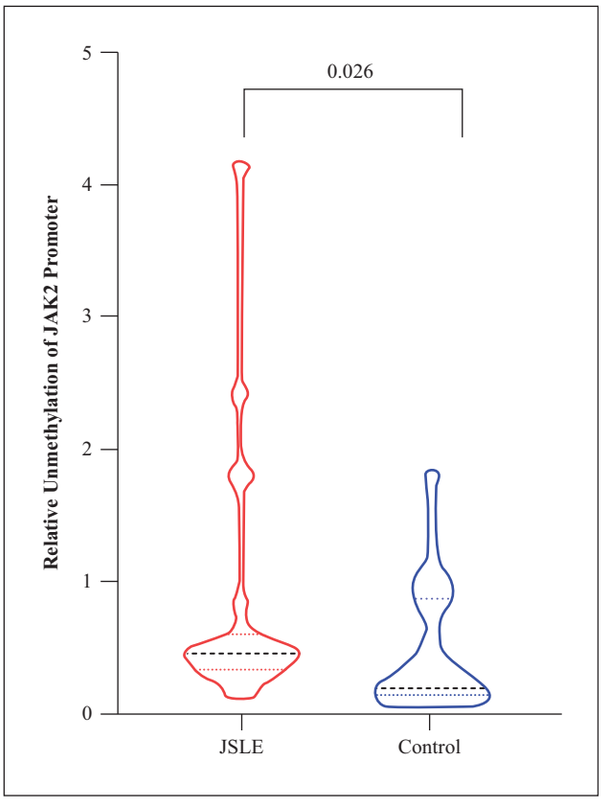

Figure 1
Relative unmethylation of the promoter of JAK2 in the peripheral blood samples of patients with JSLE and healthy controls. The graph shows increased median relative unmethylation for patients with JSLE, which indicates hypomethylation of the promoter of JAK2.

Methylation Status of the Promoter of JAK2

The methylation status of the promoter of JAK2 in the peripheral blood samples was compared and aberrant methylation was observed in patients with JSLE. The promoter of JAK2 in patients with JSLE was hypomethylated (*figure 1*). The median relative unmethylation of the promoter was significantly higher in the JSLE group compared to the control group [0.44 (0.32, 0.59) vs. 0.18 (0.12, 0.86), respectively; P-value 0.026]. *Table 3* compares the median relative unmethylation of the promoter between the groups. The risk of JSLE with JAK2 hypomethylation was higher, but it was not statistically significant [OR 3.13; 95% confidence interval (0.30, 32,47); P-value 0.32].

Methylation Status of the Promoter of SOCS3

The methylation status of the promoter of SOCS3 in the peripheral blood samples was compared and aberrant methylation was observed in patients with JSLE. The promoter of SOCS3 in patients with JSLE was hypermethylated (*figure 2*). The median relative unmethylation of the promoter was significantly lower

Table 3
Comparison of relative unmethylation of the JAK2 promoter in the peripheral blood samples of patients with JSLE and healthy controls.

Group	N	Min	Max	Mean± SD	Median (IQR)	P-value
JSLE	25	0.12	4.16	0.75±0.90	0.44 (0.32, 0.59)	0.026
Control	24	0.06	1.80	0.43±0.45	0.18 (0.12, 0.86)	

in the JSLE group compared to the control group [0.52 (0.10, 1.41) vs 1.18 (0.39, 2.19), respectively ; P-value 0.032]. *Table 4* compares the median relative unmethylation of the promoter between the groups.

DISCUSSION

Epigenetic modifications regulate the gene expression without any effect on the DNA sequence. DNA methylation is one of the central epigenetic modifications leading to gene silencing [46]. It is the result of the addition of a methyl group to the fifth carbon of cytosines located prior to a guanine nucleotide to prepare 5mC. Therefore, cytosine-phospho-guanosine dinucleotide (CpG)-rich regions or CpG islands are the main locations for DNA methylation [47]. Approximately 70% of promoters are located within the CpG islands [48] and DNA methyltransferases (Dnmts) cause promoter methylation and subsequent gene silencing [49], while inhibition of Dnmts and other mechanisms, such as activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC), cause demethylation of the promoter and subsequent gene expression [50, 51].

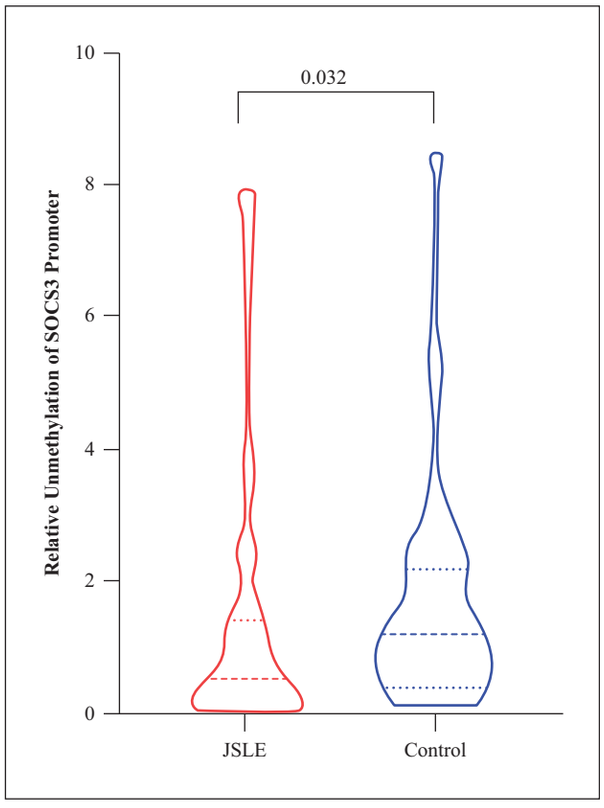

Figure 2
Relative unmethylation of the promoter of SOCS3 in the peripheral blood samples of patients with JSLE and healthy controls. The graph shows decreased median relative unmethylation for patients with JSLE, which indicates hypermethylation of the promoter of SOCS3. .

Adult-onset autoimmune diseases are caused by involvement of both genetic and epigenetic modifications [31, 52]. It has been reported that DNA methylation is one of the key players in pathogenesis of SLE. Medications such as 5-azacytidine, hydralazine, and procainamide that cause drug-induced SLE act through inhibition of Dnmt1 and development of DNA hypomethylation [53, 54]. In addition, decreased activity of the extracellular signal-regulated kinase (ERK) signaling pathway also blocks Dnmt1 and causes production of anti-double-stranded DNA (anti-dsDNA) antibodies [55]. The involvement of methylation-sensitive genes, namely, *CD70* and *CD11a*, encoding co-stimulatory molecules that enhance the acivation, as well as the autoreactive activity, of T cells and B cells, has been extensively assessed in pathogenesis of SLE. The hypomethylation of these genes and subsequent protein overexpression have been demonstrated in patients with idiopathic SLE [56, 57]. Additionally, *CD70* and *CD11a* are the key genes that showed hypomethylation upon the inhibition of Dnmt1 by blockade of the ERK signaling pathway and SLE inducing drugs [55-57]. However, other genes affected by alterations of DNA methylation have been identified; a comprehensive review article reported the hypomethylation of 17 genes and hypermethylation of five genes in adult patients with SLE [52].

JSLE is also affected by both genetic and epigenetic modifications [40, 58, 59]. Assessment of epigenetic modifications in JSLE has recently been considered to achieve an in-depth understanding of its pathogenesis. The hypomethylation of the long interspersed nuclear element-1 (LINE1), which is a retrotransposon, and the hypermethylation of FOXP3, responsible for maturation of regulatory T cells, in the peripheral blood mononuclear cells (PBMC) of patients with JSLE have already been reported [40, 41]. Given the essential role of CD70 in the pathogenesis of SLE, its methylation status has previously been assessed in JSLE as well. Although the methylation status of the CD70 promoter in PBMC of JSLE was not different from that of healthy participants in their PBMC, it might also be hypomethylated in isolated CD4+ T cells similar to adult patients [60]. Interestingly, aberrant DNA methylation was also confirmed in other juvenile autoimmune diseases. The hypomethylation of the *IL32* gene, encoding a pro-inflammatory cytokine, has also been observed in CD4+ T cells from patients with juvenile idiopathic arthritis [61].

Cytokines are important agents of the immune system and cytokine dysregulation is determinant in both adult-onset and childhood-onset SLE [39, 62]. The

Table 4
Comparison of relative unmethylation of the SOCS3 promoter in the peripheral blood samples of patients with JSLE and healthy controls.

Group	N	Min	Max	Mean± SD	Median (IQR)	P-value
JSLE	25	0.05	7.86	1.09±1.68	0.52 (0.10, 1.41)	0.032
Control	24	0.11	8.48	1.68±1.86	1.18 (0.39, 2.19)	

increased level of cytokines, including IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-21, IL-17, IFN-α, IFN-γ, and TNF-α, in SLE has been reported by several studies [16, 18, 63-67]. The cytokine signaling is dependent on the downstream molecules, that is, the JAK-STAT pathway. These components are also subject to alterations in autoimmune disorders, such as SLE. It has been shown that these molecules are involved in the regulation of T cell interferon related genes and the development of autoantibodies [68, 69], and an increased expression of JAK2 mRNA has been reported in SLE [21]. Inhibition of JAK2 and STAT3 has also led to decreased autoantibodies and pro-inflammatory cytokines and delayed or improved renal involvement in animal models of SLE [70-73]. The SOCS family members inhibit the JAK-STAT pathway in healthy individuals. However, a decreased expression of SOCS1 and SOCS3 [74] impairs their inhibitory function in SLE. It has been shown that the inhibition of SOCS3 causes subsequent activation of STAT3 and development of Th17 cells, which are important players in autoimmune diseases including SLE [75-77]. SOCS3 is the inhibitor of STAT3, the downstream of IL-6, IL-21, and IL-23, which are necessary for development of Th17 cells [78]. Histone methylation of SOCS3 promoter led to its decreased expression and development of IFN-γ producing Th17 cells and its overexpression led to inhibition of IL-17 production [79]. Therefore, JAK2 and SOCS3 are responsible for signaling of key cytokines and play essential roles in pathogenesis of SLE.

Owing to the presence of CpG islands in the promoter of JAK2 and SOCS3, these genes could be regulated via DNA methylation. The methylation status of these genes in various diseases, including cancer and coronary artery disease, has been investigated [80, 81]. However, despite their important role in pathogenesis of SLE, their methylation status has not been assessed previously.

In the current study, we have evaluated DNA methylation of the promoters of JAK2 and SOCS3 in the peripheral blood samples of patients with JSLE and healthy controls. Our results showed that hypomethylation of the promoter of JAK2 occurs in JSLE. This can be a possible mechanism for increased expression of JAK2 and subsequent increased cytokine signaling. Furthermore, we showed that the hypermethylation of the promoter of SOCS3 also associates with JSLE. This can be a possible mechanism for silencing the SOCS3 gene and blockade of its inhibitory function on the JAK-STAT pathway. In this manner, a hyperactivated cytokine signaling through the JAK-STAT pathway can be expected in JSLE. Altogether cytokines and JAK-STAT pathway plays an undeniable role in pathogenesis of JSLE and further studies are warranted to evaluate the correlation of methylation status and expression of JAK2/STAT3/SOCS3 to elucidate their role in JSLE.

Disclosure. *Ethical Standards:* The protocol of this case-control study was approved by the Ethics Committee of the National Institute for Medical Research Development (NIMAD). Written informed consents were obtained from all participants.

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