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Decreased expression of SOCS-3 mRNA in breast cancer with lymph node metastasis

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Abstract. Signal transducers and activators of transcription (STATs) play a pleomorphic role in signal transduction, similarly to an oncogene. Suppressors of cytokine signaling (SOCS) inhibit STAT pathways. In breast cancer, little is known about the correlation among STATs, SOCS, and clinicopathological/biological features. Therefore, we investigated p-STAT3 (activated form of STAT3) and SOCS-1/3 expression, and clarified their correlation. Immunohistochemical staining for p-STAT3 antigen was performed in 74 surgically resected primary breast cancers. Real-time RT-PCR was used to measure mRNA expression of SOCS-1 and SOCS-3. There were no significant correlations between p-STAT3 expression and clinicopathological/biological features. SOCS-3 mRNA expression in the lymph node-positive group was significantly lower than that in the negative group (p=0.013). Among three groups divided based on the number of involved lymph nodes (node-negative group, 1-3 involved nodes group, 4 or more involved nodes group), the group with 4 or more involved nodes had the lowest expression of SOCS-3 (p=0.043). Correlations were not seen between SOCS-1 and SOCS-3 expression and other clinicopathological/biological features, except for blood vessel invasion. There were no statistical correlations between either SOCS-1 or SOCS-3 mRNA expression and p-STAT3 expression. Reduced expression of SOCS-3 is closely related to lymph node metastasis. Therefore, SOCS-3 may be a good predictor for lymph node metastasis.

Introduction

Signal transducers and activators of transcription (STATs) are latent cytoplasmic proteins that are activated to participate in gene control by phosphorylation on a single tyrosine when cells encounter various extracellular cytokines, growth factors and hormones (1-3). Seven types of STAT protein have been identified to date; STAT1, 2, 3, 4, 5A, 5B, and 6 (1). Ligands bound to the JAK receptor-associated tyrosine kinases are subsequently phosphorylated, thus becoming activated. STATs have an SRC-homology-2 (SH2) domain through which they make contact with the receptor, dimerize, become phospho-STATs and move into the nucleus.

STAT3 plays a pleomorphic role in signal transduction. It typically acts as an oncogene. STAT3 regulates expression of VEGF and is associated with angiogenesis and tumor progression (4). Activation of STAT3 has been reported in many cancers; head and neck, breast, prostate, pancreas and leukemia (5-10). STAT3 expression is reported to be correlated with lymph node metastasis (11-13), and higher expression of STAT3 and p-STAT3 indicates a worse prognosis (10,14-16). However, in breast cancer, STAT3 activation is also associated with a better clinical outcome or decreased invasiveness; thus, the clinical role of STAT3 remains unclear (7,17).

Suppressors of cytokine signaling (SOCS) are inhibitors of STAT pathways and decrease cell sensitivity to cytokines (18-20). Eight types of SOCS protein that all contain an SH2 domain and a segment known as a SOCS-box located near the C terminal have been identified. SOCS-1 and SOCS-3 are the main inhibitors of STAT3 and they have similar structural characteristics (21). Cytokine stimulation activates the JAK-STAT pathway, leading to induction of SOCSs. These SOCS proteins then inhibit the signaling pathways that initially led to their production. SOCS proteins therefore act as part of a negative feedback loop. SOCS-1 and SOCS-3 appear to inhibit signaling by different mechanisms. SOCS-1 binds to JAKs and inhibits catalytic activity. SOCS-3 binds to JAK-proximal sites on cytokine receptors and inhibits the JAK activity (21). In various cancers, inhibition of STAT signaling suppresses cancer cell growth and induces apoptosis (22,23). SOCS-1 and SOCS-3 appears to have tumor suppressor activity (24-27). In gastric cancer, loss of SOCS-1 may be involved in lymph node metastasis and tumor progression (27). In breast cancer, however, correlations between SOCS1/3 expression and clinicopathological/biological factors, including p-STAT3, have not been investigated.

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Here, we studied p-STAT3 expression by immunohistochemistry and mRNA expression of SOCS-1 and SOCS-3 using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) in primary breast cancer samples, and investigated the relationship between p-STAT3, SOCS-1 and SOCS-3 expression and clinicopathological/biological factors.

Materials and methods

Patients and tissue samples. Primary invasive breast cancer specimens were obtained from 1 male and 73 female patients who underwent curative surgical resection at the Department of Breast Surgery, Tokyo Medical and Dental University, Tokyo, Japan, between October 2002 and April 2004. Mean patient age was 54.4 years (range 30-91 years). Informed consent was obtained from all patients before surgery. No patient had distant metastasis. Three cases recurred after surgery (two cases: bone metastasis, one case: lung metastasis). Axillary lymph-node dissection was performed in all patients, and 28 of 74 patients (37.8%) were found to have lymph node metastasis. Fresh tissue samples of about 100 mg for mRNA isolation were obtained from the center of each tumor immediately after removal. Remaining tissues were routinely fixed in formalin, and embedded in paraffin.

Examination of clinicopathological and biological features. After staining with hematoxylin and eosin, histopathological examination was performed according to the International Union Against Cancer Tumor-Node-Metastasis classification criteria (28). Blood vessel invasion (BVI) and lymphatic vessel invasion were also evaluated, and histopathological grading was based on the Elston scale (29). Cases were divided into three groups according to the number of involved lymph nodes (node-negative group, 1-3 involved nodes group, 4 or more involved nodes group). Biological features, including estrogen receptor (ER), progesterone receptor (PgR) and c-erbB-2 (HER2), were evaluated by immunohistochemistry.

p-STAT3 immunohistochemical staining. Sections (3-μm) cut from formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. After autoclaving to maximize antigen retrieval, endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in absolute methanol for 30 min. Non-specific reactivity was blocked using a solution containing 10% normal goat serum and 10% stock blocking solution. The primary antibody used for immunostaining was an anti-Phospho-Stat 3 (Tyr705) goat polyclonal antibody (#9131 Cell Signaling Technology, MA, USA) at a 1:50 dilution applied 24 h at 4˚C. Slides were then incubated with Histofine Simple Stain MAX PO G (Nichirei, Tokyo, Japan) as the secondary antibody for 30 min according to the manufacturer's instructions. Finally, sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride and were then counterstained with hematoxylin. As a negative control, non-immune goat IgG (Santa Cruz Biotechnology) was used as a substitute for the primary antibody. Nuclear staining intensity was graded on the following scale: -, no staining; +, weak staining; ++, moderate staining; +++, strong staining. Cases in which more than 10% of tumor cells were stained ++ or +++ were considered positive (Fig. 1).

Table I. Association between p-STAT3 expression and clinicopathological/biological features in breast cancer.

Patient and tumor characteristics	No. of patients	p-STAT3 Negative 35	p-STAT3 Positive 39	p-value
Age				
<50	32	16	16	0.68
≥51	42	19	23	
Histological type				
IDC	66	30	36	0.46[a]
Others	8	5	3	
Tumor size				
t1	38	20	18	0.35
t2,3	36	15	21	
Lymph node metastasis				
Negative	46	23	23	0.72
Positive	28	12	16	
Lymph node metastasis				
0	46	23	23	0.78
1,2,3	20	9	11	
>3	8	3	5	
Lympatic vessel invasion				
Negative	62	29	33	1
Positive	12	6	6	
Blood vessel invasion				
Negative	64	30	34	1
Positive	10	5	5	
Distant metastasis				
Absent	71	36	35	0.24[a]
Present	3	0	3	
Estrogen receptor				
Negative	21	10	11	0.97
Positive	53	25	28	
Progesteron receptor				
Negative	37	16	21	0.48
Positive	37	19	18	
HER2				
Negative	58	30	28	0.17
Positive	26	5	11	
Nuclear grade				
1	35	15	20	0.76
2	18	9	9	
3	21	11	10	
Histological grade				
1	30	17	13	0.22
2	31	11	20	
3	13	7	6	

[a]Fisher's exact probability test.

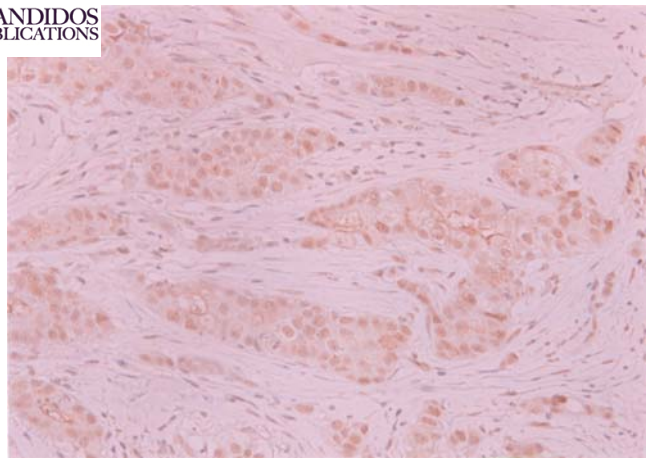

Figure 1. Immunohistochemical staining of p-STAT3. Positive staining for p-STAT3 in invasive ductal carcinoma was identified by the presence of brown coloration in the nuclei..

Real-time reverse transcriptional-polymerase chain reaction (real-time RT-PCR) for SOCS. Total RNA for each sample was isolated using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. After RNA isolation, cDNA was derived from each sample as described previously (30). Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (7300 real-time PCR System, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The thermal cycling profile consisted of: stage 1, 50˚C for 2 min; stage 2, 95˚C for 10 min; stage 3, 95˚C for 15 sec followed by 60˚C for 1 min. Stage 3 was repeated for 45 cycles. In experiments using fluorogenic probe and primer sets, Universal Master Mix was used. The 25-μl PCR reaction mixture contained 10 ng of cDNA, 15 μl of Universal Master Mix, each primer at 0.2 mmol/l, and 0.1 mmol/l fluorogenic probe.

PCR primers and probes had the following sequences: sense primer 5'-TTTTTCGCCCTTAGCGTGAAG-3', antisense primer 5'-CTGCCATCCAGGTGAAAGC-3', and probe 5'-CATCCGCGTGCACTTTCAGGCC-3' for SOCS-1; sense primer 5'-GACCAGCGCCACTTCTTCAC-3', antisense primer 5'-ACTGGATGCGCAGGTTCTTG-3', and probe 5'-CTCAGCGTCAAGACCCAGTCTGGGA-3' for SOCS-3; sense primer 5'-TGAGCGCGGCTACAGCTT-3', antisense primer 5'-TCCTTAATGTCACGCACGATTT-3', and probe 5'-ACCACCACGGCCGAGCGG-3' for ACTB. SOCS-1 and SOCS-3 real-time RT-PCR primers were designed using Primer Express ver 2.0 (Applied Biosytems). ACTB real-time RT-PCR primers are as described previously (31).

In each case, triplicate threshold cycle (C_t) values were obtained and averaged, and expression levels were then evaluated by the 2-$\Delta\Delta C_t$ method (32). The fold change in SOCS-1 and SOCS-3 was normalized against ACTB mRNA and was compared to untreated controls (calibration sample) as follows: $2^{-\Delta\Delta Ct}$ where, $\Delta\Delta C_t=(C_{t\text{-target}}-C_{t\text{-reference}})_{treated\text{-}sample}-(C_{t\text{-target}}-C_{t\text{-reference}})_{calibrator\text{-}sample}$. Calibrator-sample refers to the expression level (1X) of the target gene normalized against ACTB mRNA.

Statistical analysis. χ^2 test and Fisher's exact probability test were used to examine the association between p-STAT3 expression and clinicopathological/biological features. Comparison of mRNA levels between SOCS-1 and SOCS-3 was performed using Spearman's rank test. The relationship between mRNA expression of SOCS-1, SOCS-3 and clinicopathological/biological factors was assessed with Mann-Whitney U test and Kruskal Wallis test. The StatView 5.0 software package was used for all analysis. A $p<0.05$ was considered to be statistically significant.

Results

Correlations between p-STAT3 expression and clinicopathological/biological features. The clinicopathological and biological features, and p-STAT3 expression status for the 74 cases are listed in Table I. P-STAT3 was overexpressed in 39 of 74 cases (52.7%) and there was no significant correlation between p-STAT3 expression and clinicopathological/biological features. Three patients with distant metastasis were positive for p-STAT3 expression. There was no correlation between recurrent distant metastasis and p-STAT3 expression.

Correlations between SOCS-1 and SOCS-3 mRNA expression, and clinicopathological/biological features. Correlations between SOCS-1 and SOCS-3 mRNA expression, and clinicopathological/biological features are summarized in Table II.

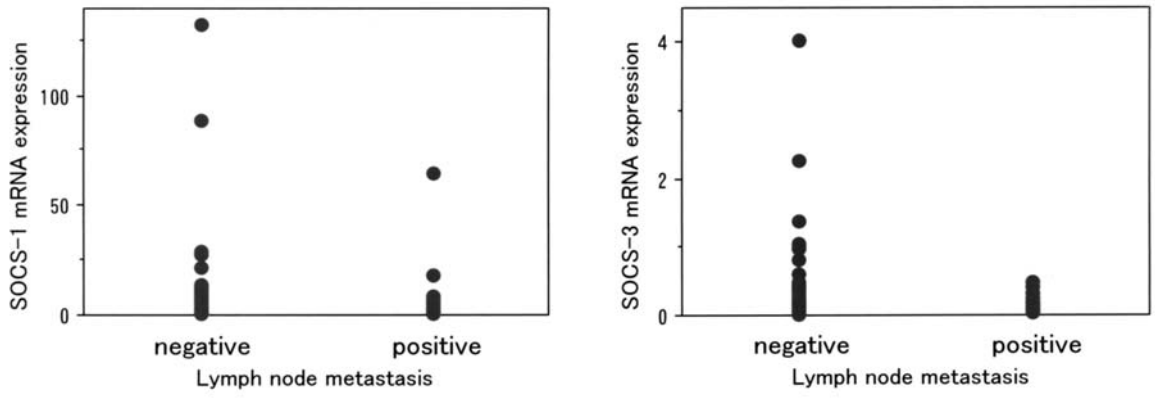

Figure 2. SOCS-1/3 mRNA expression in the node-negative and node-positive groups. SOCS-3 mRNA expression in the node-negative group is higher than that in the positive group (p=0.013).

Table II. Association between SOCS-1/3 mRNA expression and clinicopathological/biological features in breast cancer.

Patient and tumor characteristics	No.of patients	SOCS1mRNA mean ± SD	p-value	SOCS3mRNA mean ± SD	p-value
Age					
<50	32	7.7±23	0.18	0.26±0.31	0.44
≥51	42	8.9±17		0.38±0.69	
Histological type					
IDC	66	8.3±2.5	0.67	0.26±0.043	0.028
Others	8	9.2±3.7		0.86±0.47	
Tumor size					
t1	38	6.8±12	0.72	0.25±0.26	0.56
t2,3	36	10±26		0.41±0.57	
Lymph node metastasis					
Negative	46	10±23	0.2	0.43±0.68	0.013
Positive	28	5.4±12		0.16±0.11	
Lymph node metastasis					
0	46	10±23	0.18[a]	0.43±0.68	0.043[a]
1,2,3	20	6.9±14		0.17±0.12	
>3	8	1.7±1.2		0.14±0.082	
Lymph node metastasis (T2,3)					
Negative	18	18±35	0.034	0.67±1.0	0.04
Positive	18	2.2±1.6		0.15±0.10	
Lymph node metastasis (T2,3)					
0	18	18±35	0.078[a]	0.67±1.0	0.016[a]
1,2,3	10	2.5±1.8		0.16±0.11	
>3	8	1.7±1.2		0.14±0.082	
Lympatic vessel invasion					
Negative	62	7.2±14	0.36	0.33±0.59	0.45
Positive	12	14±11		0.32±0.36	
Blood vessel invasion					
Negative	64	9.3±21	0.037	0.36±0.59	0.015
Positive	10	2.1±2.2		0.12±0.081	
Distant metastasis					
Absent	71	8.6±20	0.75	0.32±0.57	0.087
Present	3	3.1±3.0		0.46±0.32	
Estrogen receptor					
Negative	21	13±31	0.43	0.30±0.30	0.12
Positive	53	6.4±13		0.34±0.63	
Progesteron receptor					
Negative	37	9.2±18	0.75	0.37±0.72	0.67
Positive	37	7.5±22		0.28±0.31	
HER2					
Negative	58	8.6±21	0.8	0.36±0.62	0.6
Positive	26	7.5±16		0.22±0.20	
Nuclear grade					
1	35	5.4±6.4	0.15[a]	0.36±0.68	0.11[a]
2	18	2.6±2.6		0.19±0.21	
3	21	18±35		0.39±0.53	
Histological grade					
1	30	5.3±6.7	0.76[a]	0.32±0.72	0.35[a]
2	31	10±28		0.37±0.50	
3	13	11±18		0.24±0.12	
p-STAT3					
Negative	35	5.0±6.4	0.69	0.20±0.19	0.12
Positive	39	11±26		0.44±0.73	

[a]Kruskal Wallis test.

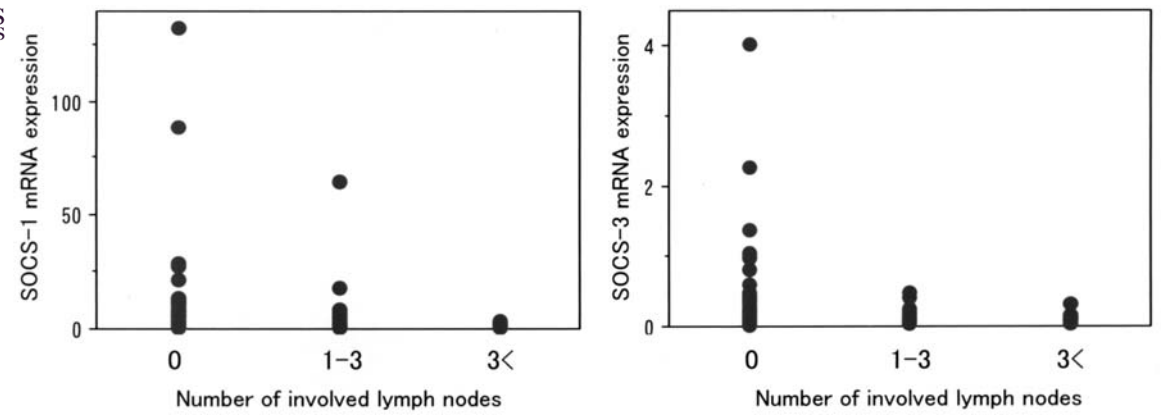

Figure 3. SOCS-1/3 mRNA expression in node-negative group, 1-3 involved nodes group, and 4 or more involved nodes group. The 4 or more involved nodes group had the lowest expression of SOCS-3 (p=0.043).

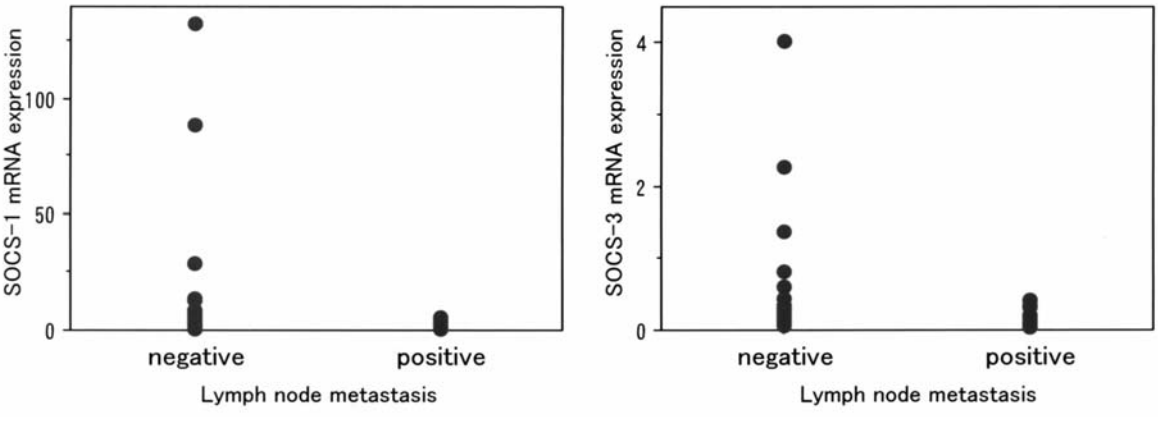

Figure 4. SOCS-1/3 mRNA expression in T2 and T3 cases in the node-negative and node-positive groups. SOCS-1 and SOCS-3 mRNA expression in the node-negative group was higher than that in the positive group (SOCS-1, p=0.034; SOCS-3, p=0.04).

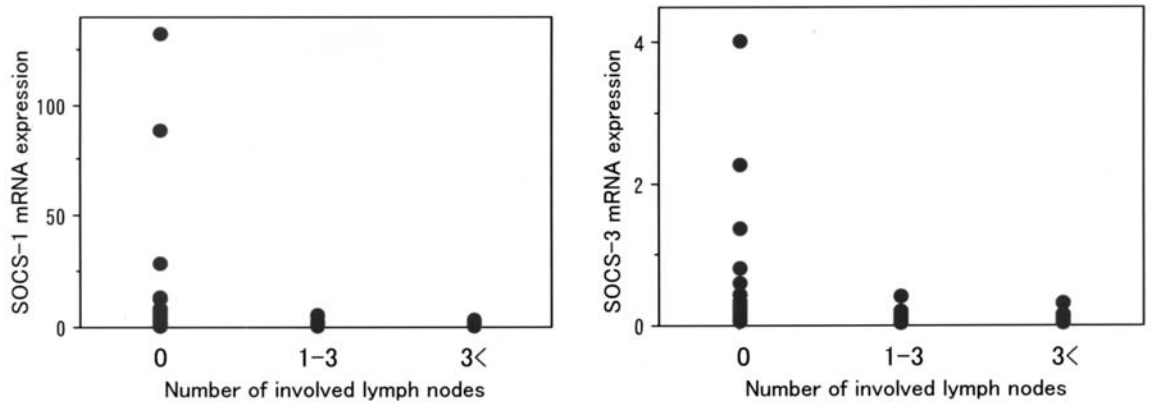

Figure 5. SOCS-1/3 mRNA expression in T2 and T3 cases in node-negative group, 1-3 involved nodes group, and 4 or more involved nodes group. The 4 or more involved nodes group had the lowest expression of SOCS-3 (p=0.016).

There was a significant correlation between SOCS-1 and SOCS-3 expression (p=0.0028). SOCS-3 mRNA expression in the lymph node-positive group was significantly lower than that in the negative group (p=0.013, Fig. 2). The group with 4 or more involved nodes had the lowest expression of SOCS-3 among the three groups (p=0.043, Fig. 3). There were no significant differences in SOCS-1 among the three groups.

In the 36 T2 and T3 cases, both SOCS-1 and SOCS-3 mRNA expression in the lymph node-positive group was significantly lower than that in the negative group (SOCS-1, p=0.034; SOCS-3, p=0.04; Fig. 4). SOCS-3 mRNA expression was significantly lower based on the number of involved lymph nodes (p=0.016, Fig. 5). There were no differences in SOCS-1 mRNA expression among the three groups (p=0.078, Fig. 5).

BVI was observed in 10 of 74 cases. Both SOCS-1 and SOCS-3 mRNA expression in the BVI-positive group was significantly lower than that in the negative group (SOCS-1, p=0.037; SOCS-3, p=0.015). The invasive ductal carcinoma group showed lower mRNA expression of SOCS-3 than other histological types. There were no significant correlations between SOCS-1 and SOCS-3 mRNA expression and other clinicopathological/biological features.

Correlations between p-STAT3, SOCS-1 and SOCS-3 mRNA expression. There were no statistical correlations between p-STAT3, SOCS-1 and SOCS-3 mRNA expression (SOCS-1, p=0.69; SOCS-3, p=0.12).

Discussion

STAT3 has been recognized as an oncogene and is considered to play an important role in the progression and evolution of neoplasms (1). We were unable to find any correlations between p-STAT3 expression and clinicopathological/biological features in breast cancer, while correlations between lymph node metastasis and p-STAT3 expression in breast cancer and colorectal cancer have been reported (11-13). Hsieh *et al* suggested that STAT3 promotes the invasion and metastasis of breast cancer by activating downstream genes, such as MMP-1, MMP-2, MMP-10, VEGF, and COX2 (11). However, others have reported the opposite; Dolled-Filhart *et al* reported that STAT3 played a role as a tumor suppressor protein in breast cancer without lymph node metastasis (7), while Dien *et al* indicated an inverse correlation between p-STAT3 expression and lymphatic and vascular invasion in breast cancer and suggested that STAT3 up-regulates tissue inhibitor of metalloproteinase-1, thereby decreasing breast cancer invasion (17). Because STAT3 is activated via a combination of different mechanisms, it may contribute to tumor progression and metastasis, as well as tumor suppression and apoptosis (7,11-13,17,33). Thus, the role of STAT3 in breast cancer still remains uncertain. Interestingly, in this study, all three cases that developed distant metastasis showed nuclear accumulation of p-STAT3 on immunostaining.

It has been reported that SOCS, particularly SOCS-1 and SOCS-3, suppress cancer cell growth and induce apoptosis by inhibiting JAK/STAT signaling (24-27,34-36). In this study, breast cancers with lymph node metastasis showed significantly lower expression of SOCS-3 when compared to those without lymph node metastasis. SOCS-3 expression in the group with 4 or more positive lymph nodes was the lowest among the three groups. We failed to find an association between SOCS-1 mRNA expression and lymph node metastasis. However, T2 and T3 tumors with lymph node metastasis exhibited significantly lower expression of both SOCS-1 and SOCS-3 mRNA than those without lymph node metastasis. SOCS-3 mRNA expression in the group with 4 or more involved nodes was the lowest among the three groups, and SOCS-1 expression also tended to be lower as the number of lymph node metastases increased. T1 tumors did not show any association between SOCS-1 and SOCS-3 mRNA expression and lymph node metastasis. The effects of SOCS-1 and SOCS-3 on inhibiting lymph node metastasis may have been more potent as the primary tumor was growing. The present results suggest that SOCS-3, and to a lesser degree, SOCS-1, play a role in preventing lymph node metastasis of breast cancer cells.

The antitumor activity of SOCS-1 has been reported by several groups. Yoshikawa *et al* showed that restoration of SOCS-1 suppressed development and progression of hepatocellular carcinoma (HCC) cells (24). Oshimo *et al* reported that loss of SOCS-1 increased responsiveness of gastric cancer cells to IL-6 signals, and was involved in lymph node metastasis and tumor progression (27). These results suggest that SOCS-1 functions as a novel anti-oncogene that suppresses inflammation-induced carcinogenesis. In contrast, SOCS-3 is little studied in clinical cancer specimens. Recently, it was elucidated that SOCS-3 may also be involved in the suppression of tumor growth and metastasis in lung cancer, HCC and neck and head squamous cell carcinoma (26,34,37). Based on these reports and our own observations, SOCS-3 may function as a tumor suppressor, similarly to SOCS-1.

Although an inverse association between p-STAT3 and SOCS-1 or SOCS-3 was expected, we did not find any such association. This suggests that that both SOCS-1 and SOCS-3 interfere with tumor growth and metastasis by regulating molecules other than STAT3.

Lymph node metastasis is one of the strongest predictors of patient survival in breast cancer, and identification of factors that predict the metastasis to lymph nodes is important in avoiding an unnecessary lymph node dissection. Sentinel lymph node biopsy is useful for determining axillary lymph node dissection (38). However, sentinel lymph node biopsy may cause surgical stress and can give false negative results. Our data suggest that both SOCS-1 and SOCS-3, particularly SOCS-3, are good predictors for lymph node metastasis in breast cancer, and that preoperative measurement of SOCS-3 expression may be useful for predicting lymph node metastasis.

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