**SHORT REPORT**

**SOCS3 deregulation contributes to aberrant activation of the JAK/STAT pathway in precursor T-cell neoplasms**

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**Summary**

Despite the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway being frequently altered in T-ALL/LBL, no specific therapy has been approved for T-ALL/LBL patients with constitutive signalling by JAK/STAT, so there is an urgent need to identify pathway members that may be potential therapeutic targets. In the present study, we searched for JAK/STAT pathway members potentially modulated through aberrant methylation and identified SOCS3 hypermethylation as a recurrent event in T-ALL/LBL. Additionally, we explored the implications of SOCS3 deregulation in T-ALL/LBL and demonstrated that SOCS3 counteracts the constitutive activation of the JAK/STAT pathway through different molecular mechanisms. Therefore, SOCS3 emerges as a potential therapeutic target in T-ALL/LBL.

**Keywords**

JAK/STAT pathway, precursor T-cell neoplasms (T-ALL/LBL), SOCS3

**Abbreviations**: dbGaP, Genotypes and phenotypes database; JAK/STAT, Janus kinase/signal transducers and activators of transcription; NCI, National Cancer Institute; T-ALL/LBL, T-cell acute lymphoblastic leukaemia / T-cell lymphoblastic lymphoma; TARGET, Therapeutically Applicable Research to Generate Effective Treatments; TSG, tumour suppressor gene; TSS, transcription start site.

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Precursor T-cell neoplasms (T-cell acute lymphoblastic leukaemia / T-cell lymphoblastic lymphoma, T-ALL/LBL) are aggressive haematological malignancies characterized by aberrant proliferation of immature thymocytes.4 The high toxicity associated with the current treatments and the low survival rate in case of relapse highlight the urgent need for novel therapies targeting the frequently deregulated pathways in these haematological disorders.2,3

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is the second most deregulated signalling pathway in T-ALL/LBL only after the NOTCH1 pathway. Consequently, a significant proportion of T-ALL/LBL patients show constitutive activation of the JAK/STAT pathway, which leads to sustained phosphorylation of STAT proteins and tumour development.4 While previous studies have reported the efficacy of inhibiting certain JAK/STAT pathway members,5,6 no specific therapy has been officially approved yet for T-ALL/LBL patients with constitutive activation of the JAK/STAT pathway.7 Therefore, characterizing the molecular alterations responsible for the sustained phosphorylation of STAT proteins is essential to identify new pathway members which could be used as potential therapeutic targets. However, most genetic alterations identified so far through whole-exome sequencing are re-pressors and activators of transcription (JAK/STAT) pathway in these samples is indicated by + or − respectively. With a methylation density higher than 40% were considered as truly hypermethylated. The presence or absence of somatic mutations affecting genes belonging to the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway in these samples is indicated by + or − respectively.

In the present study, we wondered whether epigenetic alterations and specifically an aberrant methylation profile could have an impact on the JAK/STAT pathway signalling. To this end, we focused on the CpG islands frequently appearing near the transcription start site (TSS) and colocalizing with cis-regulatory elements because tumour suppressor genes (TSG) are often silenced in cancer cells due to hypermethylation of such islands.8 We recurrently identified hypermethylation of the SOCS3 gene, a member of the SOCS family which regulates the JAK/STAT pathway in a negative feedback loop.9 Additionally, we explored the possible implications of SOCS3 in T-ALL/LBL and demonstrated that SOCS3 counteracts the constitutive activation of the JAK/STAT pathway through different molecular mechanisms. Finally, we revealed the oncogenic role of multiple JAK1 mutations and showed their ability to induce SOCS3 phosphorylation at higher levels than JAK1WT in the absence of cytokines.

Primary samples of precursor T-cell neoplasms (n = 20) were obtained through the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es) (Table S1). Human postnatal thymocytes (n = 4) were isolated from thymuses removed during cardiac surgery of paediatric patients. Institutional review board approval was obtained for these studies (CEI 98–1825) and the participants provided written informed consent in accordance with the Declaration of Helsinki.

Mutations were generated using the QuickChange Site-Directed-Mutagenesis kit (Agilent). Cell transfection was accomplished using Lipofectamine™ 2000 (ThermoFisher) while virus production and cell transduction were performed as previously described.10 Cells were counted with trypan blue and TC10 Automated Cell-Counter (Bio-Rad). Reagents and equipment from Bio-Rad were used for electrophoresis and Western blot. Immunoprecipitation of SOCS3 was accomplished using Dynabeads™ Protein G Immunoprecipitation Kit (Invitrogen). Additional information is available in Methods S1.

In search for JAK/STAT pathway genes modulated by aberrant methylation, we analysed the CpG islands associated with the TSS of genes from SHP, SOCS and PIAS families.

![Figure 1](https://onlinelibrary.wiley.com/doi/10.1111/bjh.18694)
Such genes are considered as potential negative regulators of the JAK/STAT pathway and their methylation status remains unexplored in T-ALL/LBL except for the case of SOCS5.\textsuperscript{11,12} We first evaluated the extension or size of the CpG islands (Figure S1) and their colocalization with cis-regulatory elements (Figure S1). Next, the Infinium Methylation-EPIC array was employed to assess for the presence of hypermethylated CpG dinucleotides within the selected CpG islands in three T-ALL/LBL-derived cellular models (Figure S1). Following these criteria, we identified different candidates...
potentially modulated by aberrant methylation (Table S2). Among them, we focused on SOCS3 since (i) it has been found hypermethylated in other malignancies,13,14 (ii) does not show genetic alterations in T-ALL/LBL6, and (iii) it is essential for T-cell development.9 We employed bisulphite treatment followed by DNA-pyrosequencing to study the methylation density of CpG dinucleotides within the CpG island of SOCS3 in our patient-cohort and we observed SOCS3 hypermethylation in more than 50% of the analysed samples (Figure 1A). Moreover, the methylation status of SOCS3 was significantly higher in T-ALL/LBL patients when compared to that of normal thymocytes (Figure 1B). SOCS3 hypermethylation was independent of different parameters such as the subgroup, the immunophenotype and the age (Figure S1). We corroborated that SOCS3 hypermethylation was not limited to our patient cohort by analysing its methylation density in seven T-ALL/LBL-derived cell lines. Since SOCS3 hypermethylation promotes its deregulation in other malignancies,13,14 we confirmed this also in the case for T-ALL/LBL-derived cells by analysing the expression of SOCS3 in HPB-ALL cells, which respond to interleukin-7 (IL-7) and display SOCS3 hypermethylation, in the absence or presence of the demethylating agent decitabine (Figure 1E). The results showed decitabine induced a specific effect on SOCS3 expression in HPB-ALL cells and were further validated using normal thymocytes as a negative control (Figure 1F). Moreover, we observed a negative correlation between SOCS3 methylation and expression levels when evaluating normal thymocytes, T-ALL/LBL-derived cell lines and the T-ALL/LBL tumour samples with available RNA material (Figure 1G). Using data from the TARGET cohort, we observed that SOCS3 expression follows a variable pattern, with heterogeneous levels of SOCS3 (Figure 1H) and with around 60% of T-ALL/LBL tumour samples showing downregulation of SOCS3 relative to normal thymocytes (Figure 1I). Remarkably, T-ALL/LBL patients with reduced SOCS3 expression exhibited significantly higher levels of blasts in bone marrow at diagnosis (Figure 1J). Additionally, SOCS3 expression was independent of the immunophenotype but not of the molecular classification (Figure S1).

FIGURE 2 Functional consequences of SOCS3 deregulation in T-ALL/LBL. (A) Western blot for p-STAT5, STAT5, PIM1 and SOCS3 in HPB-ALL cells untransduced or transduced with SOCS3 and treated with IL-7 (10 ng/ml) for different time periods. (B) The methylation density of CpG dinucleotides was compared between patients with and without Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway mutations. * p < 0.05; ** p < 0.01. (C) Electrophogram confirming the presence of a new single-point mutation in JAK1 that causes the amino acid substitution at codon 805. The mutation was heterozygous so it appears as a double peak (indicated with an arrow). (D) Western blot for JAK1, p-STAT1 and STAT1 in U4A cells untransformed (−) or transformed with empty vector (E.V.), JAK1 WT or JAK1 mutants. (E) Cell growth (left) and viability (right) assays of Ba/F3 cells untransformed (○) or transformed with JAK1 WT or JAK1 mutants. Data show the mean ± standard deviation (SD) after three independent experiments. * p < 0.05; ** p < 0.01. (F) Western blot for JAK1, p-STAT5 and STAT5 in Ba/F3 cells untransformed (−) or transformed with JAK1 WT or JAK1 mutants. (G) Western blot for JAK1, pSTAT1, STAT1 and SOCS3 in U4A cells untransformed (−) or transformed with JAK1 WT or JAK1 mutants in combination with SOCS3. (H) Western blot for JAK1, p-tyrosine and SOCS3 in U4A cells untransformed (−) or transfected with JAK1 mutants in combination with SOCS3 WT or SOCS3 Δ204-221. Lanes corresponding to cells expressing JAK1 along with SOCS3 and analysed with anti-p-tyrosine show a specific band around 25 kDa which is indicated with an arrow and may be attributed to SOCS3 (27kD). (I) Western blot for JAK1, p-tyrosine and SOCS3 in U4A cells untransformed (−) or transfected with JAK1 mutants in combination with SOCS3 WT or SOCS3 Δ204-221. Lanes corresponding to cells expressing JAK1 along with SOCS3 WT but not SOCS3 Δ204-221 and analysed with anti-p-tyrosine show a specific band which is indicated with an arrow and may be attributed to SOCS3 (27kDa). Western blots are representative examples of at least three independent experiments.
Finally, we analysed the effects of SOCS3 on the JAK1 mutations that we had comprehensively characterized as oncogenic and observed that SOCS3 attenuates the activity of these mutants, as shown by a reduction in STAT1 phosphorylation, a specific target of JAK1 (Figure 2G). The results were corroborated when evaluating the global levels of JAK1-induced tyrosine phosphorylation (Figure 2H). Notably, the appearance of a particular band at approximately 25 kDa (indicated with an arrow in Figure 2H) prompted us to examine whether it may correspond to phosphorylated SOCS3, since SOCS3 has a predicted molecular weight of 27 kDa and can be phosphorylated at two tyrosines, Tyr204 and Tyr221, by different protein tyrosine-kinases including JAK1.16 To prove such a hypothesis, we performed immunoprecipitation of SOCS3 followed by detection of phosphorylated tyrosines. Our results show that JAK1 oncogenic mutants induce SOCS3 phosphorylation to a greater extent than JAK1WT (Figure 2H, Figure S2D) and that such phosphorylation occurs specifically at Tyr204 and Tyr221, as demonstrated when the SOCS3Y204K221F mutant was used instead of SOCS3WT (Figure 2I, Figure S2E).

Our results identify the hypermethylation of the CpG island associated with the TSS of SOCS3 as a recurrent event in T-ALL/LBL. Contrary to most alterations affecting other members of the JAK/STAT pathway, we observed that SOCS3 hypermethylation has a notable incidence in T-ALL/LBL and was identified in more than 50% of the analysed samples. Additionally, we explored the possible implications of SOCS3 in T-ALL/LBL and demonstrated that SOCS3 counteracts the constitutive activation of the JAK/STAT pathway by attenuating the signalling through IL-7 and the oncogenic activity of JAK1 mutants. Moreover, we revealed the oncogenic role of JAK1 mutations JAK1D604T, JAK1L653F and JAK1F805V and their sensitivity to ruxolitinib. Finally, we show that such mutations induce SOCS3 phosphorylation at Tyr204 and Tyr221 at higher levels than JAK1WT, suggesting they could promote the aberrant hyperactivation of the JAK/STAT pathway not only by phosphorylating STAT proteins but also SOCS3. In conclusion, SOCS3 emerges as a potential therapeutic target for T-ALL/LBL patients with constitutive activation of the JAK/STAT pathway and future studies will be needed to determine the efficacy of SOCS3 mimetics17,18 against this malignancy.

AUTHOR CONTRIBUTIONS
Antonio Lahera designed, performed and analysed experiments; Antonio Lahera, José Fernández-Piqueras and María Villa-Morales conceived the project and the experimental plan; Pilar López-Nieva and Agustín F. Fernández performed and analysed the methylation studies; José L. López-Lorenzo and María Á. Cobos-Fernández helped with flow cytometry and cell culture; Hernán Alarcón contributed to the generation of DNA constructs; Pablo Fernández-Navarro. advised on statistical analyses; Sara Ruiz-García and Isabel Sastre helped with qPCR experiments; María Villa-Morales analysed data from TARGET; Laura Vela-Martín assisted with scientific figures preparation; Pilar Llamas, José L. López-Lorenzo and Javier Cornago collected human primary samples and clinical data; Antonio Lahera, José Fernández-Piqueras, María Villa-Morales and Pilar López-Nieva prepared and wrote the manuscript; Javier Santos assisted with review and editing of the manuscript; and all authors approved the manuscript in its final format.

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CONFLICT OF INTEREST STATEMENT
There are no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT
The data corresponding to the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative that support the findings of this study are available from the National Cancer Institute (NCI). Restrictions apply to the availability of these data, which were used under license for this study. Data are available at the genotypes and phenotypes database (dbGaP, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study) with the permission of the NCI. Additional data that support the findings of this study are available in the supplementary material of this article and/or from the corresponding authors upon reasonable request.

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