JAK2 V617F-dependent upregulation of PU.1 expression in the peripheral blood of myeloproliferative neoplasm patients

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Myeloproliferative neoplasms (MPN) are multiple disease entities characterized by clonal expansion of one or more of the myeloid lineages (i.e. granulocytic, erythroid, megakaryocytic and mast cell). *JAK2* mutations, such as the common V617F substitution and the less common exon 12 mutations, are frequently detected in such tumor cells and have been incorporated into the diagnostic criteria published by the World Health Organization since 2008. However, the mechanism by which these mutations contribute to MPN development is poorly understood. We examined gene expression profiles of MPN patients focusing on genes in the JAK–STAT signaling pathway using low-density real-time PCR arrays. We identified the following 2 upregulated genes in MPN patients: a known target of the JAK–STAT axis, *SOCS3*, and a potentially novel target, *SPI1*, encoding PU.1. Induction of PU.1 expression by *JAK2* V617F in JAK2-wildtype K562 cells and its downregulation by *JAK2* siRNA transfection in *JAK2* V617F-positive HEL cells supported this possibility. We also found that the ABL1 kinase inhibitor imatinib was very effective in suppressing PU.1 expression in BCR-ABL1-positive K562 cells but not in HEL cells. This suggests that PU.1 expression is regulated by both JAK2 and ABL1. The contribution of the two kinases in driving PU.1 expression was dominant for JAK2 and ABL1 in HEL and K562 cells, respectively. Therefore, PU.1 may be a common transcription factor upregulated in MPN, including chronic myelogenous leukemia, polycythemia vera (PV), and essential thrombocythemia (ET). PU.1 is a transcription factor required for myeloid differentiation and is implicated in erythroid leukemia. Therefore, expression of PU.1 downstream of activated JAK2 and ABL1 may explain why *JAK2* mutations or *BCR-ABL1* fusion are frequently observed in MPN patients.

1. Patients

Twenty-six patients diagnosed with MPN at Shiga Medical Center for Adults in 2008 and 2009 and 11 healthy volunteers were enrolled in this study. For each patient, the presence of a *JAK2* V617F mutation was determined by the following 3 methods: allele-specific PCR, quantitative allele-specific PCR, and direct sequencing. All PV and 57% of ET patients were positive for *JAK2* V617F mutation (Table 1).

Category	Age	Female	Male	Mutation Burden (%) [0]	WBC (x10³/L) [3.4~9.2]	RBC (x10⁶/L) [F 3500~5000; M 4000~5600]	Hb (g/dL) [F 10.9~14.7; M 13.2~17.1]	Ht (%) [F 32.5~44.7; M 38.6~50.7]	PLT (x10⁴/L) [14.8~35.2]
PV	63 ± 14	6	6	64 ± 24	16 ± 6	6330 ± 1131	15 ± 2	48 ± 6	627 ± 270
ET	63 ± 10	9	5	12 ± 14	7 ± 2	4013 ± 646	13 ± 1	39 ± 4	732 ± 259
ET+	65 ± 11	6	2	21 ± 13	7 ± 1	4208 ± 550	13 ± 1	40 ± 2	613 ± 227
ET–	61 ± 10	3	3	0 ± 0	7 ± 2	3753 ± 722	13 ± 2	37 ± 5	891 ± 222
Group 1	63 ± 15	3	6	76 ± 7	18 ± 5	6634 ± 1070	16 ± 2	50 ± 5	578 ± 285
Group 2	65 ± 11	9	2	22 ± 13	8 ± 3	4593 ± 946	13 ± 1	41 ± 3	652 ± 223
Group 3	61 ± 10	3	3	0 ± 0	7 ± 2	3753 ± 722	13 ± 2	37 ± 5	891 ± 222
Control	47 ± 9	4	7						

Table 1. Sample Category Statistics. Mean value and standard deviation of age, mutation burden, white blood cell count, red blood cell count, hemoglobin, hematocrit, and platelet count at the time of blood sampling are shown. Numbers of females and males are shown. The normal range of each test is indicated in the brackets with female (F) or male (M)-specific ranges if available. Categories based on disease are: PV for polycythemia vera; ET for total cases of essential thrombocythemia; ET+ for V617F-positive ET, ET– for V617F-negative ET; and control for healthy volunteers. Group 1, group 2, and group 3 represents cases with a V617F mutation burden 50%–100%, 1%–50%, and 0%–1%, respectively.

2. Expression profiles determined using qPCR array for JAK–STAT pathway

mRNA expression profiles in the peripheral blood of patients and controls were analyzed by a qPCR array (SABiosciences, PHAS-039) containing primers for JAK–STAT-related 84 genes:

A2M, SH2B2, BCL2L1, CCND1, CDKN1A, CEBPB, CRK, CRP, CSF1R, CSF2RB, CXCL9, EGFR, EPOR, F2, F2R, FAS, FCER1A, FCGR1A, ISG15, GATA3, GBP1, GHR, HMGA1, IFNAR1, IFNG, IFNGR1, IL10RA, IL20, IL2RA, IL2RG, IL4, IL4R, IL6ST, INSR, IRF1, IRF9, JAK1, JAK2, JAK3, JUN, JUNB, MMP3, MPL, MYC, NFKB1, NOS2, NR3C1, OAS1, OSM, PDGFRA, PIAS1, PIAS2, PPP2R1A, PRLR, PTPN1, PTPRC, SH2B1, SIT1, SLA2, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SOCS1, SOCS2, ***SOCS3****, SOCS4, SOCS5, SP1,* ***SPI1****, SRC, STAM, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6, STUB1, TYK2, USF1, YY1* with 5 housekeeping genes: *B2M, HPRT1, RPL13A, GAPDH, ACTB.*

We identified 14 genes that exhibited more than 2-fold higher expression in Group 1 than in the control (Table 2). Among them, 3 genes (*ACTB* encoding β actin, *SOCS3*, *SPI1*) were also upregulated in Group 2. We focused on *SOCS3* and *SPI1* for the subsequent analysis.

Gene	Group 1	Group 2	Group 3
1 *ACTB*	10.9	7.1	(-3.1)
2 *SOCS3*	7.5	2.2	(-1.7)
3 *JAK2*	4.4	(2.7)	(2.0)
4 *JAK3*	4.2	(2.0)	(1.7)
5 *FAS*	4.1	(2.4)	2.7
6 *SPI1*	3.9	2.1	(1.6)
7 *STAT5B*	3.6	(1.5)	(-1.5)
8 *IFNGR1*	2.7	(1.6)	(1.1)
9 *SP1*	2.6	(1.9)	(1.3)
10 *IRF9*	2.6	(1.6)	(-1.1)
11 *STUB1*	2.4	(1.6)	(1.8)
12 *GAPDH*	2.3	(-1.2)	2.0
13 *JUNB*	2.2	(-1.1)	(-1.3)
14 *FCGR1A*	2.1	(1.3)	-2.2

Table 2. Upregulated genes in the JAK–STAT signaling pathway in MPN patients. Selected genes analyzed by JAK–STAT-related PCR array that showed statistically significant ($p < 0.05$) upregulation between the group 1 and the control group are shown. The magnitude of differences for groups 2 and 3 are also shown. Positive and negative numbers indicate up- and downregulation, respectively. Numbers in parentheses indicate a lack of statistical significance ($p > 0.05$) in comparison with the control group.

3. Confirmation by individual qPCR assay

We confirmed upregulation of *SOCS3* and *SPI1* in patients by individual qPCR assay (Figure 1). Upregulation of *SOCS3* and *SPI1* genes were proportional to the *JAK2* V617F mutation burden.

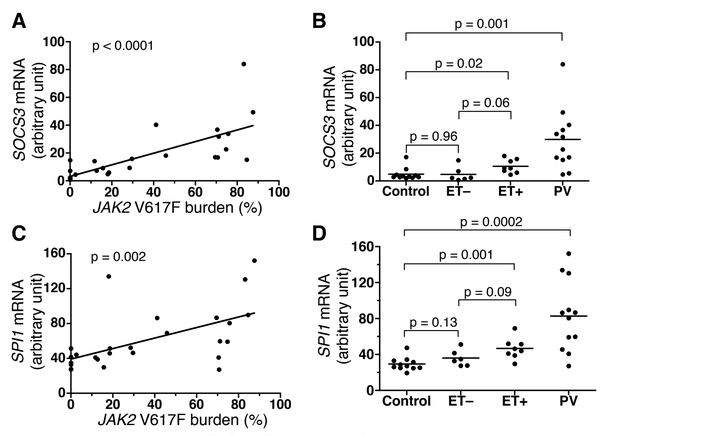

Figure 1. Expression of *SOCS3* and *SPI1* mRNA in MPN patients. A. *SOCS3* mRNA levels in peripheral blood of MPN patients determined by qPCR (not PCR array) were calibrated with quantities of *HPRT1* mRNA and plotted against *JAK2* V617F mutation burden. The values are represented with an arbitrary unit. The line and p value for the slope were calculated based on a linear regression model. B. *SOCS3* mRNA levels were plotted by disease category. Control represents the healthy volunteers, and ET– and ET+ represent V617F-negative and -positive ET patients, respectively. PV represents PV patients. The p values were calculated by a t-test. C. *SPI1* mRNA levels plotted as in A. D. *SPI1* mRNA levels plotted as in B.

4. Signal transduction for *SPI1* expression

SOCS3 upregulation in MPN patients was expected as JAK–STAT pathway is known to induce *SOCS3* expression. In contrast, *SPI1* induction by JAK–STAT signaling has not been reported in human. Therefore, we set up a series of experiments analyzing effects of JAK2 over- and downregulation on *SPI1* expression in two human cell lines: K562 cells, derived from chronic myelogenous leukemia, which have a wild-type *JAK2* gene and *BCR-ABL1* fusion gene; and HEL cells, derived from erythroid leukemia, which have a homozygous *JAK2* V617F mutation.

i) JAK2 overexpression

Wild-type and V617F-type JAK2 was introduced by a retroviral vector with puromycin-resistance gene into K562 and HEL cells. JAK2 overexpression was confirmed in puromycin-resistant K562 cells but not HEL cells. *SPI* mRNA amount was quantitated by qPCR and calibrated with ribosomal 18S RNA amount. *SPI1* induction by V617F-type JAK2 in K562 cells was observed (Figure 2).

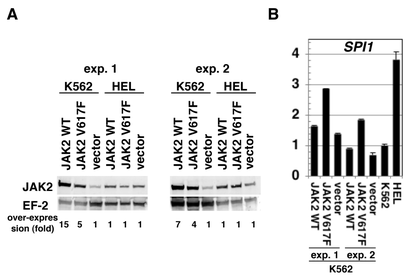

Figure 2. Induction of *SPI1* mRNA in K562 cells overexpressing V617F-type JAK2
A. Western blots showing the amounts of JAK2 protein in K562 and HEL cells infected with retrovirus vector encoding either wild-type (JAK2 WT) or V617F-type JAK2 (JAK2 V617F) or a mock vector (vector) and maintained in the presence of puromycin. The intensities of bands were calibrated with the band intensities of elongation factor 2 (EF-2) protein. Fold over-expression is shown below as the value for the mock infectant as 1. The results of two independent infections are shown under experiments (exp.) 1 and 2. B. *SPI1* mRNA levels in K562 cells prepared by the retroviral infection shown in A, along with those in non-infected K562 and HEL cells. The results were calibrated with *18S* ribosomal RNA amount and represented with an arbitrary unit.

ii) JAK2 knockdown

Three kinds of *JAK2* siRNA were electroporated into HEL and K562 cells. *SOCS3* and *SPI1* mRNA was quantitated by qPCR. One of siRNAs (siRNA3) downregulated *SOCS3* and *SPI1* expression in HEL cells but not in K562 cells.

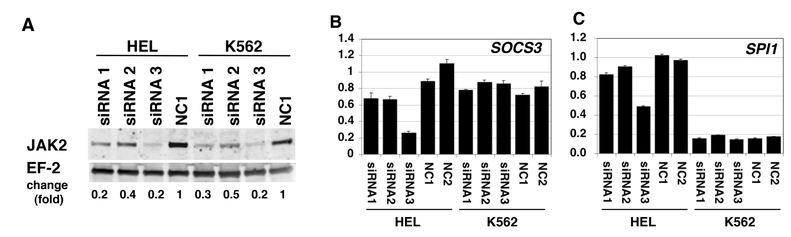

Figure 3. Reduction of *SOCS3* and *SPI1* mRNA in HEL cells transfected with *JAK2* siRNAs
A. Western blots of JAK2 protein in HEL and K562 cells treated with siRNAs against *JAK2* are shown. EF-2 was detected as a loading control. Cell lysates were prepared 24 h after siRNA transfection. Proteins derived from 1×10^5 cells were loaded onto each lane. Three types of siRNA against *JAK2* (siRNA1–3) and a negative control siRNA (NC1) were used. Fold change represents a ratio of band intensity of JAK2 and that of EF-2. B. *SOCS3* mRNA amount determined by qPCR. RNA was prepared 48 h after siRNA transfection. The values are expressed with an arbitrary unit as the mean of NC1 and NC2-treated HEL cells as 1. Error bars represent standard errors for triplicate measurements. C. *SPI1* mRNA amount shown as in B.

iii) Pharmacological inhibition of JAK2 and ABL1 kinases

Effects of JAK2 inhibition by a specific inhibitor, AG490, on *SOCS3* and *SPI1* expression was examined. As ABL1 can phosphorylate STAT3/5A/5B, effects of ABL1 inhibitor, imatinib, were examined similarly. AG490 inhibited *SOCS3* but not *SPI1* expression in HEL cells. Imatinib suppressed both *SOCS3* and *SPI1* expression in K562 cells (Figure 4).

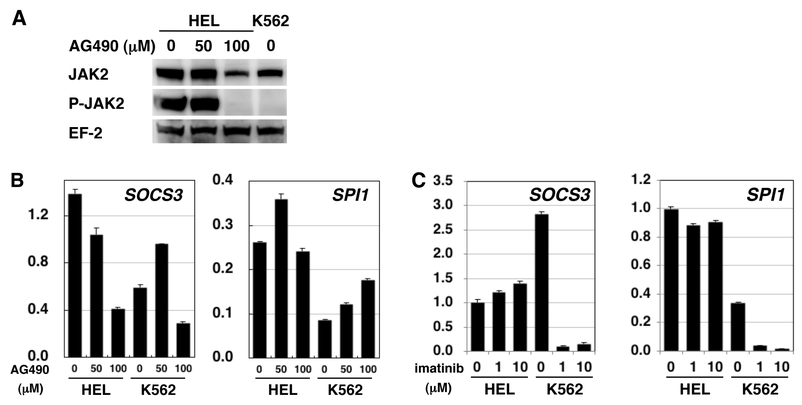

Figure 4. Effect of pharmacological inhibition of JAK2 and ABL1 kinases on *SOCS3* and *SPI1* expression
A. Western blots of total JAK2 and tyrosine 1007/1008-phosphorylated JAK2 (P-JAK2) are shown. EF-2 was detected as a loading control. Cell lysates were prepared 24 h after addition of indicated concentrations of AG490 to the culture medium. Proteins derived from 5×10^5 cells were loaded onto each lane. B. *SOCS3* and *SPI1* mRNA amount determined by qPCR in cells 24 h after addition of indicated concentrations of AG490. The values are expressed with an arbitrary unit. Data at 48 h were similar (not shown). C. *SOCS3* and *SPI1* mRNA amount determined by qPCR in cells 48 h after addition of indicated concentrations of imatinib. The values are expressed with an arbitrary unit.

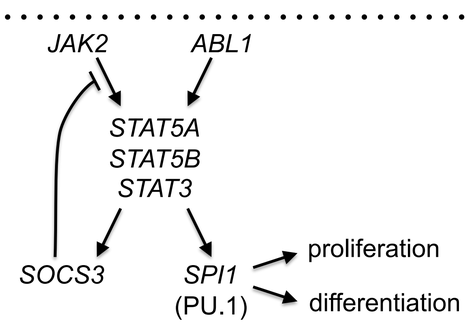

Figure 5. Proposed signaling pathways leading to *SPI1* and *SOCS3* gene expression.
Activation of JAK2 by ligand-induced dimerization of cytokine receptors or V617F mutation leads to activation of signal transducer and activator of transcription (STAT) 3/5 transcription factors, which activates transcription of known target *SOCS3* and a novel target *SPI1*, encoding a hematopoietic transcription factor PU.1. As STAT3/5 can be activated by ABL1, *SPI1* and *SOCS3* expression may be dependent on ABL1 activity activated in chronic myelogenous leukemia. The negative feedback inhibition of JAK2 by SOCS3 may account for the lack of *SPI1* inhibition by AG490 in HEL cells.

CONCLUDING REMARKS

We examined the expression profiles of 84 JAK–STAT-related genes in peripheral blood of MPN patients and identified 2 upregulated genes. One is a known target, *SOCS3*, and the other is a potentially novel JAK–STAT target, *SPI1*, encoding the transcription factor PU.1. In addition to JAK2, ABL1 kinase may induce *SPI1* expression. Molecular analysis of patient samples such as those reported here will facilitate further understanding of hematological diseases and eventually lead to improved patient care.