Methylation of the suppressor of Cytokine Signaling 1 Gene (SOCS1) in Philadelphia-negative myeloproliferative neoplasms

E.B. YASIN1, R. ALSERIHI2, H. ALKHATABI2,4,5, H.M.H. QUTOB1, R. QAHWAJI2, S.W. KATTAN3, K.A. GHOLAM6, E. HUSSEIN7, A.S. BAREFAH4,8, M.E. ALGHUTHAMI9, A. ABUZENADAH2,10

1Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Rabigh, Saudi Arabia
2Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
3Medical Laboratory Department, College of Applied Medical Sciences, Taibah University, Yanbu, Saudi Arabia
4Hematology Research Unit (HRU), King Fahad Medical Research Center (KFMRC), King Abdulaziz University, Jeddah, Saudi Arabia
5Center for Artificial Intelligence in Precision Medicine (CAIPM), King Abdulaziz University, Jeddah, Saudi Arabia
6Department of Blood Transfusion Service, King Abdul Aziz University Hospital, Jeddah, Saudi Arabia
7Department of Haematology and Immunohaematology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan
8Department of Haematology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
9GCC Accreditation centre (GAC), Jeddah, Saudi Arabia
10King Fahad Medical Research Center (KFMRC), King Abdulaziz University, Jeddah, Saudi Arabia

Abstract. – OBJECTIVE: Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway activation is initiated by mutations in the JAK2 gene. This activation is in turn, a vital pathogenetic mechanism in myeloproliferative neoplasms (MPNs). However, several factors affect the pathogenesis of MPNs other than the JAK2 gene mutations, such as the downregulation of cytokine signaling (SOCS) proteins, which are potent inhibitors of the JAK/STAT pathway. Therefore, we hypothesized that the regulation of SOCS protein system might be a possible pathogenetic mechanism of MPNs through activating the JAK/STAT pathway.

PATIENTS AND METHODS: Our study aimed to investigate the status of the Suppressors of cytokine signaling 1 (SOCS1) in 125 MPNs specimens at the level of mutated points. The acquired mutations, aberrant expression, and/or CpG island hypermethylation of SOCS1 were analyzed among Philadelphia-negative myeloproliferative neoplasm patients.

RESULTS: SOCS1 was identified in 20.0% of all patients with Philadelphia-negative myeloproliferative neoplasm. At the diagnosis, the prevalence of methylation was 41.0% for Polycythaemia Vera (PV), 27.7% for Essential Thrombocythaemia (ET), and 6.6% for Primary Myelofibrosis (PMF). The methylation was not detected in 20 healthy adult people. A significant association was found between disease groups (p=.077). The presence of methylated SOCS1 was found to be significantly correlated with age (p=.005), total RBCs count (p=.019), hemoglobin (Hb) concentration (p=.002), and Hematopoietic cell transplant (HCT) (p=.007) in PV patients. However, the presence of methylated SOCS1 was found to be significantly associated with age (p=.012), total RBCs count (p=.022), Hb concentration (p=.024), HCT (p=.033), and platelets count (p=.037) in ET patients. Moreover, the presence of methylated SOCS1 was significantly associated with Hb concentration (p=.046) and HCT (p=.040) in PMF patients.

CONCLUSIONS: We concluded that the activation of the JAK/STAT signaling pathway in alternative or with JAK2 mutations leads to SOCS1 hypermethylation, which could represent a potential therapeutic target.

Key Words: JAK2, SOCS1, Hypermethylation, Myeloproliferative neoplasms.

Corresponding Author: Elrashed B. Yasin, Ph.D, MLS; e-mail: eyasin@kau.edu.sa, rashed2t@hotmail.com
Introduction

Janus kinase 2 (JAK2) is an acquired mutation in patients with polycythemia vera (PV). It results from valine replacement by phenylalanine at position 617. JAK2 mutations account for 50% of patients with myeloproliferative neoplasms (MPNs), essential thrombocythemia (ET), and primary myelofibrosis (PMF). It was observed that proteins produced from the JAK2V617F led to erythrocytosis and an eventual myelofibrotic transformation in mouse models. This demonstrates the vital pathogenesis role of JAK2V617F mutation in MPNs. Furthermore, the JAK2V617F mutation is associated with increased early hematopoietic stem cells with both myeloid and lymphoid potential.

JAK2 is a cytoplasmic tyrosine kinase that is essential for type I cytokine receptors, such as those for erythropoietin, thrombopoietin, interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). Murine JAK2 homozygous knockdown causes embryonic mortality at day 12.5 due to the lack of definitive erythropoiesis. It seems that the acquired mutation affects different elements of the signaling pathways for phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), extracellular signal-related kinase (ERK), and mitogen-activated protein kinase (MAPK). These signaling pathways were activated in the JAK2V617F mutation without necessitating cytokines. Thus, such signaling pathways play a pivotal role in the pathophysiology of MPNs without accounting for JAK2V617F mutations. Furthermore, different phenotypes were seen in patients with the JAK2V617F-positive disease, which could modulate JAK2 kinase activity.

Suppressors of cytokine signaling 1 (SOCS1) and SOCS3 are considered attractive candidates because they negatively regulate the JAK/STAT pathway. SOCS1 and SOCS3 are promptly activated by several cytokines, including erythropoietin, interleukin-3, and GM-CSF. An increase in SOCS1 and SOCS3 expression levels causes a reduction in JAK and STAT phosphorylation and STAT dimerization. Consequently, such cytokines are imported into the nucleus, reducing target gene transcription. Unlike the other members of the SOCS family, SOCS1 and SOCS3 contain a 12-amino acid region termed the kinase inhibitory region (KIR), which interacts with and inhibits the catalytic JH1 domain of JAK proteins. SOCS1 binds phosphorylated JAK2 directly, while SOCS3 inactivates JAK2, but enables bound to a cytokine receptor, e.g., erythropoietin receptor. Moreover, JAK2 targets proteosome-mediated degradation through SOCS proteins. In vivo, it was shown that SOCS1/-/- mice have a low level of B-cell numbers due to interferon-g signaling deficiency, while SOCS3/-/- mice die in utero due to up-regulation of SOCS3, which blocks fetal liver erythrocytosis. These findings show that SOCS3 plays a vital role in controlling definitive erythropoiesis.

Irregular CpG island hypermethylation of tumor suppressor genes is a well-recognized mechanism that can lead to the development of tumors. Some hematologic malignancies, including myelodysplasia and chronic myeloid leukemia, have been involved in the methylation and down-regulation of SOCS1. However, some diseases have obtained conflicting results. An increase in SOCS3 promoter methylation and a decrease in its gene expression have been identified in patients with solid tumors such as in lung cancer and head and neck squamous cell carcinoma.

Our study aimed to examine the contribution of the aberration(s) of SOCS1 in the development of MPNs. A cohort of patients with MPNs were recruited to examine the coding region and splice sites of SOCS1 for acquired mutations. Then, we determined the transcript level of SOCS1 and the status of the CpG islands methylation within the promoter and exon 2. Finally, we evaluated the association of gene methylation with clinical manifestations and other laboratory variables.

Patients and Methods

Patients

MPN specimens (N = 125) were collected retrospectively from the King Abdulaziz University Hospital (Jeddah, Kingdom of Saudi Arabia) covering the period from January 2016 to December 2020 (Bioethical approval code: 01-CEGMR-Bioeth-2019). The samples were categorized into type of hematological disease as follows: 68 with PV, 46 with ET, and 11 with PMF; clinical data such as gender, age, tumor grade, and lymph node status were considered; and follow-up results were retrieved from the patients’ records, after obtaining the relevant ethical approvals as per the rules of the Helsinki Declaration.
Bisulfite DNA Modification and MethyLight Assay

Around 0.5 microgram of DNA was used for bisulfite conversion using the Qiagen Epitect Bisulfite Conversion kit (Qiagen, Germantown, MD, USA). DNA methylation analysis was conducted using MethyLight as described elsewhere. The methylation levels of the SOCS1 were analyzed using the primer-probe combinations. Forward primer sequence: GCGTCGAGTTCGTGGTTATTT; Reverse primer sequence: CCGAAACTCATCTTCACGCTAA; and 6FAM-Probe oligo sequence: ACAATTCCGCTAACGACTATCGCGCA-BHQ1, all made according to previously published reports. A probe targeting bisulfite-modified Alu repeat sequences was used to normalize input DNA. The specificity of the reaction was ascertained using ssil-treated and bisulfite-modified positive control DNA (Qiagen, Germantown, MD, USA) and the negative control DNA (Qiagen, Germantown, MD, USA). The percentage of fully methylated reference (PMR) was calculated by dividing the gene – Alu ratio of a sample by the gene: Alu ratio of the positive control DNA and multiplying by 100. Samples with a PMR of more than 10 were considered positive for methylation, whereas the samples with a PMR of less than 10 were considered negative (i.e., unmethylated). A PMR above 10 is considered positive as it indicates a likely hypermethylation-mediated loss of expression for the genes analyzed.

Statistical Analysis

The extracted data were analyzed using IBM SPSS Statistics version 20 (SPSS Corp., Armonk, NY, USA). The correlation between methylation events and clinicopathologic factors was determined by using Fisher exact test. The p-values less than (<) 0.05 were considered statistically significant in all statistical tests used in this study.

Table I. SOCS1 methylation status among myeloproliferative neoplasm (MPN).

<table>
<thead>
<tr>
<th>Disease subtypes</th>
<th>Total No. of subjects</th>
<th>SOCS1 unmethylated type N (%)</th>
<th>SOCS1 methylated type N (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>68 (54.4%)</td>
<td>55 (59.0%)</td>
<td>13 (41.0%)</td>
<td>.358</td>
</tr>
<tr>
<td>ET</td>
<td>45 (36.8%)</td>
<td>39 (72.3%)</td>
<td>7 (27.7%)</td>
<td>.279</td>
</tr>
<tr>
<td>PMF</td>
<td>11 (8.8%)</td>
<td>6 (93.4%)</td>
<td>5 (6.6%)</td>
<td>.323</td>
</tr>
<tr>
<td>Total</td>
<td>125 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-value significant at < 0.05.

Results

The 125 patients with Philadelphia-negative myeloproliferative neoplasm were analyzed according to the accessibility of specimens and clinical data. Among these patients, 68 (54.4%) were diagnosed with PV, 46 (36.8%) were diagnosed with ET, and 11 (8.8%) were diagnosed with PMF. The mean age at diagnosis was 46.97±7.00, 48.87±7.58, and 47.45±9.45 years for PV, ET, and PMF, respectively. SOCS1 was identified in 20.0% of all patients with MPNs. At the diagnosis, the prevalence of methylation was 41% for PV, 27.7% for ET, and 6.6% for PMF. The methylation was not detected in 20 healthy adult people. A significant association was found between the disease group (p=.077) (Table I).

The association of hematological parameters of PV patients and SOCS1 methylation are summarized in Table II. The presence of methylated SOCS1 was found to be significantly associated with age (p=.005), total RBCs count (p=.019), Hb concentration (p=.002), and HCT (p=.007) in PV patients. No significant associations were detected between total WBC count, or platelets count, and methylated SOCS1 in PV patients (see Table II).

The presence of methylated SOCS1 was found to be significantly associated with age (p=.012), total RBCs count (p=.022), Hb concentration (p=.024), HCT (p=.033), and platelets count (p=.037) in ET patients. No significant association was detected between total WBC count, or platelets count, and methylated SOCS1 in ET patients (Table III).

The presence of methylated SOCS1 was found to be significantly associated with Hb concentration (p=.046) and HCT (p=.040) in PMF pa-
patients. No significant associations were detected between total age ($p = .107$), WBC count ($p = .162$), total RBCs count ($p = .141$), or platelets count ($p = .235$), and methylated SOCS1 in PMF patients (Table IV).

**Discussion**

The study has described the hypermethylation of the SOCS1 locus in patients with Philadelphia-negative myeloproliferative neoplasm (PV, ET, and PMF). Methylation of CpG island has been occasionally assessed in patients with MPNs, where it has been reported that SOCS1 methylation was found in 15% of Myeloproliferative Disorders (MPD) patients. We observed methylation of the exon 2 CpG island in 20% of MPNs patients. However, the primers used in the previous study on patient samples corresponded to the CpG island within exon 2, without assessing the SOCS1 promoter region, whereas the 20 samples extracted from healthy individuals showed an unmethylation pattern. Subsequently, these results suggest that methylation of the SOCS1 exon 2 CpG island, but not the promoter

### Table II. Associations of hematologic parameters with the SOCS1 methylation in PV patients.

<table>
<thead>
<tr>
<th></th>
<th>SOCS1 unmethylated</th>
<th>SOCS1 methylated</th>
<th>Total</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>55</td>
<td>13</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>47.11 ± 7.58</td>
<td>46.38 ± 3.79</td>
<td>46.97 ± 7.00</td>
<td>.005</td>
</tr>
<tr>
<td>WBC ($\times 10^9$/L, mean ± SD)</td>
<td>23.00 ± 32.37</td>
<td>12.85 ± 11.98</td>
<td>21.06 ± 29.78</td>
<td>.176</td>
</tr>
<tr>
<td>RBC ($\times 10^9$/L, mean ± SD)</td>
<td>6.75 ± 1.17</td>
<td>7.15 ± 1.35</td>
<td>6.82 ± 1.21</td>
<td>.019</td>
</tr>
<tr>
<td>Hb (g/dL, mean ± SD)</td>
<td>16.29 ± 3.14</td>
<td>16.38 ± 3.79</td>
<td>16.31 ± 3.25</td>
<td>.002</td>
</tr>
<tr>
<td>Hct (%, mean ± SD)</td>
<td>50.93 ± 8.04</td>
<td>52.08 ± 9.62</td>
<td>51.15 ± 8.29</td>
<td>.007</td>
</tr>
<tr>
<td>Platelet ($\times 10^9$/L, mean ± SD)</td>
<td>364.22 ± 226.92</td>
<td>746.46 ± 951.49</td>
<td>437.29 ± 476.01</td>
<td>.211</td>
</tr>
</tbody>
</table>

$p$-value significant at $< 0.05$.

### Table III. Associations of hematologic parameters with the SOCS1 methylation in ET patients.

<table>
<thead>
<tr>
<th></th>
<th>SOCS1 unmethylated</th>
<th>SOCS1 methylated</th>
<th>Total</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>39</td>
<td>7</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>48.59 ± 7.36</td>
<td>50.43 ± 9.22</td>
<td>48.87 ± 7.58</td>
<td>.012</td>
</tr>
<tr>
<td>WBC ($\times 10^9$/L, mean ± SD)</td>
<td>35.49 ± 56.13</td>
<td>49.86 ± 70.49</td>
<td>45.13 ± 62.07</td>
<td>.281</td>
</tr>
<tr>
<td>RBC ($\times 10^9$/L, mean ± SD)</td>
<td>4.74 ± 1.02</td>
<td>4.43 ± 1.27</td>
<td>4.69 ± 1.05</td>
<td>.022</td>
</tr>
<tr>
<td>Hb (g/dL, mean ± SD)</td>
<td>10.87 ± 1.74</td>
<td>11.71 ± 2.49</td>
<td>11.00 ± 1.86</td>
<td>.024</td>
</tr>
<tr>
<td>Hct (%, mean ± SD)</td>
<td>35.59 ± 5.85</td>
<td>39.43 ± 7.22</td>
<td>36.17 ± 6.23</td>
<td>.033</td>
</tr>
<tr>
<td>Platelet ($\times 10^9$/L, mean ± SD)</td>
<td>1119.13 ± 461.13</td>
<td>1256.29 ± 420.02</td>
<td>1140.00 ± 453.39</td>
<td>.037</td>
</tr>
</tbody>
</table>

$p$-value significant at $< 0.05$.

### Table IV. Associations of hematologic parameters with the SOCS1 methylation in PMF patients.

<table>
<thead>
<tr>
<th></th>
<th>SOCS1 unmethylated</th>
<th>SOCS1 methylated</th>
<th>Total</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>40.00 ± 4.09</td>
<td>56.40 ± 4.34</td>
<td>47.45 ± 9.45</td>
<td>.107</td>
</tr>
<tr>
<td>WBC ($\times 10^9$/L, mean ± SD)</td>
<td>49.67 ± 39.92</td>
<td>84.60 ± 48.19</td>
<td>65.55 ± 45.37</td>
<td>.162</td>
</tr>
<tr>
<td>RBC ($\times 10^9$/L, mean ± SD)</td>
<td>4.74 ± 1.02</td>
<td>3.80 ± 0.45</td>
<td>5.00 ± 2.05</td>
<td>.141</td>
</tr>
<tr>
<td>Hb (g/dL, mean ± SD)</td>
<td>10.87 ± 1.74</td>
<td>10.80 ± 1.64</td>
<td>10.80 ± 1.46</td>
<td>.046</td>
</tr>
<tr>
<td>Hct (%, mean ± SD)</td>
<td>35.59 ± 5.85</td>
<td>35.20 ± 6.26</td>
<td>32.91 ± 6.43</td>
<td>.040</td>
</tr>
<tr>
<td>Platelet ($\times 10^9$/L, mean ± SD)</td>
<td>40.00 ± 4.09</td>
<td>198.60 ± 74.87</td>
<td>334.82 ± 313.55</td>
<td>.235</td>
</tr>
</tbody>
</table>

$p$-value significant at $< 0.05$. 
region, is a variable feature in the blood cells of ordinary individuals. The relevance of SOCS1 exon 2 CpG island methylation for leukemogenesis and the pathogenesis of the MPNs is, therefore, unclear and should be considered with caution.

In most MPNs, an increase in SOCS1 mRNA level has been identified in the bone marrow of MPNs patients using formalin-fixed bone marrow trephines. However, the number of PV samples was limited to 13 (compared to 68 in our analysis), and a different control gene and SOCS1 primers were used. In addition, our study failed to reveal a significant rise in granulocyte expression in patients with PV.

Several cytokines, including many interleukins, erythropoietin, and GM-CSF, but not thrombopoietin, can activate SOCS1 expression. Although JAK2 is targeted by SOCS1, leading to reduced phosphorylation of JAK2 and STAT5, it is uncertain whether STAT5A or STAT5B transcriptionally triggers SOCS1 directly. Indeed, the SOCS1 promoter contains binding sites for STAT1, STAT3, and STAT6. In JAK2V617F mutations, the SOCS1 expression in MPNs has shown no significant change, while the SOCS3 has shown a revolution in myelofibrosis, resulting in the stabilization of the SOCS1 transcript level. Accordingly, these findings suggest that a rise in SOCS1 transcription within MPNs may be independent of activated JAK2/STAT5.

Current data support the hypothesis that developed alteration can modulate JAK2 kinase activity and modify the MPNs phenotype. Several possible elucidations could reveal the different phenotypes of JAK2V617F-positive MPNs patients, the transformation of varying progenitor cells, inherited genetic differences and acquired genetic or epigenetic modifications. Mitotic recombination of 9p24, leading to duplication of the JAK2V617F mutations (homozygosity), has been detected in erythroid progenitors of most PV patients, but not in ET. By contrast, neither mutations in Myeloproliferative Leukemia (MPL) nor methylation of SOCS3 have been detected in PV, suggesting that homozygosity for JAK2V617F may be sufficient for developing PV. The role of SOCS3 methylation in patients with JAK2V617F-positive PMF remains to be elucidated.

The mechanisms underlying JAK2V617F-negative cases of MPNs are thought to reflect abnormalities affecting cytokine receptor signaling. In support of this, JAK2V617F-negative ET patients frequently demonstrate features of JAK2V617F-positive MPNs such as erythropoietin independent erythroid colonies, abnormal megakaryocyte morphology, and overexpression of Polycythemia Rubra Vera gene (PRV-1). On the other hand, acquired mutations within the MPL gene have been identified in 8% and 4% of PMF and ET patients, respectively. However, these findings have not been observed among PV patients. Hypermethylation of SOCS3 represents another acquired aberration affecting one component of a signaling pathway within JAK2V617F-negative cases of PMF. This observation raises the possibility of using demethylating agents as a potential therapy in patients with myelofibrosis.

Conclusions

The JAK/STAT signaling pathway can be activated through SOCS1 hypermethylation instead of, or together with, JAK2 mutations. These modifications might consider a prospective therapeutic target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We would like to thank King Abdulaziz University Hospital for the approval to collect the samples.

Ethical Approval

Ethical approval was obtained from the Ethical Committee of the Center of Excellence in Genomic Medicine Research (CEGMR) (Bioethical approval code: 01-CEGMR-Bioeth-2020).

Informed Consent Statement

Each participant was asked to sign a written ethical consent before the interview.

Availability of Data and Materials

All data and materials were included in the article.

Funding

This research work was funded by King Abdulaziz University, DSR through Institutional Fund Projects under grant No. IFPRC-034-140-2020.
Authors’ Contribution
E.B.Y. participated in revising the clinical data and the disease diagnosis and drafted the manuscript. E.B.Y., H.A., R.A., H.Q., R.Q., E.N., S.H., K.G., and M.A. participated in data analysis, helped design tables, critical review, and drafted the manuscript. E.B.Y., H.A., R.A., S.H., H.Q., E.N., and K.G. carried out the experiment, including DNA extraction, Methylation studies, A.B., K.G., and M.A. performed data collection. E.B.Y. participated in designing the study, provided required reagents for the experiment, and helped in drafting the manuscript. A.A. helped in proving the reagents, kits, and other logistics to perform the study. All authors have read and agreed to the published version of the manuscript.

ORCID ID
Elrashed B. Yasin: https://orcid.org/0000-0002-4087-8112.

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