SOCSI and SHPI hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway

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Abstract

SOCS1 and SHP1 negatively regulate the 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, PCR (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*, 27/34 (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 de-methylation of *SHP1* on days 2-5, with consequent increasing re-expression of *SHP1* as shown by reverse-transcription PCR. Concomitant with increasing *SHP1*, a parallel down-regulation of phosphorylated 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.

Keywords: *SOCS1*, *SHP1*, Jak/STAT, multiple myeloma, methylation
Introduction

Multiple myeloma is characterised by neoplastic proliferation of monoclonal plasma cells. These neoplastic plasma cells are thought to be derived from a post-germinal centre 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 (IL)-6. The natural course of the disease may progress through monoclonal gammopathy of undetermined significance (MGUS), smouldering 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.

The binding of cytokines to their receptors results in the dimerization of receptor complexes and activation of the Janus family of protein tyrosine kinases (JAKs), followed by phosphorylation of the cytoplasmic signal transducers and activators of transcription (STATs). Upon phosphorylation, STATs form homo- or hetero-dimers, 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. This Jak/STAT pathway is subject to negative regulation by three family of proteins, the protein inhibitors of activated STATs (PIAS), the suppressors of cytokine signalling (SOCS), and the SH2-containing phosphatases (SHP).

The SOCS family comprises at least eight members characterized by the presence of a central src homology (SH2) domain and a conserved carboxy terminal “SOCS box”. SOCS members are cytokine-inducible negative regulators of the cytokine signalling. 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. Human *SOCS1* is a single-exon gene encoding 211 amino acids, and lies
within a CpG island spanning 2.5 kb. The expression of *SOCS1* impairs cellular response to IL-6 through direct interaction with Jak proteins.\textsuperscript{13}

SHP-1 (also known as HCP, SHPTP1 and PTP1C), a member of the SHP family of proteins, is a 68-kd, cytoplasmic protein tyrosine phosphatase (PTP).\textsuperscript{14} The human *SHP-1* gene consists of 17 exons and spans approximately 17 kb of DNA. It contains two tandem Src homology (SH2) domains, a catalytic domain, and a C-terminal tail of about 100 amino acid residues.\textsuperscript{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, as it antagonises the growth-promoting and oncogenic potentials of protein tyrosine kinase.\textsuperscript{14}

DNA methylation, catalysed 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.\textsuperscript{15-17} In many cancers, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression of these genes.\textsuperscript{18} This may serve as an alternative epigenetic mechanism of gene inactivation.\textsuperscript{19,20}

Dysregulation of the IL-6/Jak/STAT pathway has been shown to be involved in hematologic cancers. For instance, activation of STAT3 is frequent in primary human myeloma or myeloma cell line,\textsuperscript{13} and blocking of either IL-6 receptor, Jak or STAT3 inhibits the expression of Bcl-X\textsubscript{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-ALL.\textsuperscript{21} Recently, hypermethylation of *SOCS1* has been separately reported in hepatoma.\textsuperscript{22,23} In this study, we investigated the potential involvement of *SOCS1* and *SHP1* promoter hypermethylation in the pathogenesis of myeloma.
Materials and methods

**Patient, diagnosis and treatment.** The diagnosis of myeloma was made according to standard criteria with marrow plasmacytosis, presence of paraprotein and osteolytic bone lesions. Complete staging work-up includes bone marrow examination, skeletal survey, serum and urine protein electrophoresis, serum immunoglobulin (IgG, IgA, & IgM) levels, renal function tests and serum calcium level. Monoclonal immunoglobulins were identified by cellulose acetate or agarose-gel electrophoresis. When an abnormal band or equivocal pattern was detected, immuno-electrophoresis or immunofixation was performed. All blood tests were repeated every six months and skeletal survey was repeated yearly. Primary treatment included either VAD (vincristine 0.4 mg/day x 4, Adriamycin 9 mg/m²/day x 4, and dexamethasone 40 mg/day, days 1-4, 9-12, 17-20) or MP (melphalan 0.15 mg/kg/day x 7, prednisolone 60 mg/day x 7). 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.

**Methylation-specific polymerase chain reaction (MSP).** High molecular weight genomic DNA was isolated by standard protocols from diagnostic bone marrow aspirates, and from a myeloma cell line AF10 (kindly provided by Professor Zelig Eshhar, Department of Immunology, Weizmann Institute of Science, Israel), and the leukemic cell lines (HL60, U937, Jurkat and Raji). The methylation-specific polymerase chain reaction (MSP) for promoter methylation was performed as described. Briefly, treatment of DNA with bisulphite (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, USA). MSP primers were designed to amplify the methylated (M-MSP) and unmethylated (U-MSP) alleles. For \textit{SOCS1}, two sets of primers (MSP3’ and MSP5’) were adopted from previous studies.\textsuperscript{22,23} These two sets of MSP primers were located 5’ (MSP5’)\textsuperscript{23} and 3’(MSP3’)\textsuperscript{22} to the translation start site of \textit{SOCS1}. Both sets of primers could be identified on NT_010393.11, and the nucleotide positions of these two sets of \textit{SOCS1} primers with reference to Genbank sequence GI: 27486099 (NT_010393.11), subregion: complement (2121832..2123597) were listed in Table 1. The relative positions with respect to the translation start site were depicted in Figure 1. Notably, the forward and reverse primers of MSP3’ (adopted from Yoshikawa et al\textsuperscript{22}) mapped to nucleotide positions 400–423 and 537–559 for methylated DNA, and 391–423 and 537–565 for unmethylated DNA in Genbank sequence U88326. The MSP primers for \textit{SHP1} were listed in table 1. There are two isoforms of \textit{SHP1}, one expressed in non-hematopoietic tissues and the other in hematopoietic tissues,\textsuperscript{29} 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 normal blood donors and three normal 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, USA) with the following cycling conditions: 95°C for 12 minutes, 35/45 (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 PCR mixture contained 50 ng of bisulphite treated DNA, 0.2 mM dNTPs, 2 mM MgCl\textsubscript{2}, 10 pmol of each primer, 1 X 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% non-denaturing 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.\textsuperscript{27,30}
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
<th>Tm / cycles</th>
<th>PCR products</th>
<th>Reference</th>
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<tr>
<td><strong>SOCS1 (MSP3’)</strong> Accession number: GI:27486099 (NT_010393.11), subregion: complement (2121832..2123597)</td>
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<td>M-MSP</td>
<td>TTC GCG TGT ATT TTT AGG TCG GTC (nt: 1081 – 1104)</td>
<td>CGA CAC AAC TCC TAC AAC GAC CG (NT: 1218 – 1240)</td>
<td>63°C / 35</td>
<td>159 bp</td>
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<td>U-MSP</td>
<td>TTA TGA GTA TTT GTG TGT ATT TTT AGG TTG GTT (nt: 1072 – 1104)</td>
<td>CGA CAC AAC TCC TAC AAC GAC CG (nt: 1218 – 1246)</td>
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<td>M-MSP</td>
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<td>CTA CTA ACC AAA CTA AAA TCC ACA (nt: 335 – 312)</td>
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<td>149 bp</td>
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<tr>
<td>U-MSP</td>
<td>GTT GTA GGA TGG GTT TGT TTT TGT (nt: 186 – 209)</td>
<td>CTA CTA ACC AAA CTA AAA TCC ACA (nt: 335 – 312)</td>
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<td>158 bp</td>
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Tm: annealing temperature; nt: nucleotide; bp: base pairs
M-MSP: methylation specific polymerase chain reaction for the methylated allele
U: MSP: methylation specific polymerase chain reaction for the unmethylated allele
Figure 1. Schematic diagram of the location of the two sets of MSP primers (MSP5' and MSP3') for SOCS1 in relation to the translation start site.

**DNA sequencing.** The identity of the methylated and unmethylated sequences was confirmed by automated DNA sequencing. PCR products were gel purified, sequenced bi-directionally, and analysed on an automated DNA sequence analyser (3700 ABI Prism, PE Biosystem).

**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, USA) 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 cells/ml, and treated with 3 µM of 5-AC (Sigma, St. Louis, MO, USA) 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 µg of total cellular RNA was reversely-transcribed with M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA), and 2 µl of the resulted cDNA was amplified by PCR with SHP-1 specific primers (forward: 5’-GGC ACT GGG AGC TGC ATC TGA GGC-3’ and reverse 5’-CTC GCA CAT GAC CTT GAT GTG-3’29). The identity of the RT-PCR product was confirmed by DNA sequencing.
**Western Blot.** Whole cell lysate was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to a PVDF membrane (PALL Corporation, Pensacola, FL, USA). The membrane was blocked with 5% bovine serum albumin (Sigma) and immunoblotted with antibodies against phosphorylated STAT3 (Tyr705) (Cell Signalling Technology, Beverly, MA, USA) and non-phosphorylated STAT3 (Zymed Laboratories, South San Francisco, CA, USA). 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 two-sided.
Results

Patients. Thirty-four patients (male: 19, female: 15) at a median age of 62 (25 – 87) years were studied. In these patients, the bone marrow plasma cells ranged from 38%-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 were 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 (10 – 78) g/L. Eighteen patients received three courses of VAD and then MP till plateau phase or maximum response; whereas 16 patients received MP till maximum response or plateau phase. Eight patients underwent HSCT (allogeneic: 2, autologous: 6) 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 six of twelve normal peripheral blood DNA, two of three normal marrows, and the methylated positive control DNA (Figure 2A). In one of the normal marrow samples and four of the six 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. Firstly, the authenticity of methylation was confirmed by the finding of multiple unconverted cytosine molecules at “CpG” dinucleotides (Figures 2B). Secondly, not all CpG dinucleotides within this amplified region were methylated. Thirdly, there was variable methylation of the individual CpG dinucleotides in different samples. Fourthly, some CpG dinucleotides showed comparable signal intensity of “C” and “T”, suggesting hemizygous methylation (arrowed “NG” dinucleotides in (Figures 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 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’.
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, six normal peripheral blood (N2, N4, N7, N8, N9, and N10) and two marrows (Ma1 and Ma3) showed methylation signals. MW: molecular weight control; B: blank; P: positive control of methylated DNA; U: U-MSP; M: M-MSP; N: normal peripheral blood; Ma: normal marrow.

B. Sequencing of SOCS1 in five bisulphite converted peripheral blood controls (N7 – 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 germline sequence of the wild-type DNA (WT). Methylated cytosine residues in CpG dinucleotide remained as “C” whereas unmethylated cytosine read as “T” after bisulphite 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.

**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).
**Figure 3.** DNA sequencing of *SHP1* in bisulphite converted methylated positive control DNA.

**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* methylation (Figure 4B). Sequencing of the *SHP1* M-MSP product from eight methylated myeloma samples confirmed authentic *SHP1* amplification with the expected sequence changes.
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: 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 five of the myeloma samples (MM1 – 5) were methylated.

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 de-methylation of SHP1 that started from day 2 onwards, as shown by positive U-MSP with increasing amplification intensity. The progressive de-methylation of SHP1 was associated with a parallel re-expression of SHP1 mRNA. This resulted in a corresponding down-regulation of phosphorylated STAT3. On day 5, phosphorylated STAT3 was almost undetectable. The level of non-phosphorylated STAT3 remained unchanged, showing that SHP1 re-expression 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, de-methylation leading to re-expression 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.
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 re-expression of SHP1, as shown by increasing amplification intensity of \textit{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 re-expression of SHP1, there was progressive down-regulation of phosphorylated STAT3, without significant changes in the amount of non-phosphorylated STAT3. By day 5 (D5), phosphorylated STAT3 was almost undetectable. Comparable protein loading was shown by \(\beta\)-actin. M: molecular weight marker; B: blank; D0 – D5: days 1 to 5 after 5-AC treatment; NC: normal control; PC: positive control with methylated DNA.

\textbf{Survival analysis.} The projected 3-year OS of patients with and without \textit{SHP1} methylation were 53\% and 63\% (\(p=0.93\)) (Figure 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Overall survival of myeloma patients with and without \textit{SHP1} methylation.}
\end{figure}
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 the majority of CpG dinucleotides in the amplified region. Moreover, the completeness of bisulphite conversion of the unmethylated cytosines to uracils was also demonstrated in the normal and methylated control samples. Incomplete bisulphite conversion is unlikely as 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 bisulphite 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 downregulation of, and thus silencing of SOCS1, by immunohistochemistry in hepatoblastoma. 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. Using six sets of consecutive MSP primers spanning the 5’ untranslated region (UTR) to intron one 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 towards the 3’ end of the intron one were methylated. 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. 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 \textit{HIC1} CpG island existed that marked the junction between methylated and unmethylated DNA in normal hematopoietic cells.\textsuperscript{32} Therefore, 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.

\textit{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.\textsuperscript{33,34} As a negative regulator of the signalling pathway for IL-6, \textit{SOCS1} is an attractive target of dysregulation in myeloma. However, we showed that \textit{SOCS1}, being a potential tumour suppressor in myeloma,\textsuperscript{35} was infrequently inactivated by methylation. This contrasted with a previous study using MSP3’ primers from the coding DNA region,\textsuperscript{36} which showed \textit{SOCS1} methylation in 63\% of myeloma patients. This disparity might be partly explained by overestimation of the frequency of \textit{SOCS1} methylation using MSP primers in the coding region. Moreover, a less important role of \textit{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.\textsuperscript{7,10}

On the other hand, SHP1, a protein tyrosine phosphatase important in the negative regulation of Jak/STAT signalling, has been shown to be frequently silenced by methylation in leukemias and lymphomas.\textsuperscript{37,38} In this study, we showed frequent \textit{SHP1} methylation in our myeloma patients, suggesting that it might be involved in the pathogenesis through dysregulation of the Jak/STAT pathway. Ideally, \textit{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, as
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.

In order 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-XL 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 re-expression consequent to demethylation resulted in down-regulation of the activated phosphorylated STAT3 protein. As 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 collaborate 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 anti-apoptotic proteins including bcl-XL 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 glioblastoma\textsuperscript{40} and melanoma\textsuperscript{41}. Moreover, reciprocal inactivation of either RB or p16 has been reported in small and non-small cell lung cancers\textsuperscript{42,43}. Similarly, our finding of frequent epigenetic inactivation of \textit{SHP1} but not \textit{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 \textit{p15} and \textit{p16}. Others showed frequent hypermethylation of \textit{DAP kinase} in the p14/MDM2/p53 pathway. In this study, we showed frequent epigenetic inactivation of \textit{SHP1}, which potentially resulted in constitutive activation of the IL-6/Jak/STAT pathway. It must be noted, however, that \textit{SHP1} methylation and STAT activation has only been demonstrated \textit{in vitro}. Therefore, the functional relationship between \textit{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.\textsuperscript{1} Therefore, demethylating agents and histone deacetylase inhibitors are potentially useful in the treatment of myeloma.
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