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Review Article

Methylation Status of SOCS1 and SOCS3 Genes in Patients with Acute Lymphoid Leukemia

Mahsima Shabani[1,2], Hanieh Mojtahedi[3], Maryam Sadr[3], Arezou Rezaei[2], Parivash Afradiasbagharani[3], Golshid Sanati[4], Zahra Aryan[2,5], Farzad Kompani[6], Nima Rezaei[2,4,5*]

[1] *International Hematology/Oncology of Pediatrics Experts (IHOPE), Universal Scientific Education and Research Network (USERN), Tehran, Iran*

[2] *Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran*

[4] *Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[5] *Network of Immunity in Infection, Malignancy and Autoimmunity (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran*

[6] *Division of Hematology and Oncology, Children's Medical Center, Pediatrics Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract

Background: Acute Lymphoid Leukemia (ALL) is the leading childhood cancer with a high mortality and morbidity. Studies have suggested an association of epigenetic transformations with prognosis, recurrence and immunophenotypes of ALL. SOCS1 and SOCS3 are tumor suppressors inhibiting JAK/STAT signaling pathway and the resultant aberrant cell proliferation.

Method: We aimed to assess the association between methylation status and ALL, using bone marrow and peripheral blood samples. 18 patients with ALL and 13 children with no malignancies were included. Using Bisulfite conversion, quantitative multiplex methylation-specific PCR and $2^{-\Delta\Delta Ct}$ formula, the methylated DNA in the promoters of SOCS1 and SOCS3 were measured.

Results: ALL patients had higher mean methylation in SOCS1 promoter and lower mean methylation in SOCS3 promoter, compared to the control group. However, neither of these mean differences were statistically significant.

Conclusion: This finding can set the foundation for further large-sample studies with the use of healthy children as a control group to strengthen the hypothetical association of the methylation status of SOCS1 and SOCS3 with ALL.

Keywords: ALL; Epigenetic; Leukemia; Methylation; SOCS

***Corresponding Author:** Nima Rezaei, MD, PhD
Research Center for Immunodeficiencies, Children's Medical Center Hospital, Dr Qarib St, Keshavarz Blvd, Tehran 14194, Iran. Tel: +9821-6692-9234, Fax: +9821-6692-9235
E-mail: rezaei_nima@tums.ac.ir

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Introduction

ALL is the most common type of pediatric neoplasm and the leading non-communicable disease-associated cause of mortality in children aged 5-14 years after congenital defects (1). ALL presents with clinical symptoms including anemia, thrombocytopenia, granulocytopenia, hepatomegaly, splenomegaly, and lymph adenopathy

(2). The leukemic state arises with uncontrolled and excessive proliferation of immature lymphoid precursors and replacement of normal hematopoietic cells of the bone marrow (BM) with malignant cells. Both of the main immunophenotypes; B-cell acute lymphoblastic leukemia (B-ALL) and T-cell acute lymphoblastic leukemia (T-ALL) include subtypes characterized by chromosomal alterations (3).

Beside the genetic modifications, epigenetic changes also have an essential role in the leukemogenesis and ALL pathogenesis via loss of plasticity and preservation of an unlimited self-renewal capacity (4). The epigenetic alternations consist of three main mechanisms; DNA methylation, histone modifications, and interaction with non-coding RNAs such as microRNAs (5, 6). DNA methylation as a central epigenetic modification at CpG-rich sites in promoter regions of genes also known as CpG islands has been associated with the prognosis, cytogenetic alterations, immunophenotype classifications, and relapse of ALL (6-8). Also, hypermethylation and hypomethylation can influence expression and long-term silencing of homeotic genes, regulation of cell cycle and proliferation (9, 10).

The suppressor of cytokine signaling (SOCS) family, including SOCS1 and SOCS3, are the most influential proteins in the malignancy development that induce negative regulation upon pro-inflammatory cytokines expression and activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways, which is associated with cell growth, differentiation, transformation and apoptosis (11, 12). However, the inhibitory effect of SOCS proteins to reduce STAT activation and cancer cell proliferation and survival is controversial and some evidence suggests that increased SOCS expression in cancer cells contribute to enhanced oncogenesis and cancer progression (13). The expression of SOCS1 and SOCS3 is downregulated by DNA hypermethylation leading to the expression of proinflammatory cytokines and antigen presentation of dendritic cells (14).

Given the importance of epigenetic alterations especially DNA methylation in the etiology of ALL, we aimed to assess methylation status of the promoter of SOCS1 and SOCS3 genes in the peripheral blood and bone marrow samples of patients with ALL.

Materials and methods

Sample collection and storage

A total of 18 samples were obtained from children of 1-15 years with definite pathologically and flow cytometry-assisted diagnosis of ALL in peripheral blood smear and bone marrow samples and 14 healthy individuals with no clinical history of malignancy were considered as healthy control group. Patients with suspected neoplasms and those who received prior chemo/radiotherapy were excluded from the study. 2cc blood sample were collected in EDTA tubes from each patient before induction treatment and stored at -20° C.

Ethical considerations

The research protocol of this case-control study was approved by the ethical Committee of Tehran University of Medical Sciences, Tehran, Iran, and informed written consents were obtained from the parents or legal guardians of all participants.

DNA isolation

The DNA was isolated from blood samples by the use of the Phenol: Chloroform method to extract the amount of DNA required for bisulfite treatment, and the purity and yield of DNA were determined using a NanoDrop spectrophotometer. The bone marrow samples were transferred into a 15 ml falcon tube and centrifuged for 20 min at 3000 rpm to separate the buffy coat layer. Then, 5 ml of RBC lysis buffer (Sucrose-1M Tris-HCl pH-7.6, 1M $MgCl_2$, Triton X-100 (Merck, Germany)) were added to the separated buffy coat and this step was redone until a clear WBC pellet was obtained. Next, 600 µl of WBC lysis buffer (1M Tris-HCl (pH-8.2), 0.5M Na_2-EDTA (Merck, Germany)) and 800 µl of 20% detergent Sodium Dodecyl Sulfate (SDS) and 200 µl proteinase-K (20mg/µl) (Merck, Germany) was added and incubated for 24 hours at 37°C. Following incubation, the supernatant was mixed with an equal volume of phenol in Tris-HCl 0.1 M and centrifuged at 3000 rpm for 5 min at 4°C. Next, a phenol/chloroform mixture was added to separate the proteins from the DNA and then centrifuged to separate double-stranded DNA molecules in the aqueous phase from the unwanted proteins and cellular debris. DNA was precipitated by the addition of double the volume of the supernatant of chilled 4M sodium acetate (Merck, Germany) and chilled absolute alcohol (100%) (Merck, Germany) and washed with chilled alcohol 100% twice to remove contaminants. The DNA was precipitated and transferred into 1.5 ml fresh tube and the pellet was air dried at 55° C for 10 min. The DNA precipitant was re-suspended in 150 µl of sterile water. Samples were labeled and stored in -20° C for further molecular studies.

Bisulfite treatment

Bisulfite modification of genomic DNA was carried out by the use of MethylEdge™ Bisulfite Conversion System and Converted Methylated Human Control (Promega.inc, USA), following the instructions of the manufacturer. During the process of treatment with sodium bisulfite, the cytosine residues, which were unmethylated, were converted to uracil unlike the 5-methylcytosine (5mC), which were not converted. The uracil residues were converted to thymine following PCR amplification. Bisulfite modified DNA specimens were aliquoted and stored at -20° C.

Methylation analysis

The real-time quantitative multiplex methylation-specific PCR (QM-MSP) procedure was performed to determine the methylation status of the CpG islands across the promoter regions of SOCS1 and SOCS3 in the genomic DNA of the participants.

As the first part of two sequential steps in this highly sensitive and specific MethySYBR PCR reaction, the external nested forward (EXT-F) and reverse primer (EXT-R), known as bisulfite-specific primers (BSP), for both genes were utilized to amplify distinct target alleles in a single reaction via the designed primers (**Table 1**). The final 25 µL reaction volume containing 1 µL of bisulfite-treated genomic DNA was used to perform the step 1 multiplex PCR reaction, in which the setting were 95 degrees Celsius (° C) for 5 minutes (min), followed by 30 cycles at 94 ° C for 30 seconds (s), 56 C for 30 s, and 72 ° C for 30 s, with a final extension at 72 ° C for 5 min.

At the second round of PCR, the amplicons produced in the previous step, known as the specific methylated target and nested methylation-specific forward (FM) and reverse primer (RM) for each of the two genes were used. Design of the methylation-specific primer for both genes (SOCS1 and SOCS3) was performed via UCSC database and MethBlast tool. Untreated template controls and fully converted methylated human plasmid DNA (100% methylated), were used as negative and positive control, respectively. A housekeeping gene, B-actin, was used for the comparative Ct method as an internal standard. human plasmid DNA (100% methylated), were used as negative and positive control, respectively. A housekeeping gene, B-actin, was used for the comparative Ct method as an internal standard. The real-time PCR reaction was conducted with 0.25 ml of each of the methylated primers, 1 µL of converted DNA, 5 ml SYBRVR Green Master Mix and 3.5 ml DDW with the temperature protocol of: 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 at 72 ° C for 5 min by using the Applied Biosystem's 7500 Real-Time PCR System for quantitative methylation-specific primers (MSP).

After normalization with the expression of PCR products amplified by the external nested primer as internal control, the $2^{-\Delta\Delta Ct}$ method was

Table 1. Primers sequences for amplifying SOCS1 and SOCS3 genes

SOCS1	
The external nested primer	EXT-F:TTTAAGAGGTGAGAAGGGGTTTG
	EXT-R:CTAAACTCCTTCCCCTTCCAAA
Nested methylation-specific primer	FM:CGGTTTCGTTTTTAGTCGAGG
	RM:CGCCGTACACGCAACATTA
SOCS3	
The external nested primer	EXT-F:GTAGGGAGGTGACGAGGTAG
	EXT-R:ACAAAATAACCCCGAACAAC
Nested methylation-specific primer	FM:GGAGATTTTAGGTTTTCGGA
	RM:CCCGAAACTACCTAAACGCC

used to determine the Unmethylated DNA level in target gene samples. The ΔΔCt was figured by the difference between Ct values of the MSP vs. the BSP products.

Statistical analysis

To report the DNA methylation data, we used mean ± SD. To report the difference in the mean promoter methylation levels between cases and controls, Mann-Whitney U tests were used with the significance level of 0.05. All statistical tests and calculations were performed using the software SPSS 22.0.

Results

Eighteen patients with ALL (16 BM samples and 2 PB samples) and thirteen healthy subjects (10 BM samples and 3 PB samples) were enrolled into the study. The distribution of unmethylated DNA was not normal in either group. The level of unmethylated DNA in the promoter of SOCS1 and SOCS3 genes were compared between the groups of study (**Figure 1**).

Although patients with ALL had lower unmethylated status in SOCS1 promoter compared with the control group, this difference was not significant (mean unmethylated DNA of 0.50 in ALL patients vs. 1.15 in control group, *P*=0.122). In other words, patients with ALL had slightly high er methylation in SOCS1 promoter. However, patients with ALL, although not significant had more unmethylated DNA in SOCS3 promoter compared to controls (mean unmethylated DNA of 0.18 in ALL patients vs. 0.04 in control group, *P*=0.161) (**Table 2**). Also, bone marrow (BM) samples showed higher unmethylated status in comparison with peripheral blood (PB) samples (*P*>0.05) (**Figure 2**).

Table 2. Status of unmethylated DNA of SOCS1 and SOCS3 promoters in patients with ALL and controls

Sample	Groups		N	Mean	SD	Median	Min	Max	SEM	*P*.value
Bone morrow	SOCS1	Control	10	1.154	1.553	0.157	0	4	0.491	0.122
		Patients	16	0.498	0.654	0.110	0	2.106	0.163	
	SOCS3	Control	10	0.039	0.050	0.009	0	0.13	0.016	0.161
		Patients	16	0.180	0.298	0.059	0	1.128	0.074	
Peripheral Blood	SOCS1	Control	3	0.355	0.604	0.008	0	1.053	0.349	0.999
		Patients	2	0.009	0.006	0.009	0	0.013	0.004	
	SOCS3	Control	3	0.055	0.048	0.069	0	0.096	0.027	0.801
		Patients	2	0.005	0.003	0.005	0	0.008	0.002	

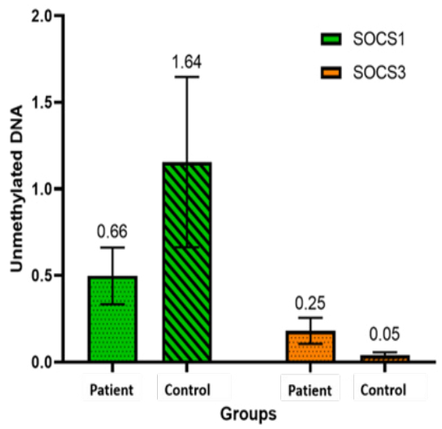

Figure 1. Comparison of unmethylated DNA of SOCS1 and SOCS3 promoters in patients with ALL and healthy controls (Mean + SEM)

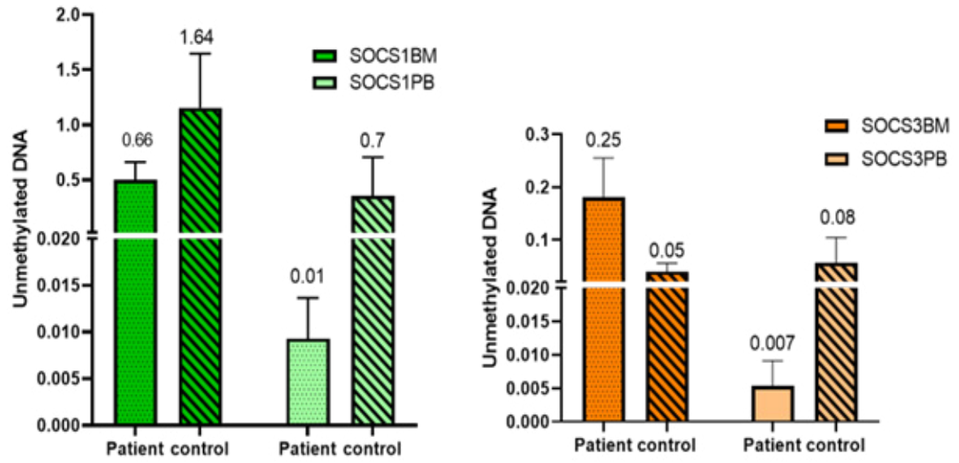

Figure 2. Comparison of unmethylated DNA of SOCS1 and SOCS3 promoters in patients with ALL and healthy controls in both sample groups Bone Morrow (BM) and Peripheral Blood (PB) (Mean + SEM)

Discussion

In summary, we have found that the methylation status of either SOCS1 or SOCS3 genes were not significantly different in ALL patients vs. control group. However, the mean value of methylated DNA in SOCS1 promoter was higher in patients with ALL.

ALL is a heterogeneous pediatric leukemia caused by an uncontrolled proliferation of altered lymphoid progenitor cells (15). The etiology of ALL is indicated by genetic alterations, sequence mutations and structural modification including differential DNA methylation on CpG-rich islands in gene promoter regions that represent the importance of epigenetic mechanisms as initiating elements (16). The genome-wide association studies (GWAS) on the entire genome of patients with ALL shows increased DNA methylation in more than 2000 CpG islands; although some of these methylation patterns were similar to undifferentiated progenitor CD34+ cells, causing de-differentiation to cells with high potential of proliferation (17). DNA methylation serves as a mediator in inflammation, tumor progression (including hepatocellular carcinoma (HCC), colorectal, and cervical) and development of hematological malignancies; where in the silencing of SOCS-1 through promoter hypermethylation leads to JAK/STAT3 activation and complete loss of tumor suppressor activity (14, 18-20). In contrast, the higher expression of SOCS1 in breast cancer is associated with earlier tumor stages (21). SOCS1 and SOCS3 are the most potent suppressors of JAK-STAT signaling pathway which is fundamental for function of cytokines such as IL-6, IFN-α, granulocyte colony-stimulating factor (G-CSF), leukemia inhibitory factor (LIF) and play a vital role in various malignant processes (22). Also, inappropriate activation of STAT proteins, especially STAT5 and STAT3 and genetic alterations in JAK2, JAK1 and JAK3, facilitate upregulation of the downstream PI3K/AKT/mTOR pathway contributing towards ALL pathogenesis (23). However, silencing SOCS1 in DCs and T cells could be therapeutic for anti-tumor immunity (24).

Several studies have ascertained that JAK-STAT pathway is involved in initiation and development of HCC, such as the IL6/JAK/STAT pathway and downregulation of SOCS-1 gene in result of the promoter methylation which is located on the CpG Island of the 5'-end this gene (16p13.3) (25, 26). Also, abnormal methylation of the SOCS1 promoter has been shown to cause proliferation of Acute Myeloid Leukemia (AML) cells by silencing of SOCS1 expression and consequent suppression of JAK2/STAT signaling pathway cells (27). Therefore, aberrant SOCS1 methylation might be a risk factor in the pathol-

ogy of various hematological malignancies, promoting leukemogenesis (28). Similarly, although not significant, we observed higher methylation of SOCS1 in patients with ALL compared to the healthy individuals, suggesting a potential role of epigenetic inactivation of SOCS1 and inhibition of JAK-STAT pathway, that should be further studied in more robust studies.

SOCS3, which is located at chromosome 17 and shares 35% homology with SOCS1, was reported to both function as a tumor suppressor and enhancer of tumor aggressiveness (29). The expression of SOCS3 seems beneficial to the malignant cells via signal down-modulation from certain growth-inhibitory and Th1-promoting cytokines as a tumor-promoting mechanism (30). Besides, melanoma cells constitutively express high levels of SOCS3, indicative of a tumor-protecting function (31). The epigenetic gene silencing of SOCS3, was demonstrated to have an important role in carcinogenesis, prostate and central nervous system tumors and non-small cell lung cancer due to increased methylation (32-34). Also, in both chronic lymphocytic leukemia (CLL) and AML, low expression of SOCS3 was detected, leading to phosphorylation of STAT3 and high expression of anti-tumor apoptosis genes and leukemogenesis (35). Moreover, IL-6/STAT3 signaling pathway induced methylation and SOCS3 epigenetic silencing via increased DNMT1 (29). In this study, contrary to SOCS1, we observed a lower mean level of methylated DNA in SOCS3 promoter of ALL subjects; but this difference was not significant.

This study was limited due to the lack of a proper control group. Since this study was not testing a hypothesis with strong background, it was not ethically acceptable to acquire bone marrow samples from healthy children and the control group consisted of patients who were referred for bone marrow biopsy for reasons other than malignancy, that could have potentially affected the methylation status of these genes. Also, the lack of significance could be partly attributed to the low sample size and a potential selection bias in recruitment of control group. Another hurdle caused by the low sample size was not classifying the ALL patients based on their immunophenptypes. Considering all that, the higher mean of SOCS1 methylation suggests a potential role of silencing of this gene in ALL that should be further studied in studies with larger sample sizes and more detailed classification.

Conclusion

In conclusion, despite the evidence of hypermethylation of SOCS1 gene promoter in patients with ALL, we have identified no statistically significant differences observed between the methylation status of SOCS1 gene promoter in the peripheral blood sample of patients with ALL compared with healthy controls in our study.

Conflict of interest

Authors approve that they have no conflict of interest.

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