Increased Expression of Suppressor of Cytokine Signaling 1 mRNA in Patients With Rheumatoid Arthritis

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The objective of this study was to investigate the associations between suppressor of cytokine signaling 1 (SOCS1) mRNA expression and *SOCS1* polymorphisms with the development of rheumatoid arthritis (RA). One hundred and eighty-one patients with RA and 96 healthy controls were enrolled in this study. The SOCS1 mRNA level in peripheral blood mononuclear cells (PBMCs) was detected by quantitative real-time polymerase chain reaction. *SOCS1* polymorphisms were determined by the polymerase chain reaction/restriction fragment length polymorphism method. We found that the expression of SOCS1 mRNA in PBMCs was significantly greater in patients with RA than in healthy controls. There were no significant differences in the expression of SOCS1 mRNA among patients with different disease activities. The increment in SOCS1 mRNA after stimulation with various cytokines was slightly lower in the patients with RA than in the healthy controls. This study also demonstrated that the *SOCS1* polymorphisms were not associated with susceptibility to RA. In conclusion, the expression of SOCS1 mRNA in PBMCs is higher in patients with RA than in healthy controls. The increment in SOCS1 mRNA expression in PBMCs after stimulation with different cytokines seems to be lower in patients with RA than in healthy controls.

Key Words: polymorphisms, rheumatoid arthritis, SOCS1
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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by destructive arthropathy. In addition to joints, internal organs may also be involved. Genetic and environmental factors may be involved in the pathogenesis of RA [1–3]. Although the detailed pathogenesis is still unclear, cytokines may play an important role in the perpetuation of arthritis [4]. A disordered balance between pro- and anti-inflammatory cytokine production can be found in rheumatoid synovitis. The production of pro- and anti-inflammatory cytokines in the synovial membrane is increased, although the balance is shifted toward pro-inflammatory cytokines.

The suppressor of cytokine signaling (SOCS) and cytokine-inducible SH2 (CIS) protein are a family of cytokine-inducible negative regulators of cytokine signaling [5]. There are eight members in the CIS/SOCS family of proteins including CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7. They have a central SH2 domain, an N-terminal domain of variable lengths and sequences, and a SOCS box in the

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C-terminal domain. The SOCS box is involved in the recruitment of the ubiquitin-transferase system and mediates protein degradation. SOCS1 and SOCS3 also have a kinase inhibitory region in the N-terminal domain, which inhibits Janus kinase (JAK) tyrosine kinase activity. Therefore, SOCS1 and SOCS3 seem to have a combined effect on kinase inhibition by kinase inhibitory region and proteosomal degradation by the SOCS box. SOCS1 and SOCS3 bind to and inhibit the catalytic activity of JAKs. Then, the complexes may be degraded by ubiquitination and proteasomal degradation mediated via the SOCS box. SOCS1 regulates the response of immune cells to cytokines, and regulates cytokine-mediated homeostasis, including innate and adaptive immunity [6]. Many cytokines activate JAK, resulting in the activation of signal transducer and activator of transcription (STAT), and subsequent nuclear localization with the induction of gene expression [7]. The binding of STAT to DNA induces the *SOCS1* expression and the production of SOCS1, which provides negative feedback to regulate the cytokine–JAK–STAT pathway [6,8]. Meanwhile, SOCS1 may also be induced by nuclear factor kappa B (NF-κB) and, in turn, suppresses NF-κB expression via negative feedback [9,10].

SOCS1 influences the signaling of many cytokines related to inflammatory diseases. The negative regulation of cytokine signaling may be impaired if the protein expression of SOCS1 is low. Therefore, SOCS1 may be associated with the development of inflammatory diseases. Isomaki et al reported elevated expression of SOCS1 in peripheral blood mononuclear cells (PBMCs) from patients with RA [11]. However, the study by Tsao et al showed that mRNA expression of SOCS1 in PBMCs was not significantly different between RA patients and healthy controls [12]. Until now, an association between *SOCS1* polymorphisms and RA has not been reported. In this study, we investigated the associations between SOCS1 mRNA expression and polymorphisms in patients with RA.

Methods

One hundred and eighty-one patients with RA (147 females and 34 males; mean age ± standard deviation, 48.3 ± 12.6 years) and 96 healthy controls (79 females and 17 males; 45.6 ± 11.8 years) were enrolled in this study. All of the patients and controls were Taiwanese. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital. The diagnosis of RA was made according to the 1987 American College of Rheumatology revised criteria for the classification of RA.

Stimulation of PBMC with various cytokines

PBMCs were isolated from 10 patients with RA and 10 healthy controls. PBMCs were cultured at 1×10^6 cells/mL in complete RPMI medium, and stimulated with 10 ng/mL of recombinant interferon (IFN)-γ, interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNFα) for 1 hour or 16 hours. Then, total RNA extraction and quantitative real-time polymerase chain reaction (PCR) were performed to determine SOCS1 mRNA expression.

Total RNA extraction, reverse transcription, and genomic DNA extraction

The PBMCs were separated by the Ficoll-Paque method. Total RNA was extracted by a NucleoSpin RNA II kit (Macherey-Nagel, GmbH, Düren, Germany). Reverse transcription was performed to obtain cDNA using a high capacity cDNA Archive kit with random primers (Applied Biosystems, City, State, Country). Genomic DNA was extracted from the buffy coat of the peripheral blood using a commercial kit (Geneaid Biotech Ltd.).

Quantitative real-time PCR

For quantitative analysis of SOCS1 mRNA in PBMC, quantitative real-time PCR was performed with the ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA, USA). In this study, RNA polymerase II (RP II) was used as the endogenous control because of its stable expression in different tissues [13]. The sequences of the primers and TaqMan probes (Applied Biosystems, Foster City, CA, USA) for SOCS1 and RP II mRNA are summarized in Table 1. The real-time PCR condition consisted of an initial incubation at 50°C for 2 minutes, enzyme activation at 95°C for 10 minutes, and 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 62°C for 1 minute. The hybridization temperature of the TaqMan probe was 62°C. All of the samples were tested in triplicate. SOCS1 mRNA expression was normalized to RP II and its relative expression was calculated using the $2^{-\Delta CT}$ method. A validation test was also performed

Table 1. Sequences of the primers and probes to detect suppressor of cytokine signaling 1 and RNA polymerase II in quantitative real-time polymerase chain reaction

Gene	Primers/probes	Sequences
SOCS1	forward	5′-GGG AGC GGA TGG GTG TAG G-3′
	reverse	5′-AGA GGT AGG AGG TGC GAG TTC-3′
	probe	5′-FAM-CGC GAT CGG GAC CCA GAG GGA GCA CCA GGA GAT CGC G-TAMRA-3′
RNA Polymerase II	forward	5′-GCA CCA CGT CCA ATG ACA T-3′
	reverse	5′-GTG CGG CTG CTT CCA TAA-3′
	probe	5′-HEX-TAC CAC GTC ATC TCC TTT GAT GGC TCC TA-BHQ-3′

SOCS1 = Suppressor of cytokine signaling 1.

to determine the PCR efficiency of the target and endogenous controls. The results showed that the amplification efficiencies of SOCS1 and RP II were approximately equal.

Polymorphisms of SOCS1

There are several polymorphisms in the promoter region of *SOCS1* and one non-synonymous polymorphism (rs 11549428; 1335 C/G, amino acid 210, His→Gln) in exon 2 of *SOCS1*. Harada et al showed that the *SOCS1* promoter polymorphism –1478 del enhanced the transcriptional level of SOCS1 [14]. The biallelic mutation in the SOCS box, which is encoded by exon 2 in *SOCS1*, might result in impaired JAK2 degradation and sustained JAK2 activation [15]. Therefore, the polymorphisms –1478 CA/del and 1335 C/G were investigated in this study. A polymorphism 1351 C/A (rs 1801729; exon 2) in the 3′-untranslated region was also determined. These polymorphisms were determined by the PCR/restriction fragment length polymorphism method. The sequences of primers and restriction enzymes are summarized in Table 2. To determine the *SOCS1* –1478 CA/del polymorphisms, PCR was done under the following conditions: initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The PCR product was digested with *Ddl*I.

The amplification conditions for the determination of the *SOCS1* 1335 G/C and 1351 C/A polymorphisms were as follows: initial denaturation at 96°C for 3 minutes, followed by five cycles of denaturation at 96°C for 1 minute; annealing at 60°C for 1 minute and extension at 72°C for 1 minute; and 25 cycles of denaturation at 96°C for 1 minute; annealing at 55°C for 1 minute and extension at 72°C for 1 minute; and a final extension at 72°C for 7 minutes. The restriction enzymes, *Hpy*188III and *Hha*I, were used to determine the *SOCS1* 1335 G/C and *SOCS1* 1351 C/A polymorphisms, respectively.

Serum cytokine concentrations

The serum concentrations of TNFα and IFNγ were determined using commercially available enzyme-linked immunosorbent assays (eBioscience Inc., San Diego, CA, USA). All of the samples were tested in duplicate.

Clinical manifestations

The disease activity score 28 (DAS28) was used to assess the disease activities of RA patients [16,17]. The disease activities of RA were defined as low activity ($3.2 \geq$ DAS28 > 2.6); moderate activity ($5.1 \geq$ DAS28 > 3.2); and high activity (DAS28 > 5.1). The Sharp score was also used to assess joint involvement [18].

Statistical analysis

Student's *t* test was used to compare SOCS1 mRNA expression between the RA patients and the controls. SOCS1 mRNA expression was compared among the patients with RA according to disease activity using one-way analysis of variance. The Mann-Whitney *U* test was used to compare the effects of cytokine stimulation on PBMC SOCS1 mRNA between the patients with RA and the controls. The χ^2 test was used to compare the genotype distributions of *SOCS1* polymorphisms between the patients with RA and the controls. Pearson's correlation test was used to evaluate the correlation between SOCS1 mRNA level and Sharp score.

Table 2. Sequences of primers and restriction enzymes for detecting suppressor of cytokine signaling 1 polymorphisms

Polymorphisms	Primers	Restriction enzymes
–1478 CA/del	5′-TGTCGTCCAGCTGCACCTC-3′ 5′-ACC ACA GGC TTC AGA GGA AC-3′	*Dde* I
1335 G/C & 1351 C/A	5′-GCT GCT GGA GCA CTA CGT G-3′ 5′-CCAGATACAGTTAAGCTGCTAC-3′	*Hpy*188III (for 1335 G/C) *HhaI* (for 1351 C/A)

Results

This study demonstrated that the expression of SOCS1 mRNA was significantly higher in the PBMCs of patients with RA than in the controls (Figure 1; $p < 0.001$).

Table 3 shows the SOCS1 mRNA expression in patients with RA according to disease activity. One-way analysis of variance revealed no differences in SOCS1 mRNA levels according to disease activity.

Figure 2 shows the SOCS1 mRNA expression in PBMCs stimulated with various cytokines (10 ng/mL) for 1 or 16 hours, from 10 patients with RA and 10 healthy controls. In the healthy controls, the expression of SOCS1 mRNA increased in response to stimulation with the cytokines, particularly with IFNγ, for 1 hour. In contrast, similar findings were not found in PBMCs from patients with RA except in response to stimulation with IFNγ. In PBMCs from patients with RA, there were no marked changes in the SOCS1 mRNA levels after stimulation with IL-1β, IL-6, and TNFα. The increments in SOCS1 expression in the RA patients were lower than those of the controls. The expression of SOCS1 mRNA, after stimulation with IFNγ for 16 hours was significantly lower in PBMCs from patients with RA than in those from healthy controls ($p < 0.05$). A similar finding was also found after stimulation with IL-1β ($p = 0.008$).

The *SOCS1* 1335G and 1351A alleles were not detected in this population of Taiwanese people (Table 4). There were no significant differences in the genotype frequencies of *SOCS1* –1478 CA/del polymorphisms between the patients with RA and the healthy controls.

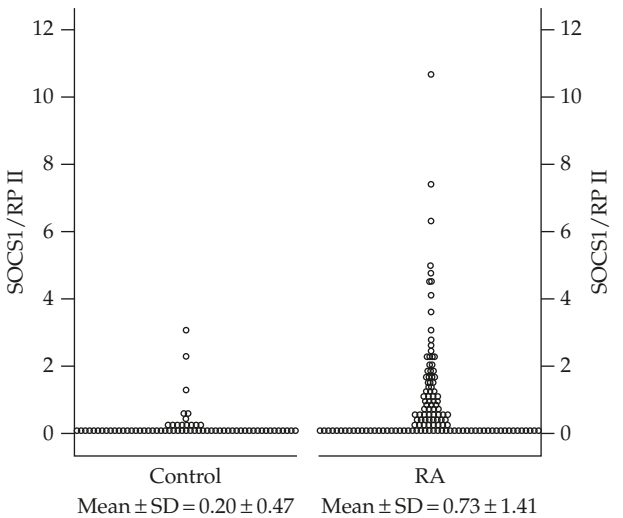

Figure 1. *Suppressor of cytokine signaling 1 mRNA expression normalized for RNA polymerase II in peripheral blood mononuclear cells obtained from patients with rheumatoid arthritis and in healthy controls. RA = Rheumatoid arthritis; SOCS1 = suppressor of cytokine signaling 1; RP II = RNA polymerase II; SD = standard deviation. (Control vs. RA 95% confidence interval = –0.77 to –0.3; p < 0.001).*

Table 3. Suppressor of cytokine signaling 1 mRNA expression in peripheral blood mononuclear cells obtained from patients with rheumatoid arthritis according to disease activity*

SOCS1 mRNA expression	SOCS1/RP II
High	0.65 ± 0.86
Moderate	0.90 ± 1.77
Low	0.74 ± 1.47

*Data presented as mean ± standard deviation. There were no significant differences in the levels of SOCS1 expression among the patients with different disease activities. RP II = RNA polymerase II; SOCS1 = suppressor of cytokine signaling 1.

Discussion

This study showed that the expression of SOCS1 mRNA in PBMCs was significantly higher in patients with RA than in healthy controls. However, the increment in SOCS1 mRNA expression in response to stimulation with IFNγ and IL-1β were significantly lower in PBMCs obtained from patients with RA than in those from healthy controls.

Many cytokines, including IL-2, IL-4, IL-6, IL-9, IL-13, IFNα, IFNβ, IFNγ and TNFα, may induce SOCS1

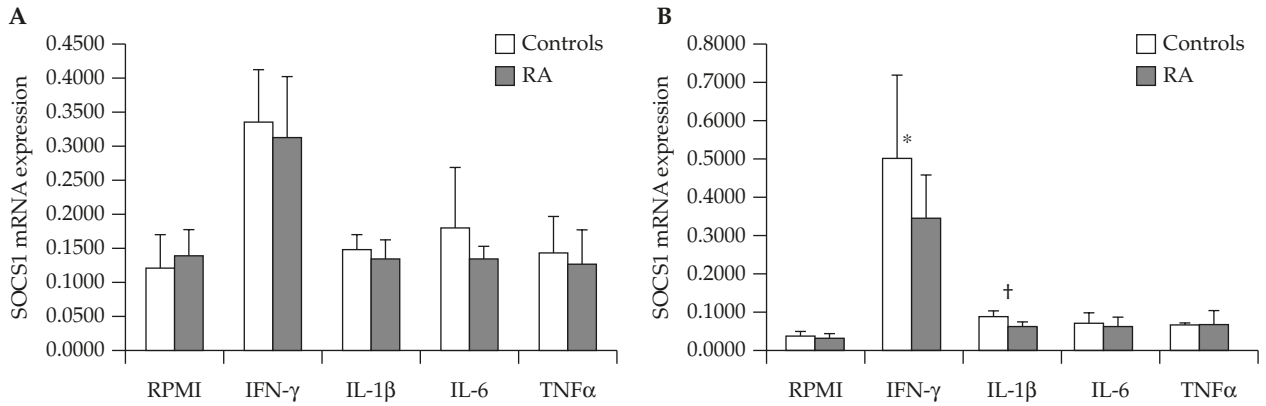

Figure 2. *Suppressor of cytokine signaling 1 mRNA expression in PBMCs from 10 patients with rheumatoid arthritis and 10 healthy controls in response to stimulation with various cytokines (10 ng/mL) for (A) 1 hour or (B) 16 hours. The Mann-Whitney U test was used for statistical analysis. *$p<0.05$ (Control vs. patients with rheumatoid arthritis); †$p=0.008$ (Control vs. patients with rheumatoid arthritis). RPMI = negative control; IFN = interferon; IL = interleukin; SOCS1 = suppressor of cytokine signaling 1; TNF = tumor necrosis factor; RA = patients with rheumatoid arthritis.*

Table 4. Frequencies of suppressor of cytokine signaling 1 polymorphisms in the patients with rheumatoid arthritis and healthy controls

SOCS1 polymorphisms	RA ($n=181$)	Healthy control ($n=96$)
–1478 CA/CA	130 (71.8)	73 (76.9)
–1478 CA/del	46 (25.4)	21 (21.8)
–1478 del/del	5 (2.8)	2 (2.1)
1335 C/C	181 (100)	96 (100)
1351 C/C	181 (100)	96 (100)

RA = Rheumatoid arthritis; SOCS1 = suppressor of cytokine signaling 1.

expression [19]. Colony stimulatory factors, hormones and growth factors may also induce the expression of SOCS1. Furthermore, SOCS1 expression is induced in macrophages by lipopolysaccharide (LPS) stimulation [9]. In turn, SOCS1 inhibits LPS-induced NF-κB and STAT1 activation [9]. Similarly, Wesemann et al showed that SOCS1 inhibited IFNγ-induced TNFα secretion and subsequent NF-κB activation [20]. These effects of SOCS1 may be mediated by SOCS1-induced degradation of the p65/NF-κB subunit [21]. Therefore, SOCS1 is induced by STAT and NF-κB, and suppresses both STAT1 and NF-κB.

SOCS1 is involved in innate immunity and plays a significant role in the pathogenesis of inflammatory diseases [22]. SOCS1 deficient mice developed multi-organ failure associated with severe inflammation [23,24]. Moreover, joint inflammation and destruction were significantly enhanced in SOCS1-deficient mice. An animal model with deletion of the STAT-binding site in gp130, an IL-6 receptor subunit, resulted in a severe joint disease resembling RA [25,26]. Fujimoto et al reported that SOCS1 expression in mutant mice with restoration of the SOCS1 gene was insufficient for effective downregulation of its target genes [27]. These mice had increased levels of serum immunoglobulins (Ig) and anti-DNA, and glomerulonephritis with glomerular IgG deposition. Therefore, dysfunction of SOCS1 may be a pathologic factor in systemic autoimmune diseases.

Our study demonstrated enhanced expression of SOCS1 in the PBMCs obtained from patients with RA, which might be caused by stimulation by cytokines or other factors. Although SOCS1 mRNA can be upregulated by IFNγ, the serum levels of IFNγ were not significantly different between the patients with RA and the controls in this study (RA, 37.00 ± 34.98 pg/mL; controls, 35.00 ± 12.95 pg/mL). Thus, the increased expression of SOCS1 in patients with RA may be caused by other factors.

The results of this study are consistent with those reported by Isomaki et al [11], but differ from those reported by Tsao et al [12]. The discrepancy between our study and the study by Tsao et al may be due to different sample sizes or different conditions of the patients with RA.

In this study, the increment in SOCS1 mRNA expression in cultured PBMCs stimulated by various cytokines seemed to be lower in PBMCs obtained from patients with RA than in those from healthy

controls. Furthermore, the SOCS1 mRNA expression after stimulation with IFNγ or IL-1β for 16 hours were significantly lower in PBMCs obtained from patients with RA than in those obtained from healthy controls. Although the mechanisms underlying the lower increment in SOCS1 expression after stimulation with cytokines are still unknown, they may be related to preactivation of PBMCs in patients with RA, a state that has been reported in earlier studies [28–30]. The lower increment in SOCS1 mRNA expression may also be related to polymorphisms or DNA hypermethylation in the promoter of *SOCS1*. However, the genotype frequencies of *SOCS1* –1478 CA/del were not significantly different between the patients with RA and the controls in this study. *SOCS1* gene silencing caused by hypermethylation of the promoter CpG islands has been reported in solid tumors and in hematological malignancies [31–36]. Decreased *SOCS1* gene expression because of DNA methylation may stimulate liver inflammation [36]. However, it is unknown whether the lower increment in SOCS1 mRNA in patients with RA is related to DNA hypermethylation of *SOCS1*.

This study showed that the expression of SOCS1 mRNA was higher in patients with moderate RA disease activity than in those with low disease activity. In contrast, the SOCS1 mRNA level was slightly lower in patients with high disease activity compared with those with moderate or low disease activity. The reasons for these findings are still unknown. Genetic or epigenetic changes in *SOCS1* may influence the expression of SOCS1 mRNA. Although there were no differences in the genotype distributions of the *SOCS1* –1478 CA/del polymorphisms according to disease activity, it cannot be excluded that other polymorphisms may be related to the different expressions of SOCS1 mRNA. Moreover, other members of the SOCS family may contribute to inflammation in RA.

We found that the SOCS1 mRNA levels were not associated with the Sharp scores of the RA patients (data not shown). Similarly, the *SOCS1* –1478 CA/del polymorphisms were not related to the Sharp scores. The reasons are still unknown. Sharp scores are related to the severity of inflammation and disease duration. However, the SOCS1 mRNA levels cannot directly reflect the inflammation status of disease. In our previous study, we found increased expression of SOCS1 mRNA in patients with systemic lupus erythematosus [37].

As a negative regulator of cytokines, SOCS1 might be used in the treatment of cytokine dysregulated disorders [8,38]. Indeed, a tyrosine kinase inhibitor peptide, acting as a mimetic of SOCS1, inhibited IFNγ signaling and suppressed the proliferation of prostate cancer cell lines [39]. Shouda et al also showed that adenovirus-mediated overexpression of SOCS3 prevented mouse antigen-induced arthritis or collagen-induced arthritis [40]. In a mouse model of arthritis, SOCS1 can counteract the onset and progression of autoimmune arthritis [41]. SOCS1 was also reported to negatively regulate acute inflammatory arthritis and CD4+T cell activation [41,42]. Therefore, upregulating SOCS1 expression may offer a new therapeutic strategy for the treatment of RA [43,44].

In summary, this study showed that there were no significant differences in the genotype frequencies of *SOCS1* polymorphisms between patients with RA and the healthy controls. The expression of SOCS1 mRNA in PBMCs was significantly increased in those from patients with RA in comparison with those from healthy controls. However, the increment in SOCS1 mRNA in cultured PBMC after stimulation with cytokines was lower in PBMCs from patients with RA than in those from healthy controls.

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類風濕性關節炎病人之 SOCS1 mRNA 表現增加

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本研究的主要目的是在探討 **suppressor of cytokine signaling**（**SOCS1**）**mRNA** 含量以及 **SOCS1** 基因多形性和類風濕性關節炎（**Rheumatoid Arthritis, RA**）的關係。本研究共包括 **181** 位 **RA** 病人和 **96** 位正常健康者。周邊血液單核球中 **SOCS1 mRNA** 含量之測定是用即時定量聚合酶連鎖反應（**real-time PCR**）方法，而 **SOCS1** 基因多形性則用 **PCR/RFLP** 方法測定。本研究結果顯示 **RA** 病人周邊血液單核球中之 **SOCS1 mRNA** 含量比正常人顯著增高。不同疾病活動性之 **RA** 病人間，其 **SOCS1 mRNA** 含量並無顯著差異。**RA** 病人之周邊血液單核球經不同細胞激素刺激後，其 **SOCS1 mRNA** 增加的幅度比正常人低。本研究亦顯示 **SOCS1** 基因多形性和是否發生 **RA** 無關。簡言之，**RA** 病人之周邊血液單核球細胞含有較高的 **SOCS1 mRNA**。經不同細胞激素刺激後，其增加的幅度似乎比正常人者低。

關鍵詞：基因多形性，類風濕性關節炎，**SOCS1**

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