Methylation of the suppressor of cytokine signaling 3 gene (*SOCS3*) in myeloproliferative disorders

Nasios Fourouclas,[1] Juan Li,[1] Daniel C. Gilby,[2] Peter J. Campbell,[1] Philip A. Beer,[1] Elaine M. Boyd,[3] Anne C. Goodeve,[2] David Bareford,[4] Claire N. Harrison,[5] John T. Reilly,[2] Anthony R. Green,[1] and Anthony J. Bench[1,3]

[1]University of Cambridge, Department of Haematology, Cambridge Institute for Medical Research, Cambridge; [2]Academic Unit of Haematology, School of Medicine and Biomedical Science, Sheffield; [3]Department of Haematology, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge; [4]Department of Haematology, Russell's Hall Hospital, Dudley, and [5]Department of Haematology, St. Thomas's Hospital, London, UK

ABSTRACT

Background

The *JAK2* V617F mutation can be found in patients with polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis. Mutation or methylation of other components of JAK/STAT signaling, such as the negative regulators suppressor of cytokine signaling 1 (*SOCS1)* and *SOCS3*, may contribute to the pathogenesis of both *JAK2* V617F positive and negative myeloproliferative disorders.

Design and Methods

A cohort of patients with myeloproliferative disorders was assessed for acquired mutations, aberrant expression and/or CpG island hypermethylation of *SOCS1* and *SOCS3*.

Results

No mutations were identified within the coding region of either gene in 73 patients with myeloproliferative disorders. No disease-specific CpG island methylation of *SOCS1* was observed. *SOCS1* expression was raised in myeloproliferative disorder granulocytes but the level was independent of *JAK2* V617F status. Hypermethylation of the *SOCS3* promoter was identified in 16 of 50 (32%) patients with idiopathic myelofibrosis but not in patients with essential thrombocythemia, polycythemia vera or myelofibrosis preceded by another myeloproliferative disorder. Confirmation of methylation status was validated by nested polymerase chain reaction and/or bisulphite sequencing. *SOCS3* transcript levels were highest in patients with polycythemia vera and other *JAK2* V617F positive myeloproliferative disorders, consistent with *SOCS3* being a target gene of JAK2/STAT5 signaling. There was a trend towards an association between *SOCS3* methylation and lower *SOCS3* expression in *JAK2* V617F negative patients with idiopathic myelofibrosis but not in *JAK2* V617F positive ones. Finally, *SOCS3* methylation was not significantly correlated with survival or other clinical variables.

Conclusions

SOCS3 promoter methylation was detected in 32% of patients with idiopathic myelofibrosis suggesting a possible role for *SOCS3* methylation in this disorder. The pathogenetic consequences of *SOCS3* methylation in idiopathic myelofibrosis remain to be fully elucidated.

Key words: *SOCS3*, *SOCS1*, hypermethylation, myeloproliferative disorders.

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Correspondence:
Anthony J. Bench, Haemato-oncology Diagnostic Service, Department of Haematology, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK.
E-mail: anthony.bench@addenbrookes.nhs.uk

The online version of this article contains a supplemental appendix.

Introduction

An acquired valine to phenylalanine mutation of Janus kinase 2 (JAK2) at position 617 is present in the vast majority of patients with polycythemia vera (PV).[1-5] Furthermore, approximately one half of patients with the related myeloproliferative disorders (MPD), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF), carry the same mutation.[6-10] *In vivo* expression of the JAK2 V617F mutant protein results in erythrocytosis and eventual myelofibrotic transformation in mouse models[2,11-14] demonstrating the crucial pathogenetic role that the *JAK2* V617F mutation plays in MPD. Furthermore, in PV, the *JAK2* V617F mutation arises in a very early hematopoietic progenitor with both myeloid and lymphoid potential.[15,16]

JAK2 is a cytoplasmic tyrosine kinase that is crucial for effective signaling through type I cytokine receptors including receptors for erythropoietin, thrombopoietin, interleukin-3, granulocyte-macrophage colony-stimulating-factor (GM-CSF) and granulocyte colony-stimulating-factor (G-CSF).[17] Homozygous knockout of murine *Jak2* results in embryonic death at day 12.5 due to a complete absence of definitive erythropoiesis.[18,19] The *JAK2* V617F mutation results in cytokine independent activation of JAK/signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathways. Acquired mutations affecting other components of these signaling pathways are likely to contribute to the pathogenesis of *JAK2* V617F-negative MPD. Indeed, mutations within the receptor for thrombopoietin (*MPL*) have been detected among *JAK2* V617F-negative cases of ET and IMF.[20-23] Furthermore, such mutations may also modulate the degree of JAK2 kinase activity leading to the variable phenotype observed among patients with *JAK2* V617F-positive disease.

Suppressor of cytokine signaling (SOCS)1 and SOCS3 are negative regulators of the JAK/STAT pathway[17] and are therefore attractive candidates. They are rapidly induced by a broad spectrum of cytokines including erythropoietin, interleukin-3, and GM-CSF.[17] Expression of *SOCS1* and *SOCS3* leads to reduced JAK and STAT phosphorylation, reduced STAT dimerization and import to the nucleus and reduced transcription of target genes.[24] Unlike the other members of the SOCS family, SOCS1 and SOCS3 contain a 12 amino acid region termed the kinase inhibitory region (KIR) which binds to and inactivates the catalytic JH1 domain of JAK proteins.[24] SOCS1 binds phosphorylated JAK2 directly, whereas SOCS3 inhibits JAK2 while bound to a cytokine receptor, e.g. erythropoietin receptor.[25,26] SOCS proteins may also target JAK2 for proteosome-mediated degradation.[17] *Socs1*$^{-/-}$ mice have low B-cell numbers due to defective interferon-γ signaling.[27] *Socs3*$^{-/-}$ mice die *in utero* due to fetal liver erythrocytosis and overexpression of *SOCS3* blocks fetal liver erythrocytosis[28] implying that SOCS3 plays a critical role in the negative regulation of definitive erythropoiesis.

Aberrant CpG island hypermethylation of tumor suppressor genes is a well recognized mechanism that can lead to tumorigenesis. Methylation and down-regulation of *SOCS1* has been demonstrated in a number of hematologic malignancies including myelodysplasia and chronic myeloid leukemia[29,30] although conflicting results have been obtained in some diseases. Methylation of the *SOCS3* promoter and reduced gene expression have been detected in patients with lung cancer, head and neck squamous cell carcinoma, hepatocellular carcinoma and Barrett's adenocarcinoma.[31-34]

We, therefore, wanted to investigate the contribution, if any, of aberration(s) of *SOCS1* and *SOCS3* in the pathogenesis of MPD. A large cohort of patients with a MPD were examined. The coding region and splice sites of *SOCS1* and *SOCS3* were assessed for acquired mutations. The methylation status of the CpG islands within the promoter and exon 2 of *SOCS1* and within the promoter of *SOCS3* were investigated. Transcript levels of *SOCS1* and *SOCS3* were also determined. Finally, we examined whether expression and/or methylation were associated with outcome and other clinical and laboratory variables.

Design and Methods

Patients' samples

This study was approved by the appropriate Regional Ethics Committees. Peripheral blood granulocytes and mononuclear cells were prepared by Ficoll gradient centrifugation.[35] Genomic DNA from 73 MPD patients [15 with PV, 25 with ET and 33 with myelofibrosis (MF)] was assessed for acquired mutations. The analysis of the methylation status of the CpG islands within the promoter and exon 2 of *SOCS1* and/or within the promoter of *SOCS3* was conducted on a total of 100 patients (15 with PV, 25 with ET and 60 with MF). Methylation status was also determined for DNA from 34 normal individuals; in 19 cases the DNA was extracted from peripheral blood, in 8 cases from purified granulocytes, and in 7 cases from bone marrow. *SOCS1* and *SOCS3* transcript levels were determined in 112 MPD patients (20 with PV, 48 with ET and 44 with MF), 6 patients with idiopathic erythrocytosis (IE) and 11 normal individuals. Ten MF patients had myelofibrosis secondary to another MPD (5 PV, 5 ET) and two MF patients showed coexisting myelodysplastic features. All patients were diagnosed according to current criteria.[36-38] *JAK2* V617F and *MPL* exon 10 status were determined by allele-specific polymerase chain reaction (PCR).[1,20]

Mutation analysis of SOCS1 and SOCS3

Genomic DNA was amplified by PCR and the coding regions and splice sites of *SOCS1* and *SOCS3* were sequenced (*Online Supplementary Table S1*).

Methylation analysis of SOCS1 CpG islands

The structure of *SOCS1* and the location of the CpG islands are shown in Figure 1A. The methylation-specific primers (MSP) and unmethylation-specific primers

(UMSP) were described by Liu *et al.*[30] (promoter region) and Yoshikawa *et al.*[39] (exon 2 region). Genomic DNA from patients, normal individuals and methylated control samples [5 peripheral blood DNA samples that had been methylated using CpG methylase (New England Biolabs, Hitchin, UK)] were modified with sodium bisulphite[40] and the CpG island regions were amplified by PCR as previously described.[30,39] PCR products were analyzed by agarose gel electrophoresis, purified and sequenced as described elsewhere.[40]

Methylation analysis of the SOCS3 *CpG island*

The structure of *SOCS3* and the location of the CpG island are shown in Figure 2A. Primers were designed using the MethPrimer program.[41] Primer sequences and their location relative to the transcription start site were: methylation specific primers; *SOCS3-MF1*, 5'-GAGGGGTCGTTGTTAGGAAC3', nt -1265; *SOCS3*-MR1, 5'ACAAAAACCGAAAAAAACGC3', nt -1176; unmethylation specific primers; *SOCS3*-UNF1, 5'-

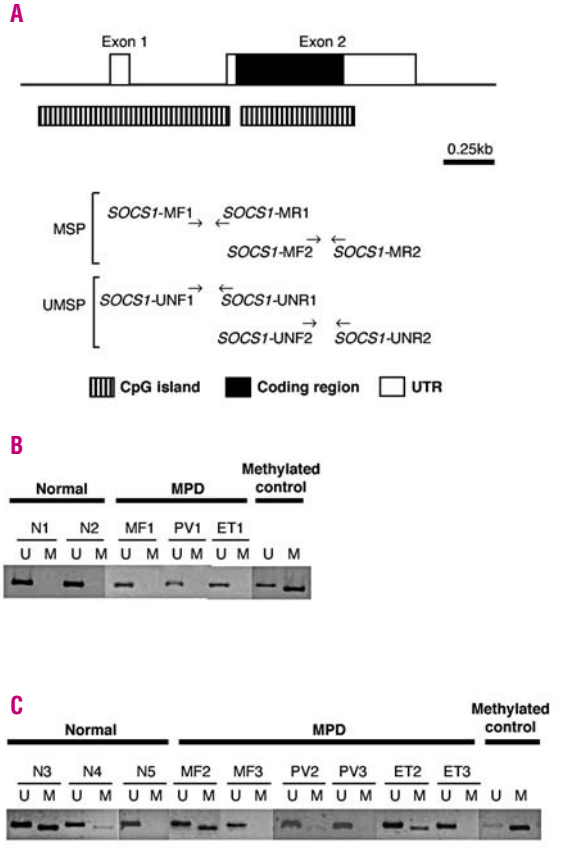

Figure 1. Methylation analysis of *SOCS1* CpG islands. (A) Structure of *SOCS1* showing the location of CpG islands, methylation-specific primers (MSP) and unmethylation-specific primers (UMSP). UTR; untranslated region. (B) Methylation-specific PCR amplification of the *SOCS1* promoter CpG island. U, unmethylated; M, methylated. N1, N2, normal; MF1, myelofibrosis; PV1, polycythemia vera; ET1, essential thrombocythemia. (C) Methylation-specific PCR amplification of the *SOCS1* exon 2 CpG island. U, unmethylated; M, methylated. N3, N4, N5, normal; MF2, MF3, myelofibrosis; PV2, PV3, polycythemia vera; ET2, ET3, essential thrombocythemia. For both CpG islands, methylated control DNA yields a PCR product within both the unmethylated and methylated-specific PCR due to mispriming of UMSP primers.

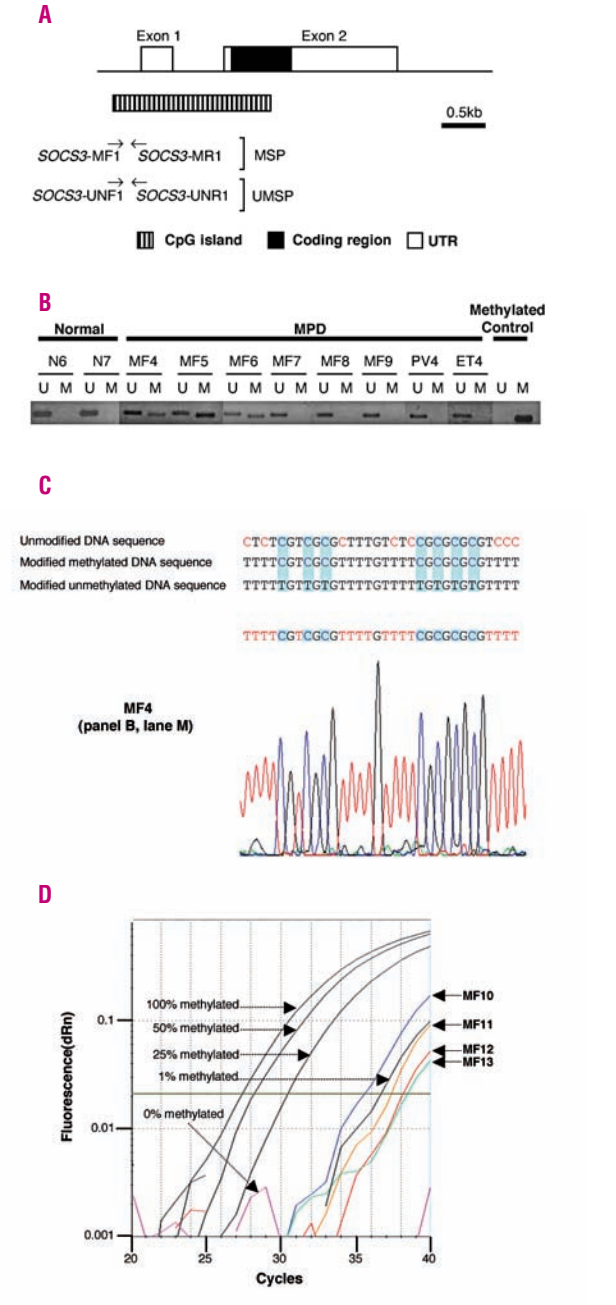

Figure 2. Methylation analysis of the *SOCS3* CpG island. (A) Structure of *SOCS3* showing the location of the CpG island, the methylation-specific primers (MSP) and unmethylation-specific primers (UMSP). UTR; untranslated region. (B) Methylation-specific PCR amplification of the *SOCS3* promoter CpG island. U, unmethylated; M, methylated. N6-N7, normal; MF4-MF9, myelofibrosis; PV4, polycythemia vera; ET4, essential thrombocythemia. A methylated CpG island was identified in samples MF4, MF5 and MF6. (C) Sequence of the methylation-specific PCR product from patient MF4. The expected sequence corresponding to methylated CpG and unmethylated CpG dinucleotides is shown. Non-CpG cytosine residues are shown in red (converted to 'T' in bisulphite-modified DNA) whilst CpG cytosine residues are shown in blue (remaining as 'C' in bisulphite-modified methylated CpG dinucleotides). Patient MF4 showed complete methylation of all CpG dinucleotides examined. (D) Real time PCR detection of *SOCS3* promoter CpG island methylation. MF10-MF13, myelofibrosis. Control samples containing methylated DNA at known concentrations (100%, 50%, 25%, 1% and 0% methylated) are indicated. The results for mononuclear cells are shown for patients MF10 and MF13 while the results using granulocyte DNA are shown for patients MF11 and MF12.

GGAGGGGTTGTTGTTAGGAAT3', nt -1266; *SOCS3*-UNR1, 5'CAAAAACAAAAACCAAAAAAA-ACA3', nt -1175. Bisulphite-modified DNA from patients, normal individuals or the methylated control (0.5 μg) was amplified by PCR in a 25 μL reaction volume containing 1X PCR buffer (Applied Biosystems, Warrington, UK), 1.5 mM $MgCl_2$, 0.2 mM dNTP and 1 μM of each primer using 1.25 units AmpliTaq Gold (Applied Biosystems) on a thermal cycler (PTC-200, MJ Research Inc., MA, USA). The PCR conditions were 94°C for 11 min followed by 40 cycles of 94°C for 30 s, an annealing temperature of 61°C for 30 s and extension at 72°C for 30 s with a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and sequencing.

SOCS3 promoter CpG island methylation was quantified using a Stratagene MX3000P sequence detection system (Stratagene, Amsterdam, The Netherlands). Bisulphite-modified DNA (0.5 μg) from purified granulocytes and/or mononuclear cells was PCR-amplified in duplicate in a 25 μL reaction volume containing 1X SYBR Green mix (Stratagene), 0.6 μM of each primer and 0.03 μM ROX reference dye (Stratagene). For methylation-specific PCR, the primers described above were utilized. For unmethylation-specific PCR, the primers were *SOCS3*-UNF1 and *SOCS3*-UNR2 (5'CAACCAAAACAACCAATAAACAC3', nt -1227). Amplification conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and an annealing temperature of 60°C (methylation-specific PCR) or 61°C (unmethylation-specific PCR) for 1 min followed by a final dissociation cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. A dilution series of 0%, 1%, 25%, 50%, 75% and 100% control methylated DNA, diluted with unmethylated DNA, was prepared. A standard curve was created by plotting ΔCt (Ct(UMSP) – Ct(MSP)) against percentage methylation for this series. This standard curve was utilized to determine percentage methylation within samples from patients. To verify that the MSP PCR products were derived from methylated DNA, nested PCR was carried out. Five microliters of each PCR product were amplified in a 25 μL reaction containing 1X SYBR Green mix (Stratagene), 0.6 μM of each primer and 0.03 μM ROX reference dye (Stratagene). Nested primers were: *SOCS3*-MF1-nested, 5'GTTGTTAGGAACGCGTCGTC3', nt -1257; *SOCS3*-MR1-nested, 5'AACAAAACGCGACGAA-AAAC3', nt -1203. Amplification conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 61°C for 1 min followed by a final dissociation cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s.

PCR products from the first round *SOCS3* methylation-specific quantitative PCR were cloned. Five microliters of each product were purified using 2 μL of ExoSAP-IT (USB Corporation, Cleveland, USA) according to the manufacturer's protocol and then diluted by the addition of 3 μL of water. The products were then cloned using the GeneJET™ PCR cloning kit (Fermentas, York, UK). Twelve colonies from each reaction were PCR-amplified using pJET1 sequencing primers (Fermentas) and sequenced.

Evaluation of SOCS1 *and* SOCS3 *expression levels*

Quantitative real-time reverse transcriptase-PCR was performed on a Stratagene MX3000P sequence detection system. RNA was prepared from 11 normal individuals, 112 MPD patients and 6 IE patients (the source of RNA being peripheral blood in 2 cases, purified granulocytes in 123 cases and purified mononuclear cells in 48 cases). Approximately 1 μg of total RNA was reverse transcribed as described elsewhere.[35] Duplicate reactions with 5 μL aliquots of cDNA were used for real-time PCR in a final volume of 25 μL containing 1X SYBR Green mix (Stratagene), 0.3 μM of each primer and 0.03 μM of ROX reference dye (Stratagene). Oligonucleotide primers were designed using the Primer3 program.[42] PCR product sizes, primer sequences and location relative to the 5' end of the mRNA were: *SOCS1* (NM_003745): 92 bp; *SOCS1*-F, 5'TGGTAGCACA-CAACCAGGTG3', nt 156; *SOCS1*-R, 5'GAGGAG-GAAGAGGAGGAAGG3', nt 247; *SOCS3* (NM_003955): 110 bp; *SOCS3*-F, 5'CAAGGACGGA-GACTTCGATT3', nt 300; *SOCS3*-R, 5'-GGAGCC-AGCGTGGATCTG3', nt 409; *ABL* (NM_005157): 118 bp; *ABL*-F, 5'GCGTGAGAGTGAGAGCAG3', nt 456; *ABL*-R, 5'CTCTCGGAGGAGACGTAG3', nt 573. Amplification conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min followed by a final dissociation cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. The threshold was set as dRn = 0.21. The mean threshold cycle (Ct) was calculated for each transcript and the *SOCS1* and *SOCS3* transcript levels relative to *ABL* were calculated using the equation $2^{-\Delta Ct(SOCS-ABL)}$.

Statistical analysis

Overall survival was assessed using the Kaplan-Meier method and groups were compared by the log-rank test. Cox proportional hazards regression analysis was carried out to assess expression levels. Calculations were performed using SigmaPlot 10 (Systat Software, London, UK) and MedCalc (Mariakerke, Belgium).

Results

Mutation analysis of SOCS1 *and* SOCS3

The coding regions and all splice sites of both *SOCS1* and *SOCS3* were amplified and sequenced. No mutation was identified in any of the 73 MPD patients (15 with PV, 25 with ET, and 33 with MF) indicating that inactivation of *SOCS1* and *SOCS3* by acquired mutation is not a frequent finding within MPD.

Methylation assessment of SOCS1 *and* SOCS3

SOCS1 and *SOCS3* CpG island methylation was then assessed in 73 patients with a MPD and in 34 normal individuals. Three CpG islands were assessed: *SOCS1* promoter and exon 2 regions (Figure 1A) and *SOCS3* promoter region (Figure 2A). No hypermethylation of the *SOCS1* promoter region was detected in normal individuals or in 68 MPD patients (Figure 1B, Table 1). However, methylation of *SOCS1* exon 2 region was observed in 53% (18/34) of normal individuals (in all cell

types assessed) and in 38% (28/73) of MPD patients (Figure 1C, Table 1). These results are in agreement with previous reports on the detection of methylation of the exon 2 region in normal individuals.[29,43]

We next assessed the CpG island within the *SOCS3* promoter. Hypermethylation of the *SOCS3* promoter was identified by methylation-specific PCR in 5/33 patients with MF but not in any of the 15 PV or 23 ET patients or 34 normal samples assessed (Figure 2B, Table 1). Methylation of the *SOCS3* promoter CpG island was confirmed in three MF patients by bisulphite sequencing (Figure 2C). All CpG dinucleotides had a methylated cytosine. Quantification of *SOCS3* promoter methylation was next assessed in purified granulocytes and/or peripheral blood mononuclear cells from a total of 37 MF patients of whom 27 had not been previously assessed (Figure 2D). The quantitative PCR had a sensitivity of approximately 0-1% and identified *SOCS3* promoter methylation in an additional 11 out of these 37 MF patients (in purified granulocytes from 7 patients, purified mononuclear cells from 3 patients and in both from 1 patient). The methylation level was low (<1%) in ten patients but was significant (>10%) in

Table 1. Methylation status of *SOCS1* and *SOCS3* CpG islands in myeloproliferative disorders patients.

CpG island	*Normal*	*MPD*		
		MF	*ET*	*PV*
SOCS1 promoter	0/34	0/33	0/20	0/15
SOCS1 exon2	18/34	17/33	6/25	5/15
SOCS3 promoter	0/34	16/60	0/23	0/15

MPD: myeloproliferative disorder; MF: myelofibrosis; PV: polycythemia vera; ET: essential thrombocythemia.

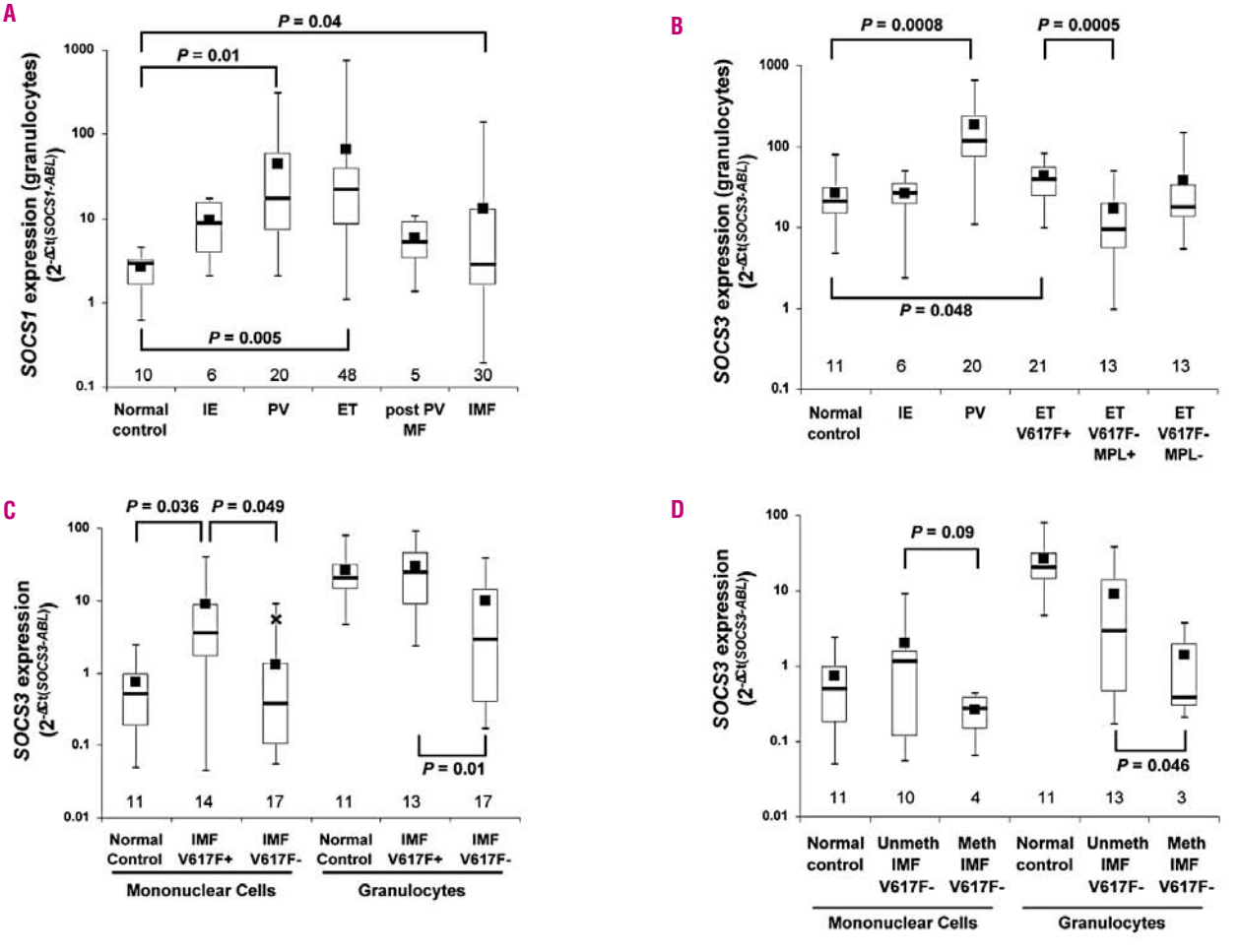

Figure 3. Expression of *SOCS1* and *SOCS3* in myeloproliferative disorders. (A) *SOCS1* expression in granulocytes. Boxes represent the interquartile range that contains 50% of the values; the horizontal line marks the median value; the filled square represents the mean value and the bars indicate the range of values. IE: idiopathic erythrocytosis; PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis; IMF: idiopathic myelofibrosis. Values at the base of the graph indicate the number of patients within each group. Bars indicate the *p* value (t-test) of differences between groups. (B) *SOCS3* expression in granulocytes. Annotation as for (A). (C) *SOCS3* expression in granulocytes and mononuclear cells from idiopathic myelofibrosis patients. The cross represents a patient carrying a *MPL* W515L mutation. Other annotations as for (A). (D) *SOCS3* expression in granulocytes and mononuclear cells from *JAK2* V617F-negative idiopathic myelofibrosis patients according to methylation status. Unmeth, Unmethylated *SOCS3* promoter; Meth, Methylated *SOCS3* promoter. Other annotations as for (A).

peripheral blood mononuclear cells from patient MF10 (Figure 2D). In each case, the methylation status was verified by nested PCR, molecular cloning and/or sequencing (*data not shown*). Overall, *SOCS3* promoter methylation was identified in 27% (16/60) of MF patients either by qualitative or quantitative methylation-specific PCR (Table 1). Of these 16 patients, 9 also carried a *JAK2* V617F mutation but none had a mutation within *MPL* exon 10.

Levels of expression of SOCS1 *and* SOCS3

SOCS1 and/or *SOCS3* expression in MPD patients may be abnormal as a result of CpG island methylation, aberrant signal transduction pathways or another unknown mechanism. We, therefore, measured *SOCS1* and *SOCS3* transcript levels within granulocytes of normal individuals, IE patients and MPD patients (20 with PV, 48 with ET, 30 with IMF, 5 with post-PV MF and 5 with post-ET MF) (Figure 3). We also measured *SOCS1* and/or *SOCS3* transcript levels in peripheral blood mononuclear cells from normal individuals and IMF patients. Granulocyte *SOCS1* transcript levels appeared to be slightly higher among IE patients than among normal individuals (Figure 3A) although this difference was not statistically significant. Granulocyte *SOCS3* expression was very similar in IE patients and in normal individuals (Figure 3B).

The transcript levels of both *SOCS1* and *SOCS3* were increased in granulocytes from *JAK2* V617F-positive PV patients (*SOCS1*: p=0.013 compared to levels in normal individuals; p=0.03 compared to levels in IE patients and *SOCS3*: p=0.0008 compared to levels in normal individuals) (Figure 3A, B). Granulocyte *SOCS1* transcript level was also increased in ET patients (p=0.005; Figure 3A) with no significant effect of *JAK2* V617F status (*data not shown*). *SOCS3* expression was only moderately raised in the granulocytes from *JAK2* V617F-positive ET patients (p=0.048) while it was similar to normal levels in *JAK2* V617F-negative ET patients (Figure 3B). We also specifically assessed *JAK2* V617F-negative ET patients known to carry a mutation within *MPL*[20] (n=13). Among the *JAK2* V617F-negative patients, *MPL* mutation status did not appear to affect expression level. However, ET patients carrying a *MPL* exon 10 mutation did show a significantly lower *SOCS3* transcript level than *JAK2* V617F-positive patients (p=0.0005; Figure 3B). Ten patients whose myelofibrosis had transformed from an MPD (5 with PV, 5 with ET of whom one was *JAK2* V617F-positive) were also assessed. The expression level of *SOCS1* was similar to that observed in normal granulocytes and was dramatically lower than that in untransformed MPD cases (Figure 3A and *data not shown*). By contrast, the expression level of *SOCS3* in the same group of patients was similar to that in patients with untransformed PV or ET (*data not shown*).

SOCS1 and *SOCS3* transcript levels were assessed in granulocytes and/or mononuclear cells from 34 IMF patients of whom 15 carried a *JAK2* V617F mutation and one carried a *MPL* W515L mutation. Granulocytes from IMF patients showed moderately raised expression of *SOCS1* (p=0.04 compared to the level in normal individuals; Figure 3A) but transcript level did not correlate with *JAK2* V617F status (*data not shown*). No significant difference was seen within peripheral blood mononuclear cells. The levels of *SOCS3* expression in mononuclear cells from *JAK2* V617F-positive IMF patients was higher than that in both normal individuals and *JAK2* V617F-negative patients (Figure 3C). This was not related to the number of myeloid precursor cells present within the mononuclear cell population. Granulocytes from *JAK2* V617F-positive IMF patients showed a higher *SOCS3* transcript level than did those from *JAK2* V617F-negative patients but a similar level to granulocytes from normal individuals (Figure 3C). Hence, in both granulocytes and mononuclear cells, *SOCS3* expression was higher in *JAK2* V617F-positive IMF patients than in *JAK2* V617F-negative patients. Compared to most other *JAK2* V617F-negative cases, the patient carrying a *MPL* W515L mutation expressed high levels of *SOCS3* within mononuclear cells (Figure 3C).

SOCS3 expression data for granulocytes and/or mononuclear cells were available for 30 patients in whom methylation status had been assessed (11 methylated; 19 unmethylated). Surprisingly, there was no difference in the mean *SOCS3* transcript level in either granulocytes or mononuclear cells between IMF patients carrying a methylated *SOCS3* promoter and those with an unmethylated *SOCS3* promoter. Since there is a correlation between *JAK2* V617F status and *SOCS3* expression level in IMF (Figure 3C), we separately analyzed IMF patients according to *JAK2* V617F status. Of the 17 patients carrying a wild type *JAK2* V617 allele, four had a methylated *SOCS3* promoter (1 within granulocytes only; 3 within mononuclear cells only). Restricting the analysis to *JAK2* V617-wild type patients only, there was a trend towards reduced *SOCS3* expression in patients carrying a methylated promoter compared to those without *SOCS3* methylation (Figure 3D; granulocytes: 1.4±2.2 v 9.0±6.4, p=0.046; mononuclear cells: 0.3±0.2 v 2.0±1.8, p=0.09). No difference was observed for *JAK2* V617F-positive patients (*data not shown*).

Correlation with clinical and laboratory factors

Of the 60 MF patients in whom *SOCS3* methylation was assessed, 50 had idiopathic myelofibrosis and, among these, two showed coexisting myelodysplastic features. In ten patients, the myelofibrosis was secondary to a pre-existing MPD (5 PV, 5 ET). All 16 patients demonstrating *SOCS3* promoter methylation were classified as having IMF suggesting that *SOCS3* methylation is not merely a reflection of myelofibrotic transformation of a previously unrecognised MPD. Within the IMF patients, there was no association between *SOCS3* promoter methylation and patient's sex, age, hemoglobin level, white cell count, Lille prognostic score, platelet count, blast count, fibrosis grade, *JAK2* V617F status or the presence of an abnormal karyotype. Survival data were available for 47 of the 50 IMF patients assessed. As previously described,[9] the presence of a *JAK2* V617F mutation was associated with shorter survival (p=0.026). However, there was no sig-

nificant difference in the length of survival between patients with *SOCS3* promoter methylation and those without (p=0.67) (Figure 4). Furthermore, *SOCS3* methylation status did not affect the survival of *JAK2* V617F-negative IMF patients or of any subgroup based on Lille prognostic score (*data not shown*).

Concerning expression levels, despite the higher level of *SOCS3* transcript seen in *JAK2* V617F-positive patients in our study, the level of *SOCS3* transcript showed no significant correlation with survival. Likewise, *SOCS1* transcript level was not correlated with length of survival. There was also no significant correlation between *SOCS1* or *SOCS3* transcript level and patient's sex, age, hemoglobin level, white cell count, Lille prognostic score, platelet count, blast count or grade of fibrosis.

Discussion

Here, we describe hypermethylation of the *SOCS3* locus in a significant proportion of patients with IMF but not in patients with PV or ET. CpG island methylation has been infrequently assessed in patients with a MPD. Jones *et al.*[44] detected methylation of the retinoic acid receptor-β gene (*RARB*) in 89% (16/18) of IMF patients and also showed reduced expression in $CD34^+$ cells. However, Jost *et al.*[45] failed to detect methylation of *RARB* in any of 23 MF patients. Hypermethylation of the *ABL1, CALCA, CDH1, CDKN4A, CDKN4B, DAPK1, MGMT, NPM1, TIMP2* and *TP73* genes has been reported, albeit usually in low percentages of patients.[45-50] No methylation of *TGFBR2* CpG islands was detected in PV or IMF.[51,52]

Recently, Jost *et al.*[45] reported methylation of *SOCS1* in 15% of MPD patients. However, the primers utilized in their study correspond to the CpG island within exon 2 and the *SOCS1* promoter region was not assessed. Although we also observed methylation of the exon 2 CpG island in 38% of MPD patients, the same pattern was detected in 53% of normal individuals. Results from our study and others in which both the promoter and the exon 2 regions of *SOCS1* have been assessed[29,43] suggest that methylation of the *SOCS1* exon 2 CpG island, but not the promoter region, is a variable feature in blood cells of normal individuals. The relevance of *SOCS1* exon 2 CpG island methylation for leukemogenesis in general and for the pathogenesis of the MPD in particular is, therefore, unclear and should be considered with caution.

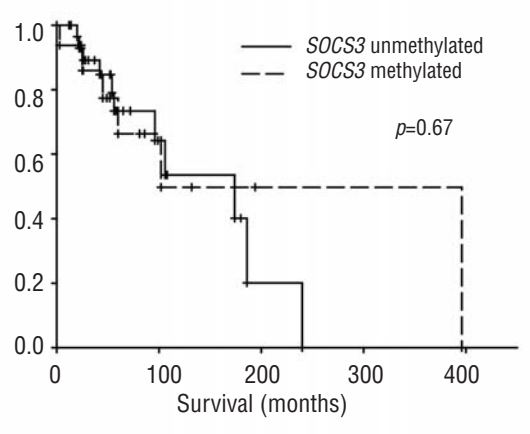

Figure 4. Survival curves of idiopathic myelofibrosis patients according to *SOCS3* promoter methylation status. Sixteen patients carried a methylated *SOCS3* allele and 31 patients had no *SOCS3* promoter methylation.

SOCS1 expression was increased, to varying degrees, in most types of MPD. Raised *SOCS1* mRNA levels have also been detected in bone marrow of MPD patients using formalin-fixed bone marrow trephines.[53] In contrast to our findings, this study failed to demonstrate significantly raised expression in granulocytes from patients with PV but only seven patients were examined (compared to 20 in our analysis) and a different control gene and *SOCS1* primers were used. *SOCS1* expression can be induced by a number of cytokines including many interleukins, erythropoietin and GM-CSF[17] but not thrombopoietin.[54] Although JAK2 is targeted by SOCS1 leading to reduced phosphorylation of JAK2 and STAT5, it is not clear whether STAT5A or STAT5B transcriptionally activate *SOCS1* directly. Indeed, the *SOCS1* promoter contains binding sites for STAT1, STAT3 and STAT6.[55] *JAK2* V617F status did not significantly affect SOCS1 expression in MPD and, unlike for *SOCS3*, transformation to myelofibrosis resulted in normalization of *SOCS1* transcript level. Taken together, these observations suggest that activation of *SOCS1* transcription within MPD may be independent of activated JAK2/STAT5.

SOCS3 represents an attractive target gene in both *JAK2* V617F-positive and *JAK2* V617F-negative MPD for a number of reasons. Firstly, SOCS3 plays a crucial negative regulatory role in erythroid differentiation as shown by the fact that $Socs3^{-/-}$ mice die as a result of erythrocytosis[28] whereas $Jak2^{-/-}$ mice die due to the absence of erythropoiesis.[18,19] Conversely, mice overexpressing transgenic *SOCS3* lack fetal liver erythrocytosis.[28] Secondly, *SOCS3* expression is induced by a number of cytokines including interleukin-3, thrombopoietin, erythropoietin and GM-CSF,[17] growth factors to which hematopoietic progenitors from MPD patients show hypersensitivity.[56] Thirdly, SOCS3 directly inhibits JAK2-related signaling by binding to a cytokine receptor and interacting with JAK2.[24] Finally, hypermethylation of *SOCS3* promoter has been demonstrated in a number of malignancies.[31-34,57] We detected *SOCS3* promoter methylation in 32% (16/50) of patients with IMF but not in patients with PV, ET or myelofibrosis preceded by another MPD. Hence, epigenetic modification of *SOCS3* represents a novel mechanism by which JAK/STAT signaling may become aberrant within MPD. When assessed, the level of *SOCS3* methylation was low suggesting that *SOCS3* methylation represents a secondary acquired change in IMF.

Cell lines carrying a methylated *SOCS3* promoter show no expression and treatment with the demethylating agent 5-aza-2'-deoxycytidine results in reactivation of *SOCS3* transcription confirming the link between methylation and transcriptional inactivation.[31,32,34] Hence, a reduction in *SOCS3* transcript level

would be expected in patients carrying a methylated promoter. Surprisingly, in the IMF group as a whole, *SOCS3* promoter methylation did not correlate with reduced transcript level. However, within the *JAK2* V617F-negative IMF group, there was a trend towards reduced *SOCS3* expression in those patients carrying a methylated promoter. Within the cell populations assessed, only a proportion of cells carried a methylated *SOCS3* promoter. Hence, it is possible that any affect of *SOCS3* methylation would be masked. Additionally, *JAK2* V617F-positive patients may exhibit increased expression in those cells not carrying a methylated *SOCS3* promoter. The pathogenetic consequences of *SOCS3* methylation for IMF, therefore, remain to be fully investigated. To clarify these observations, additional patients will be required and it will be important to assess *SOCS3* methylation and expression within megakaryocytes and myeloid progenitors of both *JAK2* V617F-positive and negative patients.

SOCS3 transcription is upregulated by cytokine-induced signal transduction and transcript levels are raised in PV, as shown in this study and by Kralovics *et al.*[58] Although we did not quantify *JAK2* V617F tumor burden, our data show that *SOCS3* expression is, in general, highest in those patients with increased levels of *JAK2* V617F. *JAK2* V617F-positive PV patients showed higher levels than did *JAK2* V617F-positive ET patients reflecting the increased V617F burden in PV compared to ET.[13,59-61] ET patients without a *JAK2* V617F mutation displayed a pattern similar to that of normal individuals in agreement with recent data that a proportion of *JAK2* V617F-negative ET patients do not have activation of the JAK/STAT pathway.[62] Similarly, for IMF, *SOCS3* transcript levels were higher among *JAK2* V617F-positive patients than among *JAK2* V617F-negative cases. Among the patients with ET, those who were *JAK2* V617F-positive displayed higher levels of *SOCS3* within granulocytes than those who were *MPL* mutation-positive, reflecting the lineage specificity of *MPL* expression.

In addition to increased *SOCS3* transcription, PV patients carrying a biallelic *JAK2* V617F mutation also show higher levels of phosphorylated SOCS3.[63] Although SOCS3 inhibits wild type JAK2 and targets it for degradation, it has no such effect on mutant JAK2 and SOCS3 may even enhance the proliferative effect of the mutant JAK2 V617F protein.[63] Hence, inactivation of *SOCS3*, for example by methylation, may not offer a proliferative advantage in *JAK2* V617F-positive PV patients. This may explain why methylation of *SOCS3* was observed in IMF but not in PV and also why methylation may only affect total *SOCS3* mRNA level in *JAK2* V617F-negative IMF patients.

A number of possible explanations exist for the different phenotypes of *JAK2* V617F-positive MPD patients; transformation of different progenitor cells, inherited genetic differences and additional, acquired genetic or epigenetic modifications.[64] Current data support the hypothesis that acquired changes modulate JAK2 kinase activity and modify the MPD phenotype. Mitotic recombination of 9p24, leading to duplication of the *JAK2* V617F mutation (homozygosity), has been detected in erythroid progenitors of most PV patients but not in ET.[61] By contrast, neither mutations in *MPL*[22] nor methylation of *SOCS3* (this study) have been detected in PV suggesting that homozygosity for *JAK2* V617F may be sufficient for the development of PV. The role of *SOCS3* methylation in patients with *JAK2* V617F-positive IMF remains to be elucidated.

The pathogenetic mechanisms underlying *JAK2* V617F-negative cases of MPD are thought to reflect abnormalities affecting cytokine receptor signaling. In support of this, for example, *JAK2* V617F-negative ET patients frequently demonstrate features of a *JAK2* V617F-positive MPD such as erythropoietin independent erythroid colonies, abnormal megakaryocyte morphology and overexpression of *PRV-1*.[8] Acquired mutations within the *MPL* gene have been detected in up to 8% of IMF patients and in up to 4% of ET patients but not in PV patients.[20-23] Hypermethylation of *SOCS3* represents another acquired aberration affecting one component of a signaling pathway within *JAK2* V617F-negative cases of IMF. The identification of promoter hypermethylation affecting components of signaling pathways raises the possibility of the use of demethylating agents as potential therapy in patients with myelofibrosis.[65]

Authorship and Disclosures

NF, JL, JTR, ARG and AJB conceived and designed the strategy; NF, JL, DCG, PJC, PAB, EMB, ACG and AJB carried out the research; PJC, PAB, DB, CNH, JTR and ARG identified appropriate patients and acquired clinical data; NF, JL, ARG and AJB wrote the manuscript. All authors reviewed and contributed to the manuscript. The authors reported no potential conflicts of interest.

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