Association between methylation of tumor suppressor gene SOCS1 and acute myeloid leukemia

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Introduction

Acute myeloid leukemia (AML) is characterized by malignant proliferation of hematopoietic cells of myeloid lineage. AML has poor prognosis and effective treatments are still lacking despite rigorous research efforts. For AML patients receiving initial treatment, the remission rate can reach as high as 80% after receiving normal chemotherapy, however for some patients, AML will reoccur (1), and the recurrent AML has a poorer prognosis (2). Accurate diagnosis and treatment requires cytogenetics as a prediction tool, and gene overexpression and silencing may provide necessary information.

The suppressor of cytokine signaling-1 (SOCS1) gene, a tumor suppressor gene, belongs to the suppressor of cytokine signaling (SOCS) family. SOCS1 negatively regulates cytokines via the JAK/STAT3 pathway by a negative feedback loop. The SOCS1 gene in humans is located on 16p13.3 and codes for a protein with 221 amino acids. Numerous studies have ascertained that the promoter of SOCS1 is located on the CpG island of the 5’-end this gene, and its abnormal methylation can result in silencing of SOCS1 expression (3). Silencing of SOCS1 expression is associated with carcinogenesis, especially in malignant tumors and proliferative diseases of the hematopoietic system (4).

SOCS1 suppresses the JAK2/STAT signaling pathway to negatively control the expression of cytokines (5) by several mechanisms. SOCS1 combined with phosphorylated tyrosine in the SH2 region blocks activation of JAK2 and transduction of cell signaling (6). Furthermore, the KIR region upstream of the SH2 region can directly act on the kinase with the substrate, thereby suppressing its activity. In addition, an E3 ubiquitin ligase complex can be formed in the SOCS-Box region at the SOCS1 C-terminal to cause proteasomal degradation of cytokine signal transduction proteins including JAK2 (7). In the present study, we investigated the status of SOCS1 expression in AML patients and the relationship between SOCS1 silencing resulting from methylation and AML occurrence and development.

Materials and methods

Patients. Between February 2015 and October 2017, samples from 110 patients diagnosed with AML and 10 normal controls
were obtained from the Department of Hematology at The Second Hospital of Hebei Medical University. Patient clinical characteristics are shown in Table I.

The initial treatment group (IT) included patients who were initially diagnosed, but did not receive any treatment. Patients in the relapsed/refractory group (RR) relapsed after a complete remission or were not yet relieved after treatment of two courses. Patients in the remission group (RE) included those who were fully relieved after treatment. Patients in the normal control group (NC) included those who were healthy or with nutritional anemia. The diagnosis and classification of AML were performed according to French-American-British (FAB) criteria, and included M0 (2 patients), M1 (9 patients), M2 (50 patients), M3 (36 patients) and M4 (13 patients).

The research specimens were studied from bone marrow cells which were isolated by lymphocyte separation. This study was approved by the Ethics Committee of Hebei Medical University and written informed consent was obtained from each patient.

**Cell lines.** Human AML cell lines U937 and THP-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). U937 and THP-1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Clark Bioscience, Claymont, DE, USA) and incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

**Cell viability and apoptosis assay.** Cell viability was assessed by Cell Counting Kit-8 (CCK-8; Beijing Zoman Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. U937 and THP-1 cells were plated in 96-well plates at a density of 1x10^4 cells/well to investigate the proliferation curves and viability curves effected by 5-azacytidine (5-azaC) and 5-aza-2'-deoxycytidine (5-aza-dC; both from Sigma-Aldrich; Merck, St. Louis, MO, USA) for 1 h for chemiluminescent detection. Specific bindings were visualized with Azure c500 (Azure Biosystems, Dublin, CA, USA).

**Western blot analysis.** Cells were washed three times with PBS and lysed with RIPA buffer (BestBio, Shanghai, China). Total proteins (50 µg) per sample were isolated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Billerica MA, USA). The membranes were probed with antibodies for SOCS1 (1:1000; cat. no. 3950), t-JAK2 (1:1000; cat. no. 4040), p-JAK2 (1:1000; cat. no. 4406), t-STAT3 (1:1000; cat. no. 9139), p-STAT3 (1:1000; cat. no. 52075), t-STAT5 (1:1000; cat. no. 25656), p-STAT5 (1:1000; cat. no. 4322) and β-actin (1:2000; cat. no. 3700), and then incubated with anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (1:3000; cat. no. 3700) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h for chemiluminescent detection. Specific bindings were visualized with Azure c500 (Azure Biosystems, Dublin, CA, USA).

**Cell transfection.** Full-length SOCS1 (NM-003745.1) cDNA was synthesized and cloned into pCMV3-C-GFPSpark vector (Sino Biological Inc., Beijing, China). The sequence and orientation of the SOCS1 insert was confirmed by DNA sequencing. pCMV3-SOCS1-GFPSpark was then transfected into U937 and THP-1 cells by Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated for 2 days at 37°C. The empty vector was used as control.

**Statistical analysis.** Statistical analysis was performed with SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with a Fisher's least significant difference and a Chi-squared were used to compare the data. A P-value of <0.05 was considered to indicate a statistically significant difference.

**Results**

**SOCS1 gene mRNA expression and methylation state in AML groups.** We first investigated the mRNA expression of SOCS1 in the four groups of AML patients. We found that SOCS1 gene expression was significantly lower in the IT and RR groups when compared to the RE and NC groups (P<0.05) (Fig. 1A).
Relative expression of SOCS1 mRNA in each group was found to be 0.0306±0.0137 for IT, 0.0164±0.0101 for RR, 1.3346±0.4852 for RE and 1.5983±0.3891 for NC. Fold change differences were compared to NC values. Subsequently, we compared SOCS1 methylation in the four groups. Methylation of SOCS1 was not detected in the RE and NC groups, but it was detected in 24 (48%) IT patients and 8 (80%) RR patients. The frequency of methylation was significantly higher in the IT, RR groups compared with the RE and NC group (P<0.05) (Fig. 1B). Thus, SOCS1 gene methylation was negatively correlated with mRNA expression.

Expression of the SOCS1 methylation-related gene. AML patients in the IT group were divided into a SOCS1 methylation group (ME) (24 cases) and a non-methylation group (NM) (26 cases) according to the methylation state of the SOCS1 gene. The NC and RR groups included 10 cases. We found that the mRNA of DNA methyltransferases (DNMTs) such as DNMT1 and DNMT3a in the SOCS1 ME and RR group was higher than that in the NM and NC group (P<0.05) (Fig. 1C). There was no change in the mRNA expression of DNMT3b. In addition, gene expression of methylated CpG binding protein MeCP2 was higher in the ME and RR groups than in the NM and NC group (P<0.05). MBD2 in the ME and RR groups was significantly higher than that of the NM and NC groups, however, the difference was of no statistical significance (P>0.05).

Expression of the SOCS1 protein and downstream pathway proteins. The relative expression of the SOCS1 protein in the AML IT and RR groups was significantly lower than that in the RE and NC groups (P<0.05) (Fig. 2A and B). In contrast, p-JAK2, p-STAT3 and p-STAT5 expression was significantly higher in the IT and RR groups (P<0.05) in comparison to the RE and NC groups. There was no difference in the expression of t-JAK2, t-STAT3 and t-STAT5 among the four groups.

Cell viability and the apoptosis rate of AML cell lines in response to demethylation drugs. The half maximal inhibitory concentration (IC50) of 5-azaC on U937 and THP-1 cell lines as determined by the cell viability assay was found to be 0.95 and 17.05 µmol/l, respectively, and that of 5-aza-dC was 4.79 and 43.55 µmol/l, respectively (Fig. 3A). With the increase of drug concentration and duration of drug treatment.
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(time <3 days), the viability of U937 and THP-1 cells gradually decreased (Fig. 3B). However, the apoptosis rate of U937 and THP-1 cells gradually increased with the increase of drug concentration (Fig. 3C and D). The results indicated that the viability of the two cell lines was negatively associated with the drug concentration and treatment time, while the apoptosis rate of the two cell lines was positively associated with the drug concentration.

**SOCS1 gene expression and methylation state in AML cell lines in response to demethylation drugs.** AML cell lines were treated with four concentrations of demethylation drugs. We observed an increase in SOCS1 mRNA expression in response to 5-azaC and 5-aza-dC in a dose dependent manner in both U937 and THP-1 cells (Fig. 4A). Statistical differences existed among the untreated group and the treated groups (P<0.05). Following intervention with demethylation drugs, the non-methylation strip of the SOCS1 gene in the U937 and THP-1 cell lines was light while the methylation strip was darker. As the concentration increased, SOCS1 completely transformed from a methylated state to an unmethylated state (Fig. 4B). As the concentration of the demethylation drugs increased, the mRNA expression of the SOCS1 gene increased, and the relative expression of methylation-related genes DNMT1, DNMT3a, MBD2 and MeCP2 gradually decreased. The expression of DNMT1 and DNMT3a in the U937 cells treated with 5-aza-dC (1 µmol/l) was not significantly different compared with that in the untreated group (P>0.05), while the expression of DNMT1, DNMT3a, MBD2 and MeCP2 in the 5-azaC- and 5-aza-dC-treated U937 and THP-1 cells was significantly different between the untreated group and the drug-treated groups (P<0.05) (Fig. 4C). As the concentration of the demethylation drugs increased, the relative expression of the SOCS1 protein gradually increased, while downstream
p-JAK2, p-STAT3 and p-STAT5 protein expression gradually decreased, which was negatively associated with the SOCS1 protein. The expression of the p-STAT3 protein between the untreated group and the drug-treated groups was statistically different (P<0.05) except for the 5-azaC low concentration group (1 or 10 µmol/l). Statistical differences in p-JAK2 and p-STAT5 protein expression existed among the untreated group and the treated groups (P<0.05). t-JAK2, t-STAT3 and t-STAT5 protein expression was not markedly altered (Fig. 5).
proteins ($P<0.05$) confirming that SOCS1 negatively affects the downstream JAK2/STAT signaling pathway. No significant change in the expression of t-JAK2, t-STAT3 and t-STAT5 proteins was observed.
Discussion

SOCS1, widely recognized as a tumor suppressor gene, is related to lymphatic metastasis and disease progression of liver cancer (10). Its methylation rate in hepatocellular carcinoma ranges from 39-60%. SOCS1 methylation also exists in other tumors, such as 61% in cervical cancer (11), 45% in esophageal squamous cancers (12) and 40% in hepatoblastomas (13). Recent studies have demonstrated that SOCS1 upregulates the expression of tiny RNAs in multiple myeloma, breast and prostate cancer, further confirming the effect of SOCS1 as a tumor-suppressor gene (14-16). DNA methylation refers to the process of biologically adding a methyl group to cytosine in cytosine-guanine CpG dinucleotides with S-adenosylmethionine (SAM) as a methyl donor under the catalysis of DNA methyltransferases (DNMTs). DNMTs mainly include DNMT1, DNMT3a and DNMT3b. DNA methylation needs to be read by a conserved family of proteins, namely, methyl-CpG binding proteins, which are bound by a methylated DNA-binding domain (MBD) to 5-methylcytosine (5 mC) followed by CpG. Five methylated CpG binding proteins are currently known, MeCP2, MBD1, MBD2, MBD3 and MBD4. Both MeCP2 and MBD2 can bind to methylated DNA and inhibit the transcription of methylated target genes. Our findings revealing that methylated SOCS1 is increased in AML corroborates these studies. In the initial treatment and relapsed/refractory groups, methylated SOCS1 (48 and 80%, respectively) decreased the expression of mRNA and protein, while the expression of DNA methyltransferases DNMT1, DNMT3a and CpG binding proteins MBD2 and MeCP2 was increased, indicating their participation in SOCS1 gene methylation. In contrast, in the remission and normal control groups, the SOCS1 gene was found to be in a non-methylated state and its mRNA and protein levels were highly expressed.

JAK2/STAT is a major signaling pathway for AML cell growth and proliferation. SOCS1, can directly bind with the JAK2/STAT complex and suppress this signal transduction pathway. Park et al (17) reported that SOCS1 silencing increased phosphorylation of STAT and promoted tumor development. In the present study, we found that SOCS1 protein expression in the initial treatment and relapsed/refractory groups was decreased, while the expression of its downstream p-JAK2, p-STAT3 and p-STAT5 proteins was higher than that in the remission and normal control groups. SOCS1 suppressed signal transduction of the JAK2/STAT pathway to exert its biological functions by suppressing p-JAK2, p-STAT3 and p-STAT5 proteins.

DNA methylation is a reversible change. In the present study, we performed demethylation on AML cell lines U937 and THP-1 with drugs 5-azaC and 5-aza-dC. We found that following treatment, the SOCS1 gene changed from a methylated state to a non-methylated state and this was accompanied by increased mRNA and protein expression in a drug concentration-dependent manner. In contrast, the
Figure 6. Cells overexpressing SOCS1 reveal inhibition of the JAK2/STAT pathway. (A) U937 and THP-1 cells transfected with the SOCS1-GFP vector. Cells were imaged with LSM 510 (ZEISS AG, Oberkochen, Germany). (B) Proliferation curves of transfected cells. Cell viability was determined by the CCK-8 assay. (C) The apoptosis rate of transfected cells. Flow cytometric analysis of cell apoptosis detected by Annexin V staining. This experiment was performed twice. (D) Expression of downstream pathway proteins in cells with the empty vector and after transfection of the SOCS1 gene in U937 and THP-1 cell lines. Subclone 1 and 2 are each from SOCS1-transfected AML cell lines. The control represents the empty vector, SOCS1+ represents cells transfected with SOCS1. *P<0.05.
expression of downstream p-JAK2, p-STAT3 and p-STAT5 proteins and the tumor cell viability rate was decreased, while the apoptosis rate was increased. Furthermore, the expression of the p-JAK2, p-STAT3 and p-STAT5 proteins was downregulated in cells transfected with the SOCS1 protein. This further ascertained that SOCS1 negatively regulates the downstream JAK2/STAT signaling pathway. In addition, in transfected cells, we also observed that the cell viability rate was decreased and the apoptosis rate was increased.

The relationship between SOCS1 methylation and AML should be further explored in gene methylation sequencing and with siRNA, which is the study aim in our future study.

Thus, this study revealed that SOCS1 may be used a therapeutic target and interventions that may induce expression of SOCS1 may be used for anticancer therapy (18-20). Whether SOCS1 can suppress other types of tumors should be verified by studies on different types of tumors. The demethylated SOCS1 gene may possibly become a new target for future tumor therapy and provide a new hope for tumor therapy and prognosis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
XZ and JL conceived and designed the study; XZ, LY, XL and YZ collected, analysed and interpreted the data; YP and XW designed the experimental techniques and XZ drafted the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Hebei Medical University and written informed consent was obtained from each patient.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References