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

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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.

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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[1-5]. *JAK2* mutations account for 50% of patients with myeloproliferative neoplasms (MPNs), essential thrombocythemia (ET), and primary myelofibrosis (PMF)[6-10]. It was observed that proteins produced from the *JAK2V617F* led to erythrocytosis and an eventual myelofibrotic transformation in mouse models 2[11-14]. This demonstrates the vital pathogenesis role of *JAK2V617F* mutation in MPNs. Furthermore, the *JAK2V617F* mutation in PV increases in early hematopoietic stem cells with both myeloid and lymphoid potential[15,16].

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)[17]. Murine *JAK2* homozygous knockdown causes embryonic mortality at day 12.5 due to the lack of definitive erythropoiesis[18,19]. 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 the *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[17]. *SOCS1* and *SOCS3* are promptly activated by several cytokines, including erythropoietin, interleukin-3, and GM-CSF[17]. 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[20]. 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[20]. *SOCS1* binds phosphorylated JAK2 directly, while *SOCS3* inactivates *JAK2,* but enables bound to a cytokine receptor, e.g., erythropoietin receptor[21,22]. Moreover, *JAK2* targets proteosome-mediated degradation through SOCS proteins[17]. *In vivo*, it was shown that *SOCS1*-/- mice have a low level of B-cell numbers due to interferon-g signaling deficiency[23], while *SOCS3*-/- mice die in utero due to up-regulation of *SOCS3,* which blocks fetal liver erythrocytosis[24]. 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[25,26]. 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[27-30].

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[31]. The methylation levels of the *SOCS1* were analyzed using the primer-probe combinations. Forward primer sequence: GCGTCGAGTTCGTGGGTATTT; Reverse primer sequence: CCGAAACCATCTTCACGCTAA; and 6FAM-Probe oligo sequence: ACAATTCCGCTAACGACTATCGCGCA-BHQ1, all made according to previously published reports[32-35]. A probe targeting bisulfite-modified Alu repeat sequences was used to normalize input DNA. The specificity of the reaction was ascertained using sssl-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. The Gene CLUSTER 3.0 program, visualized using JavaTree software, was used to analyze K-means clustering[36].

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 (p=.281) 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-

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

Disease subtypes	Total No. of subjects	SOCS1 unmethylated type N (%)	SOCS1 methylated type N (%)	*p*-value
PV	68 (54.4%)	55 (59.0%)	13 (41.0%)	.358
ET	45 (36.8%)	39 (72.3%)	7 (27.7%)	.279
PMF	11 (8.8%)	6 (93.4%)	5 (6.6%)	.323
Total	125 (100%)		.077	

p-value significant at < 0.05.

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

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

p-value significant at < 0.05.

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

ET	SOCS1 unmethylated	SOCS1 methylated	Total	*p*-value
No. of patients	39	7	46	
Age (years, mean ± SD)	48.59 ± 7.36	50.43 ± 9.22	48.87 ± 7.58	.012
WBC ($\times 10^9$/L, mean ± SD)	35.49 ± 56.13	98.86 ± 70.49	45.13 ± 62.07	.281
RBC ($\times 10^9$/L, mean ± SD)	4.74 ± 1.02	4.43 ± 1.27	4.69 ± 1.05	.022
Hb (g/dL, mean ± SD)	10.87 ± 1.74	11.71 ± 2.49	11.00 ± 1.86	.024
Hct (%, mean ± SD)	35.59 ± 5.85	39.43 ± 7.72	36.17 ± 6.23	.033
Platelet ($\times 10^9$/L, mean ± SD)	1119.13 ± 461.13	1256.29 ± 420.02	1140.00 ± 453.39	.037

p-value significant at < 0.05.

tients. 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[37]. Subsequently, these results suggest that methylation of the *SOCS1* exon 2 CpG island, but not the promoter

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

PMF	SOCS1 unmethylated	SOCS1 methylated	Total	*p*-value
No. of patients	6	5	11	
Age (years, mean ± SD)	40.00 ± 4.09	56.40 ± 4.34	47.45 ± 9.45	.107
WBC ($\times 10^9$/L, mean ± SD)	49.67 ± 39.92	84.60 ± 48.19	65.55 ± 45.37	.162
RBC ($\times 10^9$/L, mean ± SD)	6.00 ± 2.37	3.80 ± .45	5.00 ± 2.05	.141
Hb (g/dL, mean ± SD)	9.33 ± 1.86	10.80 ± 1.64	10.00 ± 1.84	.046
Hct (%, mean ± SD)	6.00 ± 2.37	35.20 ± 6.26	32.91 ± 6.43	.040
Platelet ($\times 10^9$/L, mean ± SD)	40.00 ± 4.09	198.60 ± 74.87	334.82 ± 313.55	.235

p-value significant at < 0.05.

region, is a variable feature in the blood cells of ordinary individuals[25,38]. {Chim, 2004 #311}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[39]. 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[17,40]. 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[41]. 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[42]. 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[42]. 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[42].

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 1 gene (PRV-1)[8]. 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 finding has not been observed among PV patients[43-46]. Hypermethylation of *SOCS3* represents another acquired aberration affecting one component of a signaling pathway within *JAK2V617F*-negative cases of PMF[47]. This observation raises the possibility of using demethylating agents as a potential therapy in patients with myelofibrosis[48].

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.

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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.

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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., A.B., 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.

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