Genes Involved in Maintaining the Bone Marrow Stroma Are Dysregulated in Patients with Myelofibrosis: Lenalidomide Treatment Up-regulates *SOCS3*

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Abstract. *Aim: The purpose of the present study was to determine whether genes involved in the organization of the hematopoietic niche were dysregulated in patients with primary myelofibrosis (MF) treated with lenalidomide. Materials and Methods: We used reverse-transcription quantitative polymerase chain reaction to study the expression of a set of genes involved in the organization of the hematopoietic niche in peripheral blood and bone marrow (BM) mononuclear cell (MNC) samples from 32 patients with primary MF who participated in a phase II trial of lenalidomide plus prednisone. Results: At baseline (before treatment) cyclo-oxygenase 2 (COX2) was significantly up-regulated, while chemokine (C-X-C motif) receptor 4 (CXCR4), paired box 5 (PAX5) C-terminus, and hypoxia inducible factor 1A (HIF1A) were significantly down-regulated in BM MNCs from patients with primary MF compared to BM MNCs from healthy individuals. After 9 months of treatment, the expression of suppressor of cytokine signaling 3 (SOCS3) was significantly increased. Conclusion: Patients with primary MF showed aberrant expression of several genes involved in maintaining BM homeostasis and our findings suggest that treatment with lenalidomide plus prednisone up-regulates SOCS3.*

Progressive bone marrow (BM) fibrosis is a key feature of primary myelofibrosis (MF), a disease characterized by clonal myeloproliferation. The BM microenvironment comprises of stromal cells, osteoclasts and endothelial cells, and communication defects between these cells upon expansion of the neoplastic clone results in a functionally-disturbed stromal niche, impaired hematopoiesis and, eventually, BM fibrosis. Lenalidomide, an agent which modulates inflammatory cytokine secretion, angiogenesis and the expression of adhesion molecules likely has effects on the BM microenvironment (1). In a phase II trial of lenalidomide plus prednisone in 40 patients with MF conducted at our Institution, 30% of patients achieved an objective response (13). Furthermore, 10 out of 11 patients who responded and had grade 4 reticulin fibrosis at baseline had reduction in fibrosis to grade 2 or less. While newer therapies, such as janus kinase (JAK) inhibitors, have been shown to significantly improve symptoms of MF and quality of life, with the exception of minor reductions in BM fibrosis in some patients after many years of treatment with ruxolitinib (8), lenalidomide is the only therapy shown to significantly reduce BM fibrosis in patients with MF. We hypothesized that lenalidomide may exert its effects, in part, by modulating the expression of genes involved in maintaining the BM stromal niche. To test our hypothesis, we measured the expression of a set of genes involved the organization of the hematopoietic niche in peripheral blood (PB) and BM mononuclear cell (MNC) samples from patients with primary MF who participated in a phase II trial of lenalidomide plus prednisone (13). Genes involved in cell–stroma interactions (secreted protein, acidic, cysteine-rich [*SPARC*], chemokine [C-X-C motif] receptor 4 [*CXCR4*]), angiogenesis (cyclo-oxygenase 2 [*COX2*]), response to hypoxia (hypoxia inducible factor 1A [*HIF1A*], and cell differentiation and signaling (paired box 5 [*PAX5*] *C-terminus*, FBJ murine osteosarcoma viral oncogene homolog [*FOS*], Kristen rat sarcoma viral oncogene homolog [*KRAS*], suppressor of cytokine signaling 3 [*SOCS3*]) were profiled.

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Table I. *Primers used for quantitative reverse transcriptase–polymerase chain reactionq (qRT-PCR).*

Primer	Gene name	Primer sequence
COX2_F:	Cyclo-oxygenase 2	5'-CCT TCCTCCTGTGCCTGATG-3',
COX2_R:		5'-ACAATCTCATTTGAATCAGGAAGCT-3'
COX2-6FAM		5'-TGCCCGACTCCCTTGGGTGTCA-MGBNFQ
SPARC_R:	Secreted protein, acidic, cysteine-rich	5'-TCTTCCCTGTACACTGGCAGTTC-3'
SPARC_F:		5'-AGCTCGGTGTGGGAGAGGTA-3'
SPARC-6FAM		5'-CAGCTGGACCAGCACCCATTGACA-MGBNFQ
HIF1A_F:	Hypoxia inducible factor 1A	5'-CTCATCCAAGAAGCCCTAACGTGTT-3'
HIF1A_R:		5'-GCTTTCTCTGAGCATTCTGCAAAGC-3'
HIF1A-6FAM		5'-CCTCAGGAACTGTAGTTCTTTGACTCAAAGCGACA-MGBNFQ
CXCR4:	Chemokine (C-X-C motif) receptor 4	Hs00607978_s1 (Applied Bioscience)
PAX5C	Paired box 5 C-terminus	Hs00277134_m1 (Applied Bioscience)
FOS_F:	FBJ murine osteosarcoma viral oncogene homolog	5'-CGAGCCCTTTGTATGACTTCCT-3'
FOS_R:		5'-GTCCATGTCTGGCACGGA G-3'
FOS-6FAM		5'-CCCAGCATCATCCAGGCCCAGTCA-MGBNFQ
KRAS_F:	Kristen rat sarcoma viral oncogene homolog	5'-TTCCTACAGGAAGCA AGT AG-3'
KRAS_R:		5'-CACAAAGAA AGCCCTCCCCA-3'
KRAS-6FAM		5'-TTGATGGAGAAACCTGTCTCTTGGCA-MGBNFQ
β-Actin_F:		5'-GATGGCCACGGCTGCTT-3'
β-Actin _R:		5'-ACCGCTCATTGCCAATGG-3'
β-Actin -6FAM		5'-ACCACCACGGCCGAGCGGCA-MGMNFQ

F: Forward primer; R: reverse primer; 6FAM: 6-carboxyfluorescein; MGBNFQ: molecular-groove binding non-fluorescence quencher.

Materials and Methods

All patients gave written informed consent and the study was approved by the Institutional Review Board (PA11-1122) and performed in accordance with the Declaration of Helsinki. BM and PB samples from six hematologically healthy individuals were purchased from Stem Cell Technologies (Vancouver, Canada). BM aspirates and PB samples were available for 13 patients with primary MF. Sequential BM and PB samples were collected at baseline and every 3 months during the course of treatment. However, samples were not available for all 13 patients at all time points due to the fact that some patients discontinued treatment or died, or the samples were of poor quality. For this reason, baseline BM samples available for nine patients and baseline PB samples available for 11 patients (13 patients total) were used for our analysis. Low-density mononuclear cells (MNCs) were isolated from BM aspirates and PB samples using gradient centrifugation with Ficoll Hypaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). Total RNA was isolated from gradient-separated MNCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription was performed with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify the expression levels of *SPARC, COX2, CXCR4, PAX5* C-terminus, *SOCS3, HIF1A* and β-actin (reference gene) using primer pairs obtained from Applied Biosystems Inc. (Foster City, CA, USA). The primer sequences used are listed in Table I. qRT-PCR was performed in duplicate for each sample. Gene expression was calculated as ΔCT values, using β-actin as the reference gene. Data are presented as mean ΔCT values with 95% confidence intervals. Student's *t*-tests were

Table II. *Baseline characteristics of 13 patients whose samples were analyzed in this study.*

Characteristic	Value
Age, median (range), years	65 (51-83)
Gender, N (%)	
Male	7 (54)
Female	6 (46)
Cytogenetics, N (%)	
Diploid	6 (46)
Abnormal	7 (54)
JAK2^{V617F} mutation, N (%)	
Positive	7 (54)
Negative	4 (31)
Not determined	1 (8)
Hemoglobin, mean (range), g/dL	10.1 (8.1-16.4)
Platelet count, mean (range), $\times 10^9$/L	244 (18-704)
WBC count, mean (range), $\times 10^9$/L	12 (1.3-28)
Spleen size, mean (range), cm	8.9 (0-22)
Performance status, median (range)	1 (0-2)
Prior therapy, N (%)	8 (69)
Number of prior therapies, median (range)	1 (0-3)
Best response (IWG-MRT)*, N (%)	
Stable disease	6 (46)
Clinical improvement	5 (38)
Partial response	1 (8)
Complete hematological response	1 (8)
Duration of response, median (range), months	9 (3-59)

IWG-MRT: International Working Group for Myelofibrosis Research and Treatment; JAK: janus kinase; *IWG-MRT criteria published in 2006 were used.
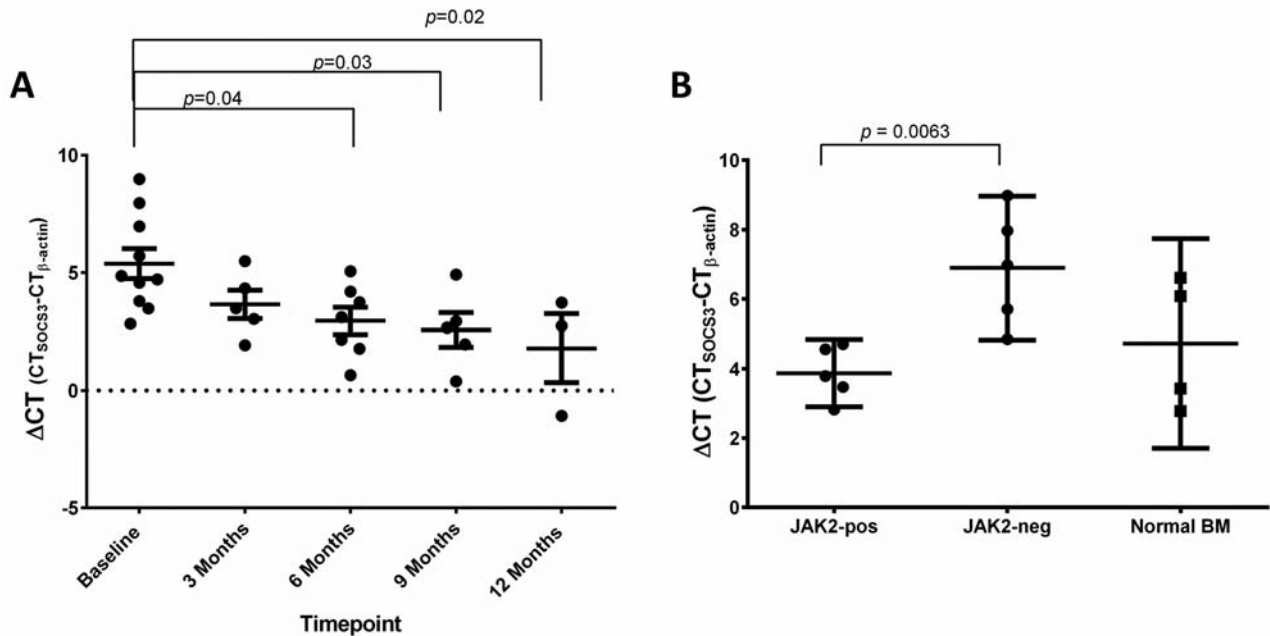
Figure 1. *Changes in suppressor of cytokine signaling 3 [SOCS3] gene expression. A: Expression of SOCS3 increased significantly with time on treatment (p=0.02 using one-way analysis of variance). Mean expression at 9, 12 and >14 months was significantly higher than at baseline, as assessed by Dunnett's multiple comparisons test. Horizontal bars represent median±standard deviation. p<0.05 was considered statistically significant. B: SOCS3 expression was significantly higher in patients with the Janus kinase 2 V617F mutation.*

Table III. *Comparison of mean relative gene expression in patients with primary myelofibrosis and healthy controls at baseline.*

| Gene | Gene name | Patients | | Controls | | |
		No. of samples analyzed	Mean ΔCT (95% CI)	No. of samples analyzed	Mean ΔCT (95% CI)	*p*-Value*
SPARC-BM	Secreted protein, acidic, cysteine-rich	9	4.953 (3.84-6.06)	6	6.207 (5.62-6.79)	0.0659
SPARC-PB		11	5.852 (4.17-7.54)	6	4.941 (3.93-5.96)	0.4113
COX2-BM	Cyclo-oxygenase 2	9	7.259 (6.27-8.25)	6	8.998 (8.08-9.92)	0.0132
COX2-PB		11	7.509 (6.32-8.70)	6	11.40 (9.74-13.1)	0.0004
KRAS-BM	Kristen rat sarcoma viral oncogene	9	11.60 (7.33-15.87)	6	6.548 (4.526-8.570)	0.0538
KRAS-PB		11	12.35 (8.24-16.46)	6	7.974 (7.05-8.90)	0.1064
FOS-BM	FBJ murine osteosarcoma viral oncogene homolog	9	2.656 (1.952-3.359)	6	2.720 (1.77-3.67)	0.895
FOS-PB		11	3.349 (2.38-4.32)	5	4.503 (3.53-5.48)	0.1197
CXCR4-BM	Chemokine (C-X-C motif) receptor 4	9	5.072 (3.98-6.17)	6	2.707 (2.30-3.11)	0.0017
CXCR4-PB		11	4.351 (3.33-5.37)	4	5.284 (4.618-5.949)	0.2575
PAX5C-BM	Paired box 5 C-terminus	6	9.539 (7.83-11.25)	4	6.473 (4.80-8.15)	0.0108
PAX5C-PB		11	9.971 (8.64-11.31)	3	7.482 (5.83-9.13)	0.0592
SOCS2-BM	Suppressor of cytokine signaling 2	8	11.47 (8.46-14.47)	4	8.828 (7.76-9.90)	0.1634
SOCS2-PB		11	13.19 (10.41-15.97)	2	9.400 (6.80-12.00)	0.7099
SOCS3-BM	Suppressor of cytokine signaling 3	10	5.384 (3.946-6.822)	4	4.724 (1.71-7.74)	0.5838
SOCS3-PB		11	5.011 (3.50-6.52)	3	5.562 (2.15-8.97)	0.6984
HIF1A-BM	Hypoxia inducible factor 1A	10	7.303 (6.34-8.27)	4	5.106 (4.20-6.01)	0.0095
HIF1A-PB		11	6.259 (5.39-7.13)	3	6.32 (5.90-6.74)	0.9383

*Differences in mean expression between patient sample and control samples were compared using unpaired *t*-tests. *p*<0.05 was considered significant. CT: cycle threshold value; CI: confidence interval; BM: bone marrow; PB: peripheral blood.

used to compare mean ΔCT values from patient samples at baseline (before treatment) and healthy controls. One-way analysis of variance was used to compare the mean ΔCT values at different time points.

Results and Discussion

Clinical characteristics of the 13 patients whose samples we studied are shown in Table II. At baseline (before treatment) *COX2* was significantly up-regulated, while *CXCR4, PAX5* C-terminus, and *HIF1A* were significantly down-regulated in BM MNCs from patients compared to healthy BM MNCs (Table III). Expression of *SPARC, KRAS, SOCS3,* and *FOS* and were not significantly different. Although *SOCS3* has been shown to be down-regulated in primary MF, in part due to hypermethylation of its promoter, we only detected a significant difference in the expression of *SOCS3* in patients with JAK2-negative primary MF and normal controls (Figure 1B). There were no significant differences in relative gene expression between BM MNC and PB samples; however, when compared with samples from normal controls, significant down-regulation of *CXCR4* and *HIF1A* in primary MF was only observed in the BM samples. This may be expected since changes in the expression of these genes are likely to be more prominent in the BM.

Treatment with lenalidomide plus prednisone had no effect on the expression of *SPARC, COX2, CXCR4, PAX5* C-terminus, or *HIF1A*. However, the expression of SOCS3 was significantly increased after 9 months of treatment (Figure 1A), suggesting that some of the clinical effects of lenalidomide may be due to a SOCS3-mediated reduction in JAK signaling. Interestingly, at baseline, *SOCS3* expression was significantly lower in patients without the $JAK2^{V617F}$ mutation than in those with the mutation (p=0.0063; Figure 1B), which is in agreement with a previous study(3). By contrast, there was no correlation between *JAK2* mutation status and the expression of the other genes. In addition, there was no correlation between expression levels and cytogenetic abnormalities.

Up-regulation of *COX2* and down-regulation of *CXCR4, PAX5C*, and *HIF1A* may reflect disruptions in the interactions between cells in the BM microenvironment in primary MF. For example, down-regulation of *CXCR4* in primary MF has been shown in several studies and is thought to contribute to the increased circulation of CD34^{+} cells in primary MF (1, 6, 15). The observed down-regulation of *HIF1A* in the BM from patients with primary MF is consistent with studies showing that a less hypoxic BM microenvironment promotes the aberrant proliferation of hematopoietic progenitor cells in MPNs (2, 9, 14). *PAX5* encodes a transcription factor that plays a key role in B-cell development (11). Thus, the reduction in *PAX5C* expression in primary MF BM compared with normal BM, may be due

to the expansion of the myeloid lineage in primary MF. Finally, the up-regulation of *COX2*, an enzyme involved in the formation of prostaglandins, which are key mediators of inflammation and angiogenesis (among other functions), is consistent with the increased inflammation and angiogenesis seen in BM in primary MF (5, 10, 12).

In T-cells from BM in multiple myeloma, lenalidomide has been shown to decrease *SOCS1* expression (4). How lenalidomide increases *SOCS3* expression in primary MF is therefore not clear. It is intriguing that SOCS3 has been shown to act as part of an E3 ubiquitin ligase complex to promote ubiquitinylation (and hence degradation) of JAK2 and the interleukin 6 receptor common chain (gp130) given the recent finding that lenalidomide exerts its action, in part, by binding to cereblon as part of an E3 ubiquitin ligase complex (7). In conclusion, we found that patients with primary MF show aberrant expression of several genes involved in maintaining BM homeostasis and our findings suggest that treatment with lenalidomide plus prednisone up-regulates *SOCS3*. These results should be validated in a larger cohort of patients treated with lenalidomide.

Acknowledgements

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