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The suppressor of cytokine signaling-1 is constitutively expressed in chronic myeloid leukemia and correlates with poor cytogenetic response to interferon-α

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A B S T R A C T

Background and Objectives. Interferon-α (IFN-α) has proven useful for treating chronic myeloid leukemia (CML). However, only 7% of patients achieve a complete cytogenetic response. Although efforts to understand the molecular basis of this resistance to IFN-α have been made, the mechanism is still unknown. Because suppressor of cytokine signaling (SOCS) proteins are negative regulators of cytokine-induced signaling, it has been hypothesized that aberrant SOCS expression could confer resistance against cytokine therapy.

Design and Methods. In order to analyze the role of SOCS-1 in the acquisition of IFN-α resistance in this setting, we examined SOCS-1 mRNA expression using reverse transcription polymerase chain reaction (RT-PCR) in 75 newly diagnosed chronic phase-CML patients who received IFN-α therapy.

Results. SOCS-1 was constitutively expressed in 49 (65%) of 75 CML patients at diagnosis. Constitutive SOCS-1 expression was more frequently observed among Hasford high-risk patients ($p = 0.05$) and was also independently associated with a shorter median progression-free survival time ($p = 0.001$) and poor cytogenetic response to IFN-α treatment ($p < 0.0001$).

Interpretation and Conclusions. Our data indicate that constitutive expression of SOCS-1 occurs at an early stage in CML pathogenesis and probably influences the clinical behavior of the disease.

Key words: SOCS-1, CML, IFN, BCR-ABL

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Until the introduction of imatinib mesylate, interferon-α (IFN-α) was the initial treatment of choice for patients with chronic myeloid leukemia (CML) who did not have a suitable bone marrow donor or who were not candidates for transplantation.[1] In the treatment of chronic phase (CP) CML, imatinib produces much better hematologic and cytogenetic responses than does IFN-α, with most patients maintaining these responses. In newly diagnosed CML, the rate of major cytogenetic response to imatinib therapy is 83%, with 68% complete responses; the respective response rates to IFN-α plus Ara-C are 20% and 7%.[2] Although efforts to understand the molecular basis of this resistance to IFN-α have been made,[3] the mechanism is still unknown.

IFN-α exerts its biological actions by binding to the high-affinity cell-surface receptor. Receptor-associated Janus family tyrosine kinase Tyk2 and Jak1 are activated upon stimulation by IFN-α, and this is followed by tyrosine phosphorylation of critical tyrosine residues of the cytoplasmic domain of the receptors by Jaks.[4] This allows receptor recruitment and Jak-mediated tyrosine-phosphorylation of signal transducer and activator of transcription (STAT) molecules. When STAT1 and STAT2 become tyrosine phosphorylated they bind to each other and, in combination with p48, form a complex called IFN-stimulated gene factor-3 (ISGF3). After translocation into the cell nucleus, this complex binds to conserved IFN-stimulated responsive element sequences within the promoter of IFN-responsive genes and initiates transcription of these genes.[5]

The suppressor of cytokine signaling (SOCS) proteins are a family of negative

regulators of cytokine signaling that inhibit cytokine action by inhibiting Jak activation.[6-7] Of the family members, SOCS-1 and SOCS-3 are the most potent inhibitors of cytokine-induced signals. Forced expression of SOCS-1 or SOCS-3 down-regulates a variety of cytokine signal pathways including IFN-α.[8] Recent data indicate that *SOCS-2* and *SOCS-3* mRNA is constitutively expressed in most blast cells of patients with CML blast crisis but not in the early stage of disease.[9-10] These results suggest that *SOCS-2* and *SOCS-3* might be involved in unresponsiveness of IFN-α therapy in patients with CML blast crisis. As primary or acquired resistance to IFN-α is often observed in chronic phase CML patients, it is important to determine whether the tumor cells from such patients also show changes in the expression of *SOCS*, specially *SOCS-1* which has never been evaluated in CML.

In the present study we demonstrate that *SOCS-1* was constitutively expressed in a group of newly diagnosed CML patients and that this overexpression correlated with cytogenetic response to IFN-α.

Design and Methods

Patients

We studied 75 patients with Philadelphia-positive chronic phase (CP)-CML, diagnosed between November 1992 and December 2001, in three Hematology Departments in Spain: Reina Sofia Hospital, Cordoba; Carlos Haya Hospital, Malaga and Hospital Clinic, Barcelona. The study was approved by the Investigational Review Boards in accordance with the policies of the Department of Health and Human Services. All patients gave informed consent for the use of their samples. The patients were selected for type of therapy (all of them received IFN-α). A diagnostic sample taken during the CP was available for analysis in all patients. Paired samples, where both a diagnostic sample and an accelerated phase (AP) or blastic crisis (BC) sample were available, were analyzed in 20 patients (11 patients in AP, 7 in myeloid BC and 2 in lymphoid BC). AP and BC were diagnosed according to standard criteria.[11-12] Risk categories were determined according to the Sokal, Kantarjian and Hasford score systems as previously described.[11,13-14] Hematologic and cytogenetic responses to IFN-α treatment were evaluated according to the criteria of the Houston group.[15] The criteria for complete hematologic response (CHR) was normalization of the peripheral white blood cell (WBC) count to less than 10×10^9/L with the disappearance of immature circulating cells, normalization of the platelet count (to less than 450×10^9/L), and disappearance of all signs and symptoms of the disease (in particular, splenomegaly).

Cytogenetic response was assessed by analyzing at least 20 metaphases and was defined as *good*, which comprised complete responses (CR, 0% Ph^+ metaphases) and major responses (MR, 1%-34% Ph^+ metaphases), or *poor*, which included patients with a minor response (MinR, 35%-94% Ph^+ metaphases) or no response (NR, 95%-100% Ph^+ metaphases). The mean duration of IFN-α administration was 29.3 months for *good* responders and 28.1 months for *poor* responders.

Samples

Heparinized bone marrow cells were collected from patients with CML, and from healthy marrow donors. Immediately after harvest, total WBC were obtained by dextran sedimentation or by red cell lysis of centrifuged buffy coat preparations. Mononuclear cells (MNC) were isolated from BC-CML and donors by sedimentation on Ficoll-Hypaque gradients. More than 90% of the MNC populations from acute phase CML were leukemic blasts. In addition, granulocyte fractions were collected from peripheral blood with Polymorphprep (Nycomed Pharma, Oslo, Norway) in five CP-CML patients and two healthy individuals.

Expression of SOCS-1 gene

Expression of the *SOCS-1* gene was analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. Total RNA was extracted from marrow and peripheral blood samples with Ultraspec (Biotecx, Houston, TX, USA) following the manufacturer's instructions. Reverse transcription was performed on 1 μg total RNA, after heating at 70°C for 5 min, with random hexamers as the reaction primer. The reaction was carried out at 42°C for 45 min in the presence of 12 U Avian Myeloblastosis virus reverse transcriptase (Boehringer-Mannhein, Germany). Complementary DNA was amplified by means of a primer set that was specific for the *SOCS-1* gene (sense, 5'-CACGCCGATTACCGGCGCATC-3'; antisense, 5'-GCTCCTGCAGCGGCCGCACG-3'). The PCR reaction was performed as follows: 94°C for 5 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and incubation at 72°C for 10 min. PCR products were resolved on 2% agarose gels. Amplification of RARα gene transcript was performed to assess RNA integrity.[16] Bone marrow (n = 15) and peripheral blood (n = 20) RNA samples from healthy donors age-matched with our CML patients (median 50 years, interquartile range 37-70) were used as controls for the *SOCS-1* RT-PCR assays.

Quantitative real-time PCR (qrt-PCR) for *SOCS-1* expression was performed in a rapid fluorescent thermal cycler with three-color fluorescence monitoring capability (LightCycler, Roche), using 1 μL of cDNA in a 20 μL reaction volume with 0.4 μmol/L of each primer (see above), and 2 μL of 10 × LightCycler Fast-

Star DNA Master SYBR Green I (Roche Molecular Biochemicals). The final Mg^{2+} concentration in the reaction mixture was adjusted to 3.5 mmol/L. The following program conditions were applied for the qrt-PCR runs: denaturation, one cycle at 95°C for 8 minutes; amplification, 45 cycles at 95°C for 5 s, 60°C for 10 s and 72°C for 15 s; melting, one cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling, one cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, which was 0.4°C/s between 40°C and 90°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as the reference gene, and it was amplified in the same run and following the same procedure as that described above for *SOCS-1*. In order to reduce the variation between different assays and samples, we used a procedure based on the relative quantification of target genes vs their controls in relation to the reference gene. Calculations were automatically performed by the LightCycler software (RealQuant, version 1.0, Roche). The normalized ratio was obtained from the equation below and expressed as a percentage of the control:

$$\text{Normalized ratio} = (E_{target})^{\Delta Cp\ target\ (control\ -\ sample)} \div (E_{ref})^{\Delta Cp\ target\ (control\ -\ sample)}$$

Efficiencies (E) of each gene were calculated from the slopes of crossover points (Cp) vs cDNA concentration plot, according to the formula $E = 10^{(-1/slope)}$. ΔCp corresponded to the difference between control Cp and sample Cp, either for the target or for the reference gene. The selected control was the Philadelphia-positive BV173 cell line which expresses high levels of *SOCS-1*.[10] It was considered to have 100 % expression.

Statistical analysis

All calculations were performed with the SPSS statistical package (SPSS, Chicago, IL, USA). Medians and interquartile ranges were calculated for age and clinical and laboratory findings at diagnosis for patients with and without *SOCS-1* expression (Table 1) and tested for any significant differences with the Mann-Whitney U test (for continuous variables) or χ^2 analysis and Fisher's exact test (for categorical variables). Overall survival (OS) was calculated from the time of diagnosis to death from any cause and was censored only for patients known to be alive at last contact. Progression-free survival (PFS) was measured from the time of CML diagnosis to the appearance of AP/BC or death without disease progression, and was censored only for those patients alive and without evidence of progression at their last follow-up. Bone marrow transplant recipients were censored at the time of transplantation for calculations of both OS and PFS. Distributions of OS and PFS curves were estimated by the method of Kaplan and Meier, with 95% confidence intervals calculated by means of Greenwood's formula. Comparisons of OS and PFS between groups were based on the log-rank test. Comparisons adjusted for significant prognostic factors were based on Cox regression models and hazard regression models. All progression and survival data were updated in February, 2003, and all follow-up data were censored at that point.

Results

Constitutive SOCS-1 expression in CML patients

The RT-PCR method revealed lack of *SOCS-1* expression in total WBC, MNC and granulocytes from normal bone marrow and blood controls. However, overexpression of *SOCS-1* was present in total WBC from 49 of 75 (65%) CP-CML patients at diagnosis and also in granulocytes from 3 of 5 (60%) of these patients. Representative data are shown in Figure 1. Normalized ratios for *SOCS-1* were determined in total WBC from healthy individuals and *SOCS-1*-expressing CML patients. Whereas no expression was observed among normal individuals, the normalized values in CML patients fell between 57% and 102%. Sequential paired samples were analyzed in 20 patients, with the second sample being taken following disease progression. Among the samples taken at diagnosis, 67% (13 of 20) showed *SOCS-1* expression compared to 90% (18 of 20) of samples taken following progression to AP or BC ($p = 0.09$). This latter result indicates that the majority of patients show *SOCS-1* expression during the course of their disease.

SOCS-1 expression and pretreatment clinical features

The clinical and laboratory characteristics of CML patients with and without *SOCS-1* expression at diagnosis are shown in Table 1. Individual factors such as sex, age, enlarged spleen, peripheral blood blast cells, platelet count, hemoglobin level and WBC count were not significantly associated with *SOCS-1* expression. Correlating *SOCS-1* expression with pretreatment risk groups, we observed a significant association between constitutive *SOCS-1* expression and high-risk patients, as assessed by the Hasford scoring system (80% of high-risk patients expressed *SOCS-1* versus 60% of low/intermediate risk patients, $p = 0.05$). A trend towards significance was also observed for the Sokal high-risk group (78% in high-risk patients versus 61% in low/intermediate-risk patients, $p = 0.09$).

Table 1. Clinical characteristics at diagnosis and outcome of 75 chronic myeloid leukemia patients according to SOCS-1 expression.

Feature	*No SOCS-1 expression (n = 26)*	*SOCS-1 expression (n = 49)*	*p*
Sex (M/F), %	54/46	51/49	NS
Age, median (int. r)	45 (35-59)	48 (37-57)	NS
Palpable spleen, %	58	62	NS
Median hemoglobin (g/L) (int. r)	116 (97.7-129.3)	118 (83.5-119.1)	NS
WBC×10^9/L, median (int. r)	142 (61.2-348.1)	117 (53.1-228.5)	NS
Median platelet count 10^9/L (int. r)	449 (281-652)	493 (264-612)	NS
Median peripheral blood blast as % WBC (int. r)	1 (0-4.1)	1 (0-3.6)	NS
Sokal score, %			
High	15	28	NS
Low/Intermediate	85	72	
Kantarjian score, %			
Stage 3	11	13	NS
Stages 1-2	89	87	
Hasford score, %			
High	7	27	0.05
Low/Intermediate	93	73	
Treatment type, %			
Interferon + Ara C	51	49	NS
Interferon alone	49	51	
Response to treatment, %			
Complete hematologic response	88	85	NS
Good cytogenetic response	54	10	< 0.0001
Disease progression, %	19	65	< 0.0001
Death, %	27	41	0.09

(int. r) indicates interquartile range.

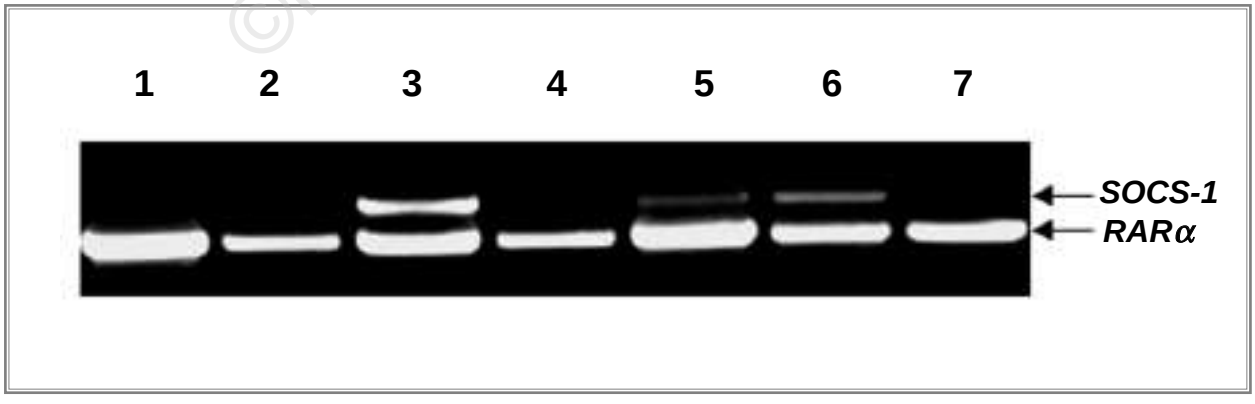

Figure 1. Expression of the *SOCS-1* gene in newly diagnosed chronic phase-CML. Representative examples of *SOCS-1* expression assessed by RT-PCR with primers for *SOCS-1* and RARα (as control) in bone marrow CML samples (lanes 1-6) and a healthy donor (lane 7). Constitutive *SOCS-1* expression is observed in lanes 3, 5 and 6.

Table 2. Multivariate regression analysis for response to interferon treatment (A) and multivariate Cox model for progression-free survival (B) in 75 CML patients.

A		
Variable	*Level of significance*	
	p	*p**
SOCS-1 expression	0.0001	–
Kantarjian score	0.05	0.1
Sokal score	0.2	0.3
Hasford score	0.5	0.6

**Significance after adjustment for SOCS-1 expression.*

B		
Variable	*Level of significance*	
	p	*p**
SOCS-1 expression	0.001	–
Kantarjian score	0.6	0.7
Sokal score	0.3	0.4
Hasford score	0.06	0.3
Cytogenetic response	0.03	0.5

**Significance after adjustment for SOCS-1 expression.*

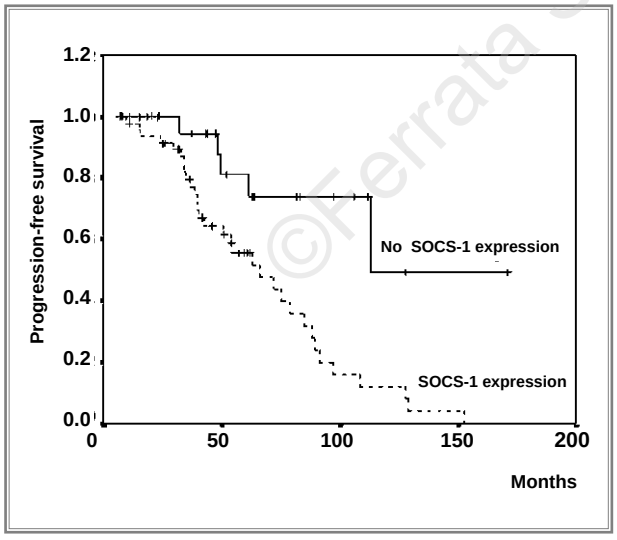

Figure 2. Kaplan-Meier analysis of the duration of the chronic phase showing prognostic significance of *SOCS-1* expression. Calculations were performed using data from 75 patients and the significance of survival differences assessed by the log-rank test. Patients who underwent stem cell transplantation and patients who died from causes unrelated to CML were censored at the time of procedure or death, respectively.

SOCS-1 expression, response to treatment and clinical outcome

CML patients in this study were treated with IFN-α-based regimens (Table 1). Seventeen patients received stem cell transplantation (8 autologous, 9 allogeneic). The type of treatment administered and the number of patients who received transplantation were similarly distributed between the two *SOCS-1*-expressing groups (Table 1). Moreover, the mean duration of IFN-α administration was not different between patients with constitutive SOCS-1 expression (29 months) and those without such expression (28.4 months). Although the CHR rate was similar in both *SOCS-1*-expressing groups, constitutive *SOCS-1* expression correlated with poor cytogenetic response to IFN-α treatment (Table 1). Thus, a *good* cytogenetic response was observed in 54% (14 of 26) of patients with no *SOCS-1* expression but in only 10% (5 of 49) of *SOCS-1* expressing patients. This difference was highly significant ($p < 0.0001$). A multivariate analysis including the clinical factors described in Table 1 demonstrated that expression of the *SOCS-1* gene was the only independent factor predicting the cytogenetic response to IFN-α (Table 2A).

Survival data were available for all patients. During the study period, patients with constitutive *SOCS-1* expression had a higher progression rate (65% versus 19%, $p < 0.0001$) and a higher mortality rate (41% versus 27%, $p = 0.09$) than did patients with absence of *SOCS-1* expression (Table 1). Kaplan-Meier analysis revealed differences in the duration of CP (Figure 2). The estimated median PFS time for patients with constitutive *SOCS-1* expression was 65.9 months (95% CI [confidence interval], 40.7-91.2) compared to 112.6 months (95% CI, 92.3-157) for patients with absent *SOCS-1* expression ($p = 0.001$). The prognostic strengths of *SOCS-1* expression, cytogenetic response, Sokal score, Kantarjian score and Hasford score were then compared. Sokal, Kantarjian and Hasford non-high-risk patients had an estimated median PFS of 75.1 months. Moreover, in contrast to the prognostic significance of *SOCS-1* expression status ($p = 0.001$), the difference in PFS between high-risk and non-high-risk (low plus intermediate risk or stages 1-2) patients using the Sokal, the Kantarjian or the Hasford scoring system was not significant ($p = 0.8$, $p = 0.7$, and $p = 0.1$, respectively). However, the estimated PFS for the patients with poor cytogenetic response was 72.2 months (95% CI, 51.5-92.9), compared with 112.6 months (95% CI, 38.7-186.5) for patients in the good cytogenetic response group ($p= 0.03$). Multivariate analysis using a forward stepping model showed that the independent prognostic importance of *SOCS-1* expression remained after adjusting for age, sex, percentage of peripheral blood blasts, platelet count,

response to IFN-α and initial Sokal, Kantarjian and Hasford scores (Table 2B). The Hasford scoring system was the only variable significantly associated with OS ($p = 0.05$).

Discussion

In the present study, we found that *SOCS-1* was constitutively expressed in most newly diagnosed CP-CML patients who were resistant to IFN-α in terms of cytogenetic response. This phenomenon is not universal to all *SOCS* proteins. Whereas high levels of *SOCS* are found in 65% of CML patients at diagnosis, *SOCS-2* and *SOCS-3* overexpression is also evident in primary cells from patients with CML, but it appears to be exclusive to the advanced stages of the disease.[9-10] The reasons for the association between different SOCS proteins and different phases of the disease are not clear. It could be argued that *SOCS-2/SOCS-3* induction may be a dose-dependent event, requiring a certain threshold of BCR-ABL expression to take place. This possibility seems plausible, as *SOCS-2* is a downstream target of *BCR-ABL*[10] and there is some evidence that BC cells express more *BCR-ABL* than CP progenitors.[17] In contrast, *SOCS-1* transcription appears to be independent from *BCR-ABL*, in fact, CML cells do not show downregulation of *SOCS-1* levels on *in vitro* exposure to imatinib mesylate.[10]

Constitutive expression of *SOCS-1* was found to be correlated with three dismal prognostic features in CML patients: overexpression of *SOCS-1* was more frequently observed among high-risk CML groups and was also associated with shorter PFS and, specially, with poor cytogenetic response to IFN-α. There are several lines of evidence that *SOCS-1* limits the action of IFN. The phenotypic characteristics of *SOCS-1*$^{-/-}$ mice have striking similarity to those observed in neonatal mice injected with IFN,[18] raising the possibility that *SOCS-1*$^{-/-}$ mice suffer from deregulated production or responsiveness to IFN. Consistent with this possibility, *SOCS-1*$^{-/-}$ mice have more activated STAT-1, a primary mediator of IFN action, and increased expression of STAT-1-responsive genes.[19] *SOCS-1*$^{-/-}$ IFN-γ$^{-/-}$ double knockout mice survive weaning and are healthy until at least 6 months of age.[19] The pathology associated with absolute *SOCS-1* deficiency can also be prevented by giving *SOCS-1*$^{-/-}$ mice twice-weekly injections of antibodies against IFN. Moreover, overexpression of SOCS-1 in HeLa and MCF-7 cell lines inhibits IFN-α-mediated antiviral and antiproliferative activities[20] and interleukin-10 also suppresses IFN-α-induced tyrosine phosphorylation of STAT-1 and IFN-α-induced ISGF3 complexes in monocytes by inducing *SOCS-1*.[21] All these observations support our findings that constitutive expression of *SOCS-1* in CML cells affects their sensitivity to IFN-α.

One of the main questions arising from our observations is that of the possible role of *SOCS-1* in the pathogenesis of CML. The SOCS proteins suppress cytokine signaling and provide a safeguard to avoid continuous stimulation of transcription of key genes when a receptor is engaged by its cytokine. The fact that CML patients show overexpression of *SOCS-1* appears paradoxical, as in this case the net effect should be the prevention of continuous up-regulation of proliferation-controlling genes. However the opposite phenomenon characterizes CML, in which deregulated cell growth is an important phenotypic feature. One possibility, also supported by experimental data, is that SOCS-1 may not be a true suppressor protein in BCR-ABL-transformed cells. Expression of SOCS-1 in Ba/F3 cells transformed by the TEL-JAK2 fusion variants inhibits interleukin-3-independent growth of these cells but does not inhibit growth of Ba/F3 cells transformed by TEL-PDGFβR, BCR-ABL, and TEL-ABL.[22-23] This result is intriguing since although several tyrosine kinase fusions activate STATs, *SOCS-1* inhibits only TEL-JAK2 of the tyrosine kinase fusions tested. This suggests that TEL-JAK2-mediated transformation is dependent on STATs, whereas other tyrosine kinase fusions, for example BCR-ABL, can bypass the requirement for STAT activation. This finding is consistent with results of murine bone marrow transplants conducted in a STAT5α,β$^{-/-}$ background, which demonstrate that STAT5 is necessary for TEL-JAK2-mediated disease but is not required for BCR-ABL-mediated disease.[24-25]

In conclusion, our results strongly suggest that constitutive expression of *SOCS-1* occurs at an early stage in the multistage process of CML and plays a role in the clinical behavior of the disease.

Contributions. JR-G and AJ-V designed the study, were responsible for the laboratory studies, and drafted the manuscript. JAC, FC, MB and DC were responsible for collecting the clinical data and collaborated in the statistical analysis. JRG and AJ-V analyzed and interpreted the results. AH and AT critically revised the manuscript and also gave their final approval. Primary responsability for the paper rests with JR-G and AJ-V, who contributed equally to the paper. The authors reported no conflict of interest.

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