

blood

2004 103: 4630-4635
Prepublished online Feb 19, 2004;
doi:10.1182/blood-2003-06-2007

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SOCS1 and *SHP1* hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway

Chor-Sang Chim, Tsz-Kin Fung, Wai-Chung Cheung, Raymond Liang, and Yok-Lam Kwong

SOCS1 and SHP1 negatively regulate the Janus kinase/signal transducer and activator of transcription (Jak/STAT) signaling pathway. The role of promoter hypermethylation leading to epigenetic inactivation of *SOCS1* and *SHP1* in myeloma was investigated. The methylation-specific polymerase chain reaction (MSP) was used to define *SOCS1* and *SHP1* methylation in 34 diagnostic myeloma samples. For *SOCS1*, MSP primers 3' to the translation start site were unreliable and gave positive results in normal controls. However, primers in the 5' promoter region were specific, although no myeloma

samples showed methylation. For *SHP1*, 27 of 34 (79.4%) myeloma samples showed *SHP1* hypermethylation. The biologic significance of *SHP1* methylation was investigated in the U266 human myeloma line. U266 contained completely methylated *SHP1*. Furthermore, there was constitutive STAT3 phosphorylation. Treatment with 5-azacytidine led to progressive demethylation of *SHP1* on days 2 to 5, with consequent increasing reexpression of *SHP1* as shown by reverse transcription-polymerase chain reaction (RT-PCR). Concomitant with increasing *SHP1*, a parallel down-regulation of phos-

phorylated STAT3 occurred, so that by day 5 phosphorylated STAT3 was barely detectable. The overall survivals of patients with and without *SHP1* methylation were similar. *SHP1* methylation leading to epigenetic activation of the Jak/STAT pathway might have a tentative role in the pathogenesis of myeloma, which should be further confirmed by functional studies in primary myeloma samples. (Blood. 2004;103:4630-4635)

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Introduction

Multiple myeloma is characterized by neoplastic proliferation of monoclonal plasma cells. These neoplastic plasma cells are thought to be derived from a postgerminal center B cell, which migrates to the bone marrow, adheres to the marrow stroma, and triggers subsequent bone resorption and a paracrine cytokine loop that involves interleukin-6 (IL-6).^{1,2} The natural course of the disease may progress through monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma, intramedullary myeloma, and eventually extramedullary myeloma. MGUS is a precursor of myeloma, and the median interval from discovery of the M protein to the development of myeloma might be up to 10 years.^{3,4}

The binding of cytokines to their receptors results in the dimerization of receptor complexes and activation of the Janus family of protein tyrosine kinases (Jak's),⁵⁻⁷ followed by phosphorylation of the cytoplasmic signal transducers and activators of transcription (STATs). Upon phosphorylation, STATs form homodimers or heterodimers, migrate to the nucleus, and activate gene transcription. In myeloma, binding of the IL-6 to its membrane receptor induces dimerization of receptor subunit gp130 and results in activation of Jak by cross-phosphorylation.⁸ This Jak/STAT pathway is subject to negative regulation by 3 families of proteins, the protein inhibitors of activated STATs (PIAS), the suppressors of cytokine signaling (SOCS), and the SH2-containing phosphatases (SHP).^{6,7,9}

The SOCS family comprises at least 8 members characterized by the presence of a central Src homology (SH2) domain and a conserved carboxy-terminal "SOCS box."^{6,7} SOCS members are

cytokine-inducible negative regulators of the cytokine signaling. An important member is SOCS1. The *SOCS1* gene can be induced by a multitude of cytokines, including IL-1, IL-3, IL-6, erythropoietin, granulocyte-macrophage colony-stimulating factor, and γ -interferon.¹⁰⁻¹² Human *SOCS1* is a single-exon gene encoding 211 amino acids and lies within a CpG island spanning 2.5 kilobase (kb). The expression of *SOCS1* impairs cellular response to IL-6 through direct interaction with Jak proteins.¹³

SHP1, a member of the SHP family of proteins, is a 68-kd, cytoplasmic protein tyrosine phosphatase (PTP).¹⁴ The human *SHP1* gene consists of 17 exons and spans approximately 17 kb of DNA. It contains 2 tandem Src homology (SH2) domains, a catalytic domain, and a C-terminal tail of about 100 amino acid residues.¹⁴ In contrast to the ubiquitous expression of the structurally related *SHP2*, *SHP1* is primarily expressed in hematopoietic cells, and considered a putative tumor suppressor gene in lymphoma and leukemias, because it antagonizes the growth-promoting and oncogenic potentials of protein tyrosine kinase.¹⁴

DNA methylation, catalyzed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide, leading to a conversion to methylcytosine.¹⁵⁻¹⁷ In many cancers, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression of these genes.¹⁸ This may serve as an alternative epigenetic mechanism of gene inactivation.^{19,20}

Dysregulation of the IL-6/Jak/STAT pathway has been shown to be involved in hematologic cancers. For instance, activation of

From the Department of Medicine, Queen Mary Hospital, Hong Kong.

Submitted June 19, 2003; accepted February 7, 2004. Prepublished online as *Blood* First Edition Paper, February 19, 2004; DOI 10.1182/blood-2003-06-2007.

Supported by the Kadoorie Charitable Foundation.

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STAT3 is frequent in primary human myeloma or myeloma cell line,¹³ and blocking of either IL-6 receptor, Jak, or STAT3 inhibits the expression of Bcl-X_L and promotes apoptosis in myeloma cells. Moreover, a TEL-Jak2 fusion protein is expressed as a consequence of t(9;12)(p24;p13) in some T-cell acute lymphoblastic leukemias (T-ALLs).²¹ Recently, hypermethylation of *SOCS1* has been separately reported in hepatoma.^{22,23} In this study, we investigated the potential involvement of *SOCS1* and *SHP1* promoter hypermethylation in the pathogenesis of myeloma.

Patients, materials, and methods

Patients, diagnosis, and treatment

The diagnosis of myeloma was made according to standard criteria with marrow plasmacytosis, presence of paraprotein, and osteolytic bone lesions.²⁴ Complete staging work-up included bone marrow examination, skeletal survey, serum and urine protein electrophoresis, serum immunoglobulin (IgG, IgA, and IgM) levels, renal function tests, and serum calcium level.²⁵ Monoclonal immunoglobulins were identified by cellulose acetate or agarose-gel electrophoresis. When an abnormal band or equivocal pattern was detected, immunoelectrophoresis or immunofixation was performed. All blood tests were repeated every 6 months, and skeletal survey was repeated yearly. Primary treatment included either VAD (vincristine, 0.4 mg/d, 4 times daily; adriamycin, 9 mg/m²/d, 4 times daily; and dexamethasone, 40 mg/d, days 1 to 4, 9 to 12, and 17 to 20) or MP (melphalan, 0.15 mg/kg/d, for 7 days; prednisolone, 60 mg/d, for 7 days). On reaching a plateau phase, eligible patients received either allogeneic or autologous hematopoietic stem cell transplantation (HSCT). Patients who were not candidates for HSCT, and who showed disease progress from the plateau phase, received melphalan or cyclophosphamide depending on initial treatment. Involved field radiotherapy was given to relieve local symptoms. Patients gave informed consent to treatment, and the study was approved by the institutional review board of Queen Mary Hospital, Hong Kong.

Methylation-specific polymerase chain reaction (MSP)

High-molecular-weight genomic DNA was isolated by standard protocols from diagnostic bone marrow aspirates, myeloma cell line AF10 (kindly provided by Professor Zelig Eshhar, Department of Immunology, Weizmann Institute of Science, Israel), and leukemic cell lines (HL60, U937, Jurkat, and Raji). The methylation-specific polymerase chain reaction

(MSP) for promoter methylation was performed as described.²⁶⁻²⁸ Briefly, treatment of DNA with bisulfite (which resulted in conversion of unmethylated cytosine to uracil, but unaffected methylated cytosine) was performed with a commercially available kit (CpGenome DNA modification kit; Intergen, New York, NY). MSP primers were designed to amplify the methylated (M-MSP) and unmethylated (U-MSP) alleles. For *SOCS1*, 2 sets of primers (MSP3' and MSP5') were adopted from previous studies.^{22,23} These 2 sets of MSP primers were located 5' (MSP5')²³ and 3' (MSP3')²² to the translation start site of *SOCS1*. Both sets of primers could be identified on NT_010393.11, and the nucleotide positions of these 2 sets of *SOCS1* primers with reference to GenBank sequence GI: 27486099 (NT_010393.11), subregion: complement (2121832 to 2123597) are listed in Table 1. The relative positions with respect to the translation start site are depicted in Figure 1. Notably, the forward and reverse primers of MSP3' (adopted from Yoshikawa et al²²) mapped to nucleotide positions 400 to 423 and 537 to 559 for methylated DNA and 391 to 423 and 537 to 565 for unmethylated DNA in GenBank sequence U88326. The MSP primers for *SHP1* are listed in Table 1. There are 2 isoforms of SHP1, one expressed in nonhematopoietic tissues and the other in hematopoietic tissues,³⁰ which use different promoters, P1 and P2, located upstream of exon 1 and exon 2, respectively. MSP primers were selected for the hematopoietic-specific P2 promoter. For all experiments, DNA from the peripheral blood of 12 healthy blood donors and 3 healthy bone marrow donors was used as negative control, and methylated DNA (CpGenome Universal Methylated DNA; Intergen) was used as positive control. MSP was performed in a thermal cycler (9700; PE Biosystems, Foster City, CA) with the following cycling conditions: 95°C for 12 minutes, 35 to 45 (Table 1) cycles of 95°C for 45 seconds, specific annealing temperature (Table 1) for 30 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. The polymerase chain reaction (PCR) mixture contained 50 ng bisulfite-treated DNA, 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 2 mM MgCl₂, 10 pmol of each primer, 1 × PCR Buffer II, and 2.5 units of AmpliTaq Gold (PE Biosystems) in a final volume of 50 μL. Ten microliters of PCR products were loaded onto 6% nondenaturing polyacrylamide gels, electrophoresed, and visualized under ultraviolet light after staining with ethidium bromide. Previous experiments had shown that the MSP had a sensitivity of 10⁻³ to 10⁻⁵ for detecting the methylated allele.^{27,31}

DNA sequencing

The identity of the methylated and unmethylated sequences was confirmed by automated DNA sequencing. PCR products were gel purified, sequenced bidirectionally, and analyzed on an automated DNA sequence analyser (3700 ABI Prism; PE Biosystems).

Table 1. Methylation-specific polymerase chain reaction (MSP): primer sequences and reaction conditions

Gene	Forward primer: 5'-3'	Reverse primer: 5'-3'	Temperature, °C (no. cycles)	PCR products, bp	Reference no.
SOCS1: MSP3'*					
M-MSP	TTC GCG TGT ATT TTT AGG TCG GTC (nt: 1081-1104)	CGA CAC AAC TCC TAC AAC GAC CG (nt: 1218-1240)	63 (35)	159	Yoshikawa et al ²²
U-MSP	TTA TGA GTA TTT GTG TGT ATT TTT AGG TTG GTT (nt: 1072-1104)	CGA CAC AAC TCC TAC AAC GAC CG (nt: 1218-1246)	63 (35)	174	
SOCS1: MSP5'*					
M-MSP	GTT GTA GGA TGG GGT CGC GGT CGC (nt: 186-209)	CTA CTA ACC AAA CTA AAA TCC ACA (nt: 335-312)	63 (35)	149	Nagai et al ²³
U-MSP	GTT GTA GGA TGG GGT TGT GGT TGT (nt: 186-209)	CTA CTA ACC AAA CTA AAA TCC ACA (nt: 335-312)	63 (35)	149	
SHP1†					
M-MSP	GAA CGT TAT TAT AGT ATA GCG TTC (nt: 6857-6880)	TCA CGC ATA CGA ACC CAA ACG (nt: 6995-7015)	60 (40)	158	Oka et al ²⁹
U-MSP	GTG AAT GTT ATT ATA GTA TAG TGT TTG G (nt: 6857-6882)	TTC ACA CAT ACA AAC CCA AAC AAT (nt: 6993-7015)	59 (35)	158	

Tm indicates annealing temperature; M-MSP, methylation-specific polymerase chain reaction for the methylated allele; U-MSP, methylation-specific polymerase chain reaction for the unmethylated allele; nt, nucleotide; and bp, base pairs.

*Accession no.: GI: 27486099 (NT_010393.11), subregion: complement (2121832 to 2123597).

†Accession no.: X82818.



Figure 1. Schematic diagram of the location of the 2 sets of MSP primers (MSP5' and MSP3') for *SOCS1* in relation to the translation start site.

5-Azacytidine (5-AC) treatment of the myeloma cell line U266

The human myeloma cell line U266 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI supplemented with 10% fetal calf serum. For treatment with 5-AC, U266 cells were seeded in 25 cm² culture flasks at a density of 10⁶/mL and treated with 3 μM 5-AC (Sigma, St Louis, MO) for 5 days, with fresh medium containing 5-AC replenished on day 2. U266 cells were harvested on days 1, 2, 4, and 5 for genomic DNA, total cellular RNA, and whole cell protein extractions.

Reverse transcription polymerase chain reaction (RT-PCR) for *SHP1*

One microgram of total cellular RNA was reverse transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Rockville, MD), and 2 μL of the resulted cDNA was amplified by PCR with *SHP1*-specific primers (forward: 5'-GGC ACT GGG AGC TGC ATC TGA GGC-3'; and reverse: 5'-CTC GCA CAT GAC CTT GAT GTG-3'³⁰). The identity of the RT-PCR product was confirmed by DNA sequencing.

Western blot

Whole-cell lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (PALL, Pensacola, FL). The membrane was blocked with 5% bovine serum albumin (Sigma) and immunoblotted with antibodies against phosphorylated STAT3 (Tyr705) (Cell Signaling Technology, Beverly, MA) and nonphosphorylated STAT3 (Zymed Laboratories, South San Francisco, CA). Equal loading of protein (7 μg for each lane) was confirmed by immunoblotting with an anti-β-actin antibody (Sigma).

Statistical analysis

Overall survival (OS) was measured from the date of diagnosis to the date of death or last follow-up. For patients undergoing HSCT, OS was censored at the time of transplantation. Survival curves were computed by the Kaplan-Meier method and compared by the log-rank test. All *P* values were 2-sided.

Results

Patients

Thirty-four patients (19 male, 15 female) at a median age of 62 years (range, 25 to 87 years) were studied. In these patients, the bone marrow plasma cells ranged from 38% to 89% (median, 56%). The types of myeloma were IgG (*n* = 21, 62%), IgA (*n* = 7, 21%), light chain (*n* = 5, 14%), and IgD (*n* = 1, 3%). The Durie-Salmon staging was stage 1 (*n* = 5, 14.7%), stage 2 (*n* = 12, 35.3%), and stage 3 (*n* = 17, 50%). Six patients (17.6%) had impaired renal function at diagnosis. The median diagnostic paraprotein level was 29.8 g/L (range, 10 to 78 g/L). Eighteen patients received 3 courses of VAD and then MP until plateau phase or maximum response, whereas 16 patients received MP until maximum response or plateau phase. Eight patients underwent HSCT (2 allogeneic, 6 autologous) during the plateau phase.

MSP for *SOCS1*

With primers MSP3' (selected from the CpG islands inside exon 1 of the *SOCS1* gene), M-MSP was positive in 6 of 12 normal

peripheral blood DNAs, 2 of 3 normal marrows, and the methylated positive control DNA (Figure 2A). In 1 of the normal marrow samples and 4 of the 6 normal peripheral blood samples showing positive methylation signals, DNA sequencing of the M-MSP product confirmed authentic *SOCS1* amplification (Figure 2B). Several observations were made from the sequence analysis of these M-MSP products from normal marrow and blood samples. First, the authenticity of methylation was confirmed by the finding of multiple unconverted cytosine molecules at "CpG" dinucleotides (Figures 2B). Second, not all CpG dinucleotides within this amplified region were methylated. Third, there was variable methylation of the individual CpG dinucleotides in different samples. Fourth, some CpG dinucleotides showed comparable signal intensity of C and T, suggesting hemizygous methylation (NG dinucleotides in Figure 2B). Sequence analysis of the M-MSP product from the methylated control DNA, on the other hand, showed complete methylation of all the CpG islands (Figure 2C). With the primer set MSP5' (selected from the 5' promoter region), none of the normal peripheral blood and marrow samples showed

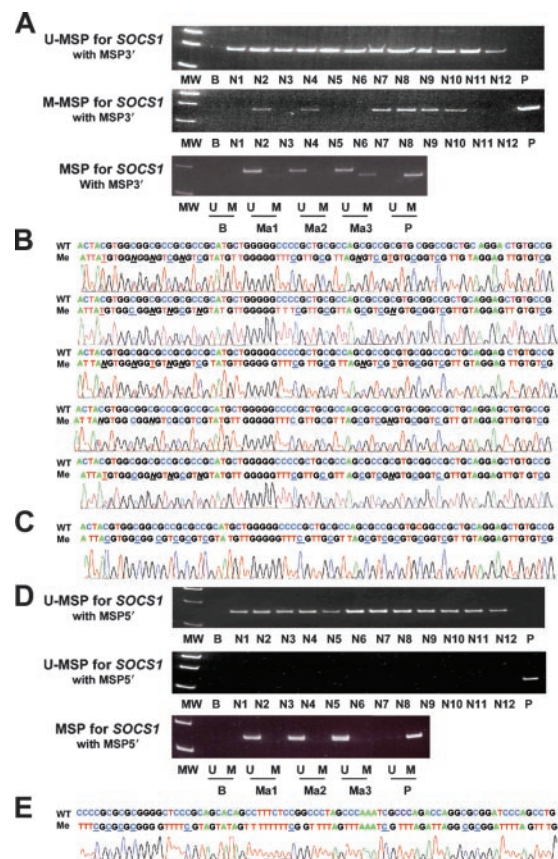


Figure 2. Methylation-specific polymerase chain reaction (MSP) for *SOCS1*. (A) MSP for the unmethylated allele (U-MSP) and methylated allele (M-MSP), showing that with the MSP3' primers, 6 normal peripheral bloods (N2, N4, N7, N8, N9, and N10) and 2 marrows (Ma1 and Ma3) showed methylation signals. MW indicates molecular weight control; B, blank; P, positive control of methylated DNA; U, U-MSP; M, M-MSP; N, normal peripheral blood; and Ma, normal marrow. (B) Sequencing of *SOCS1* in 5 bisulfite-converted peripheral blood controls (N7 to N10 and Ma3) showing methylation signals with primer set MSP3'. The DNA sequence of the "methylated" (Me) PCR product was aligned and compared with the germ line sequence of the wild-type DNA (WT). Methylated cytosine residues in CpG dinucleotide remained as C, whereas unmethylated cytosine read as T after bisulfite conversion. Underlined N suggested hemizygous methylation in a CpG island. Note the presence of many TG islands, representing unmethylated CpG dinucleotides. (C) Sequencing of the methylated PCR product with the MSP3' primers of the methylated positive control. (D) MSP for *SOCS1* using the MSP5' primers. None of the normal peripheral blood and marrow samples showed positive methylation signals. (E) Sequencing of the methylated PCR product with MSP5' primers from the methylated control DNA, showing complete methylation.

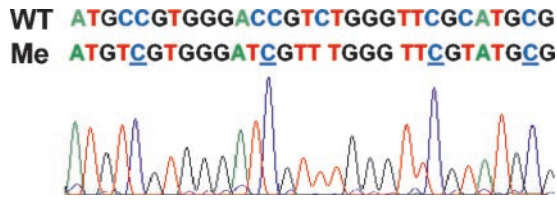


Figure 3. DNA sequencing of *SHP1* in bisulfite-converted methylated positive control DNA.

positive M-MSP (Figure 2D). The methylated control DNA, however, showed positive methylation (Figure 2D) with the expected sequence alterations (Figure 2E). These data suggested that methylation occurred in the 3' coding region, but not the 5' promoter region, of *SOCS1* in normal DNA. Therefore, analysis of the 5' region of *SOCS1* might truly reflect promoter methylation, so that MSP of the myeloma samples was performed with primer set MSP5' instead of MSP3'.

MSP of *SHP1* in control DNA

M-MSP was negative in all of the normal peripheral blood and marrow samples but positive for the methylated control DNA. Sequencing of the M-MSP product from the methylated control DNA showed the expected nucleotide changes (Figure 3).

MSP of *SOCS1* and *SHP1* in cell lines and diagnostic myeloma samples

For the *SOCS1* gene with the primers MSP5', the cell lines AF10, HL60, U937, Jurkat, and Raji showed negative M-MSP but positive U-MSP that verified DNA integrity (Figure 4A). None of the myeloma samples showed *SOCS1* methylation (Figure 4A). For the *SHP1* gene, AF10, U937, Jurkat, and Raji showed hemizygous methylation, while HL60 was totally unmethylated (Figure 4B). On the other hand, 27 of 34 (79.4%) myeloma samples showed *SHP1*

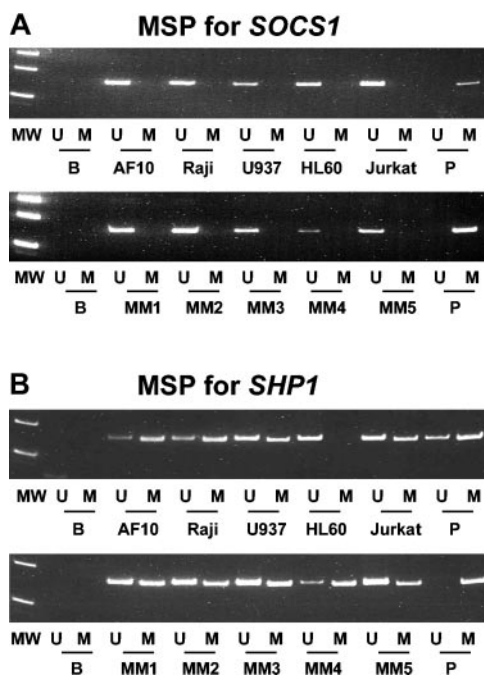


Figure 4. MSP of cell lines and primary myeloma samples. (A) MSP for *SOCS1* with primers MSP5', showing that methylation was absent in the cell lines and primary myeloma samples tested. B indicates blank; P, positive control; MM, primary multiple myeloma samples. (B) MSP for *SHP1*, showing that with the exception of HL60 all the cell lines were methylated. All 5 of the myeloma samples (MM1 to MM5) were methylated.

methylation (Figure 4B). Sequencing of the *SHP1* M-MSP product from 8 methylated myeloma samples confirmed authentic *SHP1* amplification with the expected sequence changes.

5-AC treatment of the myeloma cell line U266

To study the biologic significance of *SHP1* methylation, the myeloma cell line U266 was chosen as an in vitro model. U266 showed complete methylation of *SHP1*, with a consequent absence of *SHP1* mRNA expression as shown by RT-PCR (Figure 5). A high level of phosphorylated STAT3 was constitutively expressed. Treatment with 5-AC led to a progressive demethylation of *SHP1* that started from day 2 onward, as shown by positive U-MSP with increasing amplification intensity. The progressive demethylation of *SHP1* was associated with a parallel reexpression of *SHP1* mRNA. This resulted in a corresponding down-regulation of phosphorylated STAT3. On day 5, phosphorylated STAT3 was almost undetectable. The level of nonphosphorylated STAT3 remained unchanged, showing that *SHP1* reexpression interfered with phosphorylation of STAT3. Therefore, the biologic consequence of *SHP1* gene methylation was repression of *SHP1* expression and, hence, unopposed STAT3 phosphorylation. On the other hand, demethylation leading to reexpression of *SHP1* resulted in down-regulation of phosphorylation of STAT3. These results implied that the epigenetic control of *SHP1* expression might be critically involved in the regulation of STAT3 phosphorylation.

Survival analysis

The projected 3-year OSs of patients with and without *SHP1* methylation were 53% and 63%, respectively ($P = .93$) (Figure 6).

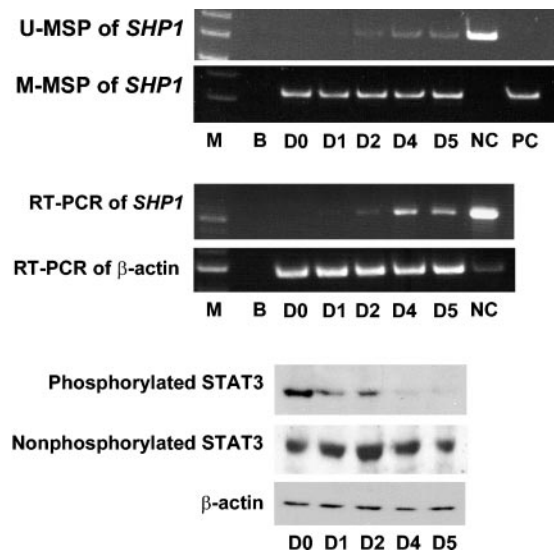


Figure 5. 5-Azacytidine (5-AC) treatment of the myeloma line U266. The U266 cell line was totally methylated at *SHP1*, as shown by positive amplification only in M-MSP and not U-MSP at day 0 (D0) before treatment with 5-AC. On treatment with 5-AC, positive amplification appeared in U-MSP on day 2 (D2), indicating *SHP1* demethylation. The U-MSP amplification became progressively stronger until day 5 (D5), indicating increasing demethylation of *SHP1*. The progressive demethylation of *SHP1* was associated with increasing reexpression of *SHP1*, as shown by increasing amplification intensity of *SHP1* mRNA by reverse transcription PCR (RT-PCR). Western blot analysis of STAT3 showed that before treatment (D0), phosphorylated STAT3 was constitutively expressed. With progressive demethylation and reexpression of *SHP1*, there was progressive down-regulation of phosphorylated STAT3, without significant changes in the amount of nonphosphorylated STAT3. By day 5 (D5), phosphorylated STAT3 was almost undetectable. Comparable protein loading was shown by β -actin. M indicates molecular weight marker; B, blank; D0 to D5, days 1 to 5 after 5-AC treatment; NC, normal control; PC, positive control with methylated DNA.

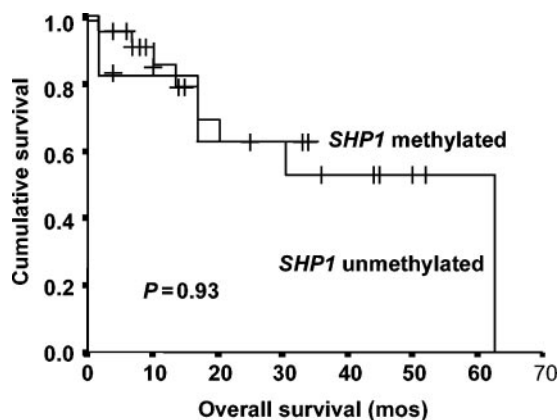


Figure 6. Overall survival of myeloma patients with and without *SHP1* methylation.

Discussion

For the *SOCS1* gene, with primer set MSP3' designed inside exon 1, our study showed methylation signals in about half of the normal blood and marrow DNA. The possibility of mispriming was excluded by sequencing, which showed extensive methylation of most of the CpG dinucleotides in the amplified region. Moreover, the completeness of bisulfite conversion of the unmethylated cytosines to uracils was also demonstrated in the normal and methylated control samples. Incomplete bisulfite conversion is unlikely, because all non-CpG cytosines in these normal controls were converted to uracil and thus read as "T" in sequencing. These served as internal controls for the completeness of bisulfite conversion. Moreover, all non-CpG cytosine molecules in the methylated positive control were also successfully converted and thus also read as "T" in sequencing. Therefore, this region contained genuine methylated CpG islands in normal samples. On the other hand, with primer set MSP5' selected from the 5' promoter region, none of the normal blood and marrow samples showed methylation. Indeed, methylation as detected by MSP5' primer has been demonstrated to be associated with down-regulation of, and thus silencing of, *SOCS1* by immunohistochemistry in hepatoblastoma.²³ Therefore, detection of methylation in CpG islands is more accurate in the 5' promoter than the exon 1 region. This observation supported the occurrence of a methylation boundary in normal cellular DNA, as demonstrated in normal cellular DNA of certain genes.^{32,33} Using 6 sets of consecutive MSP primers spanning the 5' untranslated region (UTR) to intron 1 of the *E-cadherin* gene in normal breast tissues, Graff et al showed that, while CpG dinucleotides in the 5'UTR promoter region were unmethylated, CpG dinucleotides toward the 3' end of the intron 1 were methylated.³² This notion of methylation boundary had been further illustrated in another study of high-resolution methylation mapping of 45 CpG dinucleotides in the *HIC1* gene.³³ The results showed that in normal DNA significant CpG methylation occurred beyond exon 3 but not in intron 2, while for leukemic samples variable methylation of CpG dinucleotides occurred both 5' and 3' to exon 3. Therefore, a boundary sequence within the *HIC1* CpG island existed that marked the junction between methylated and unmethylated DNA in normal hematopoietic cells.³³ The presence of a methylation boundary underscores the importance of selecting MSP primers from the 5'UTR region. Conversely, MSP primers selected inside the coding regions might overestimate the frequency of methylation.

SOCS1 binds to the conserved regulatory tyrosine in the activation loop of the Jak2 kinase (JH1) domain through its SH2 domain and inhibits Jak kinase activity.^{34,35} As a negative regulator of the signaling pathway for IL-6, *SOCS1* is an attractive target of dysregulation in myeloma. However, we showed that *SOCS1*, being a potential tumor suppressor in myeloma,³⁶ was infrequently inactivated by methylation. This contrasted with a previous study using MSP3' primers from the coding DNA region,³⁷ which showed *SOCS1* methylation in 63% of myeloma patients. This disparity might be partly explained by overestimation of the frequency of *SOCS1* methylation using MSP primers in the coding region. Moreover, a less important role of *SOCS1* in the pathogenesis of myeloma has also been demonstrated in *SOCS1*-deficient mice, which died of a myeloproliferative disease instead of plasma cell dyscrasia.^{7,10}

On the other hand, *SHP1*, a PTP important in the negative regulation of Jak/STAT signaling, has been shown to be frequently silenced by methylation in leukemias and lymphomas.^{29,38} In this study, we showed frequent *SHP1* methylation in our myeloma patients, suggesting that it might be involved in the pathogenesis through dysregulation of the Jak/STAT pathway. Ideally, *SHP1* methylation is best demonstrated by purifying the myeloma cells by sorting for CD38- or CD138-positive cells. However, we have shown that the sensitivity of MSP for various genes ranges from 10^{-3} to 10^{-5} . Moreover, because *SHP1* methylation was undetectable in normal hematopoietic cells, and thus specific for the neoplastic plasma cells, our results were valid without a purified myeloma cell population.

To demonstrate that *SHP1* methylation would lead to down-regulation of *SHP1* with consequent activated phosphorylation of STAT3, immunohistochemical studies would be required to show concurrent down-regulation of *SHP1* and up-regulation of phosphorylated STAT3 in the same cellular populations. However, this is technically difficult, the results are not always easy to interpret, and a qualitative relationship between *SHP1* down-regulation and STAT3 activation cannot be demonstrated. Moreover, almost all the primary myeloma samples would be contaminated by residual normal hematopoietic cells that expressed *SHP1*, so that performing Western blot analysis to show a relationship between *SHP1* and phosphorylated STAT3 would be unreliable. Therefore, the U266 myeloma cell line was used as an in vitro model. A previous study had shown that STAT3 was constitutively activated in U266, which expressed high levels of Bcl-X_L and was resistant to Fas-mediated apoptosis.¹³ Our study further showed that *SHP1* methylation was implicated in the constitutive activation of STAT3. The biallelic *SHP1* gene methylation led to down-regulation of *SHP1* and constitutive phosphorylation of STAT3. *SHP1* reexpression consequent to demethylation resulted in down-regulation of the activated phosphorylated STAT3 protein. Because IL-6 is a major growth factor for myeloma cells, and IL-6 signaling acts through the Jak/STAT pathway, our results strongly suggest that *SHP1* methylation might be an important epigenetic mechanism that collaborates with IL-6 in promoting the growth of the neoplastic plasma cells. However, a recent study showed that increased nuclear expression of phosphorylated STAT3, found in about 48% of myeloma patients, might not be related to the abundance of antiapoptotic proteins including Bcl-X_L and Mcl-1.³⁹ Furthermore, the expression of cyclin D1 and phosphorylated STAT3 appeared to be mutually exclusive. Therefore, mechanisms additional to constitutive activation of STAT3 might be involved in the pathogenesis of myeloma. Further experiments will thus be needed to define how modulation of the Jak/STAT pathway might interact with apoptotic mechanisms in myeloma.

In cancer, one hit of the cell cycle control pathway may be sufficient to result in dysregulation of cellular proliferation. For instance, selective, but not concurrent, inactivation of either p16, RB, or cyclin D1 has been reported in glioblastoma⁴⁰ and melanoma.⁴¹ Moreover, reciprocal inactivation of either RB or p16 has been reported in small and non-small cell lung cancers.^{42,43} Similarly, our finding of frequent epigenetic inactivation of *SHP1* but not *SOCS1*, both being negative regulators of the Jak/STAT pathway, in myeloma also lent support to this notion.

Previous studies in myeloma have demonstrated frequent epigenetic inactivation of the INK4/CDK/RB pathway by hypermethylation of *p15* and *p16*. Others showed frequent hypermethylation of death-associated protein (*DAP*) kinase in the p14/MDM2/p53 pathway. In this study, we showed frequent epigenetic inactivation of *SHP1*, which potentially resulted in constitutive activation of the IL-6/Jak/STAT pathway. Notably, however, *SHP1* methylation and STAT activation have only been demonstrated in

vitro. Therefore, the functional relationship between *SHP1* methylation and constitutive STAT activation in primary myeloma samples remains speculative and must be further evaluated. However, our findings together with results from previous studies suggest that gene hypermethylation might be a frequent genetic aberration in myeloma and may collaborate with translocation and other molecular aberrations in pathogenesis.¹ Therefore, demethylating agents and histone deacetylase inhibitors are potentially useful in the treatment of myeloma.

Acknowledgments

We thank Professor Z. Eshhar, Department of Immunology, Weizmann Institute of Science, Israel, for provision of the AF10 cell line and thank the Department of Pathology, Queen Mary Hospital, for pathologic diagnoses.

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