Suppressor of cytokine signaling-1 gene therapy induces potent antitumor effect in patient-derived esophageal squamous cell carcinoma xenograft mice

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Chronic inflammation is involved in cancer growth in esophageal squamous cell carcinoma (ESCC), which is a highly refractory cancer with poor prognosis. This study investigated the antitumor effect and mechanisms of SOCS1 gene therapy for ESCC. Patients with ESCC showed epigenetics silencing of SOCS1 gene by methylation in the CpG islands. We infected 10 ESCC cells with an adenovirus-expressing SOCS1 (AdSOCS1) to examine the antitumor effect and mechanism of SOCS1 overexpression. SOCS1 overexpression markedly decreased the proliferation of all ESCC cell lines and induced apoptosis. Also, SOCS1 inhibited the proliferation of ESCC cells via multiple signaling pathways including Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and focal adhesion kinase (FAK)/p44/42 mitogen-activated protein kinase (p44/42 MAPK). Additionally, we established two xenograft mouse models in which TE14 ESCC cells or ESCC patient-derived tissues (PDX) were subcutaneously implanted. Mice were intra-tumorally injected with AdSOCS1 or control adenovirus vector (AdLacZ). In mice, tumor volumes and tumor weights were significantly lower in mice treated with AdSOCS1 than that with AdLacZ as similar mechanism to the in vitro findings. The Ki-67 index of tumors treated with AdSOCS1 was significantly lower than that with AdLacZ, and SOCS1 gene therapy induced apoptosis. These findings demonstrated that overexpression of SOCS1 has a potent antitumor effect against ESCC both in vitro and in vivo including PDX mice. SOCS1 gene therapy may be a promising approach for the treatment of ESCC.

Esophageal cancer is a highly refractory cancer; worldwide, almost 450,000 new cases of esophageal cancer are diagnosed annually. Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related mortality in the world.1 In Asian countries, esophageal squamous cell carcinoma (ESCC) is a major histological form of esophageal cancer. Patients with ESCC are faced with poor prognoses, despite undergoing multidisciplinary treatments, including surgery, radiotherapy and chemotherapy.2,3 One of the reason of poor prognoses is the complexities of pathophysiology of esophagus. The lack of a serosa layer in the esophagus and the location of the tissue in a very narrow mediastinal space allows early tumor invasion into the neighboring organs, such as the trachea, bronchus, lung, and aorta

Key words: esophageal squamous cell cancer, suppressor of cytokine signaling, signal transducer and activator of transcription 3, patient-derived xenograft model

Abbreviations: BSA: bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; ESCC: esophageal squamous cell carcinoma; FAK: focal adhesion kinase; JAK: Janus kinase; MAPK: mitogen-activated protein kinase; MOI: multiplicity of infection; MSP: methylation-specific polymerase chain reaction; PBMCs: human peripheral blood mononuclear cells; PDX: patient-derived xenograft; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SH2: src homology 2; siRNA: small-interfering RNA; SOCS: suppressor of cytokine signaling; STAT3: signal transducer and activator of transcription 3

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What's new?
Chronic inflammation is thought to play a role in esophageal squamous cell carcinoma (ESCC). A protein called SOCS1 reduces inflammatory signaling, and the gene for SOCS1 is inactivated in many ESCC tumors. In this study, the authors found that, when SOCS1 levels were increased in mice via gene therapy, the proliferation of ESCC xenografts decreased, and apoptosis was induced in these cells via several pathways. SOCS1 gene therapy may thus be a promising approach for the treatment of ESCC.

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AAAATAACCCAGAAGCTG-3′ (reverse); sequences of corresponding primers specific for unmethylated DNA were 5′-TATTTTTGTTGATGTGATGTT-3′ (forward) and 5′-AAACTCAACACAACCACCTC-3′ (reverse).25 The lengths of the PCR products were 132 bp for the methylated reaction and 122 bp for the unmethylated reaction. PCR products were resolved on 2.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination. To ensure that the PCR primers were specific for the detection of methylated or unmethylated bisulfite-converted DNA, completely methylated or unmethylated bisulfite-converted DNAs (EpiTect control DNA; Qiagen) were used for control experiments.

**Real-time PCR analysis**

After serum starvation for 12 h, ESCC cell lines and human peripheral blood mononuclear cells (PBMCs) were treated with 10 ng/mL recombinant human interferon (IFN)-γ (PeproTech, Rocky Hill, NJ) for 15 min. cDNAs were synthesized using a SuperPrep Cell Lysis & RT Kit for qPCR (TOYOBO, Osaka, Japan) according to the manufacturers’ instructions. The forward and reverse primers were as follows: human SOCS1 forward primer, 5′-AGACCCCTTCTCACCTCTG-3′ and reverse primer, 5′-GCACAGCAGAAATAAACGC-3′; β-actin forward primer 5′-GGGGGCACCACAAGGACA-3′ and reverse primer, 5′-CTCTTAAAGTGCAGCAGATTTCC-3′.26 SOCS1 mRNA expression was quantified by real-time reverse transcription RT-PCR using Thunderbird SYBR qPCR Mix (TOYOBO). The RT-PCR conditions were as follows: one cycle at 95°C for 10 min and then 40 cycles of 96°C for 10 s, 68°C for 15 s, and 72°C for 15 s. PCR products were measured continuously using the My IQ Single-Color Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA).

**Enzyme-linked immunosorbent assay (ELISA)**

Cell lines were cultured in six-well plates at a density of 2.5 × 10^5 cells per well in RPMI 1640 containing 0.5% FBS and 1% PS. The concentrations of IL-6 in the cell culture supernatant were measured after 24 h using human IL-6 Quanti-Elisa from BD Transduction Laboratories (San Jose, CA). My IQ Single-Color Real-Time Detection System (Perkin-Elmer Life Sciences, Boston, MA).

**Western blotting analysis**

ESCC cell lines and tumor tissues from xenograft mouse models were harvested and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1× phosphatase inhibitor cocktail [Nacalai Tesque], and 1× protease inhibitor cocktail [Nacalai Tesque]), followed by centrifugation at 13,200 rpm for 15 min at 4°C. The supernatants were then stored at −80°C until use. Protein concentrations were determined with a DC Protein Assay kit (Bio-Rad Laboratories) using bovine serum albumin (BSA) as the concentration standard.

Proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with gels purchased from Wako Pure Chemical Industries (Osaka, Japan) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 1% BSA in Tris-Buffered Saline containing 0.1% Tween 20 and incubated with the respective antibodies against different targets. The following antibodies were used: anti-phospho-STAT3 (1:1,000 dilution), anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) (1:1,000 dilution), anti-cleaved caspase-3 (1:500 dilution); all from Cell Signaling Technology, Danvers, MA; anti-STAT3 (1:1,000 dilution), anti-GAPDH (1:2,000 dilution); all from Santa Cruz Biotechnology, Santa Cruz, CA, anti-phospho-focal adhesion kinase (FAK) (Tyr397; 1:1,000 dilution), anti-FAK (1:1,000 dilution); all from BD Transduction Laboratories, San Jose, CA, and anti-β-actin (1:1,000 dilution; Sigma, St. Louis, MO). Next, the membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG or horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Finally, the signals were visualized using an enhanced chemiluminescence (ECL) reaction system (Perkin-Elmer Life Sciences, Boston, MA).

**Flow cytometric analysis**

The expression of coxsackievirus and adenovirus receptor (CAR) in ESCC cell surface molecules was assessed by flow cytometry analysis. Cells were incubated in a 1:80 dilution of anti-CAR monoclonal antibody RmC (Merck Millipore) or with buffer only for 1 h at 4°C. After being washed with PBS-BSA, cells were incubated in a 1:50 dilution of FITC-labeled goat anti-mouse F(ab)2-fragments (DAKO, Glostrup, Denmark) for 1 h at 4°C. The number of CAR per cell surface was quantified as antibody binding capacity by indirect immunofluorescence assay using QIFIKIT (DAKO) according to the manufacturer’s instructions.

**Adenoviral vectors**

A replication-defective recombinant adenoviral vector expressing the mouse SOCS1 gene was provided by Dr. Hirokazu Mizuguchi (Osaka University, Osaka, Japan) and was constructed using an improved in vitro ligation method, as described previously.33,34 An adenoviral vector expressing the LacZ gene was constructed using similar methods. Expression of these genes was regulated by the CMV promoter/enhancer and intron A. The viruses were amplified in 293 cells and purified using CsCl2 step gradient ultracentrifugation followed by CsCl2 linear gradient ultracentrifugation. The purified viruses were dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and 10% glycerol and were stored at −80°C. Viral particle and biological titers were determined using a spectrophotometric method and QuickTiter (Adenovirus Titer Immunooassay Kit; Cell Biolabs, San Diego, CA), respectively. After incubation of ESCC cells with 3x10^5 viral particles/mL for 1h, the cells were washed with PBS and cultured for 24 h. After fixation, the cells were stained with X-Gal (Roche) and visualized under UV illumination. The number of stained cells was counted for each group, and the mean number of stained cells was calculated. The results were analyzed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA).
for 24 h, cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 10 to 160.

**Cell proliferation assay**

ESCC cell lines were plated in 96-well plates at a density of 2 × 10^3 cells per well and incubated for 24 h. Cell proliferation was evaluated with WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt] assays (Cell Counting Kit-SF; Nacalai Tesque) at the indicated times after treatment. The absorption of WST-8 was measured at a wavelength of 450 nm with a reference wavelength of 630 nm using a microplate reader (Model 680; Bio-Rad Laboratories). The growth rate was expressed as the percentage of absorbance for treated cells versus control cells. Experiments were performed with six replicate wells for each sample, and the data are presented as averages.

**Caspase-3/7 activity assay**

ESCC cell lines were plated into 96-well white plates at a density of 2 × 10^3 cells per well and treated with AdLacZ or AdSOCS1 for 48 h. The activities of caspase-3 and −7 in cell culture were detected using Caspase Glo 3/7 Assays (Promega, Madison, WI) according to the manufacturer’s instructions. Luminometer readings were determined by 1 h after addition of the reagent using a Spectra Max Gemini EM Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Small-interfering RNA (siRNA) transfection**

The following siRNA ON-TAR-GET Plus SMART pools were purchased from Thermo Scientific Dharmacon (Lafayette, CO): nontargeting siRNA (D-001810–10-20) and human PTK2 siRNA (L-003164–00-0010). ESCC cell lines were seeded in antibiotic-free medium. The next day, cells were transfected with nontargeting or PTK2 siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**ESCC cell xenograft and patient-derived xenograft (PDX) mouse models**

All animal experiments were conducted according to the institutional ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation (Osaka, Japan). We established two types of mouse xenografts; mice were inoculated subcutaneously with ESCC cells or human ESCC tissues. In the ESCC cell xenograft model, female ICR mice (Charles River Japan, Yokohama, Japan). This study received IRB approval in Osaka University Hospital, and include protocol number (09011–2). Mice were observed daily for tumor growth, and tumors were passaged once or twice.

**Immunohistochemistry**

Subcutaneously implanted tumors were harvested and embedded in paraffin for immunohistochemical analysis using anti-SOCS1 antibodies (Abcam, Cambridge, MA), anti-CXADR (Atlas Antibodies, Stockholm, Sweden) and anti-Ki-67 antibodies (Novocea Strauss Laboratories, Newcastle, UK). Terminal dUTP nick-end labeling (TUNEL) assays (with DAPI nuclear counterstaining) were carried out using an ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions.

**Statistical analysis**

Data are shown as means ± standard deviations (SDs) from the indicated number of experiments. In xenograft mouse models, data are shown as means ± standard errors of the means (SEMs). To test for statistically significant differences between two groups, unpaired Student’s t-tests were used. Two-sided p values <0.05 were considered significant. These analyses were carried out using JMP version 11.0 (SAS Institute, Cary, NC).

**Results**

**SOCS1 gene CpG islands were methylated in ESCC cell lines and human ESCC tissue**

SOCS1 gene methylation is frequently observed in primary ESCC. Therefore, we first screened human ESCC/normal tissues and ten ESCC cell lines for methylation of SOCS1 gene. MSP analysis was performed using primers selected from the CpG islands within exon 1 of SOCS1 gene, and we detected SOCS1 methylation in four human ESCC tissues and all ten ESCC cell lines, but not in normal human tissue (Figs. 1a and 1b). Real-time PCR analysis showed that all ESCC cell lines failed to upregulate SOCS1 or showed very low expression of SOCS1 in response to IFN-γ, whereas human PBMCs markedly upregulated SOCS1 expression in response to IFN-γ (Fig. 1c).

**Production of IL-6, constitutive activation status and coxsackievirus and adenovirus receptor (CAR) expression in ESCC cell lines**

Previous studies have revealed that IL-6 levels are elevated in cancer tissues of patients with ESCC and that serum IL-6 levels are associated with patient prognosis. In this study, we quantified the levels of IL-6 in 24-hr culture supernatants from ESCC cell lines by sandwich ELISA. Ten ESCC cell lines were evaluated, and serum IL-6 levels were elevated in 7 of these 10 ESCC cell lines (i.e., TE1, TE4, TE5, TE6, TE8, TE10, and TE15), but not in TE9, TE11, and TE14 cells (Fig. 2a). Next, we evaluated the constitutive activation status of
Similar to STAT3, FAK, and p44/42 MAPK signaling pathways are also involved in the proliferation of esophageal cancer. Similar to STAT3, FAK, and p44/42 MAPK signaling pathways are also involved in the proliferation of esophageal cancer.36,37 As shown in Figure 2b, STAT3 was constitutively phosphorylated at high levels in seven ESCC cell lines (TE1, TE4, TE5, TE6, TE8, TE10, and TE15 cells). In contrast, p-STAT3 levels in three ESCC cell lines (TE9, TE11, and TE14) were low. These results suggested that self-production of IL-6 may be crucial for aberrant STAT3 phosphorylation in ESCC cell lines. In the expression of CAR which functions as a primary receptor for adenovirus, although the levels of it differed respectively, we confirmed it in all ESCC cell lines (Fig. 2c).

Proliferation inhibition of SOCS1 gene therapy using adenovirus vector in ESCC cells

We used replication-defective recombinant adenoviral vectors harboring SOCS1 gene in cell proliferation assays. As shown in Figure 2d, WST-8 assays revealed that adenovirus-mediated SOCS1 gene delivery (AdSOCS1) markedly decreased the proliferation of all ESCC cell lines in a concentration-dependent manner. Although some cancer cell lines did not exhibit reduced proliferation in response to AdSOCS1 in our previous reports,29–31 all 10 ESCC cell lines examined in this study showed marked growth inhibition in a concentration-dependent manner in the presence of AdSOCS1.

For analysis of the growth inhibition mechanism of AdSOCS1 in ESCC, we selected three ESCC cell lines exhibiting different in levels of p-STAT3: TE4 and TE8 cells had high levels of p-STAT3, whereas TE14 cells had low levels of p-STAT3. Immunoblotting analysis showed that the level of p-STAT3 (Tyr705) was decreased in response to overexpression of SOCS1 protein in these cell lines (Fig. 3a). In addition, total and phosphorylated FAK (Tyr397) was downregulated by overexpression of SOCS1 protein in these cell lines. TE8 and TE14 cells also showed reduced phosphorylation of p44/42 MAPK, which functions downstream of FAK signaling (Fig. 3b).

To evaluate the induction of apoptosis by overexpressed SOCS1 protein, we measured the levels of caspase-3/7 activity using luminescence assays after infection with AdSOCS1 or AdLacZ. The levels of caspase-3/7 activity in AdSOCS1-infected cells were significantly higher than those in AdLacZ-infected cells (Fig. 3c). In addition, overexpression of SOCS1...
was associated with increased expression of cleaved caspase-3, as shown by western blotting (Fig. 3). These results indicated that SOCS1 induced apoptosis in ESCC cells.

**JAK inhibition and FAK downregulation suppressed cell proliferation in ESCC cells**

To confirm whether the activities of JAK/STAT and FAK/p44/42 MAPK signaling regulated the proliferation of three ESCC cell lines (TE4, TE8, and TE14 cells), these cells were treated with a JAK inhibitor I or FAK-specific siRNA. Cell proliferation assays showed that JAK inhibitor I suppressed the proliferation of TE4 and TE8 cells, which had high levels of p-STAT3, but showed reduced effects in TE14 cells, which had lower levels of p-STAT3 (Fig. 4a). As shown in Figure 4b, p-STAT3 (Tyr705) levels were decreased in response to JAK inhibitor I in three ESCC cell lines, similar to the results...
with AdSOCS1. However, JAK inhibitor I did not influence FAK/p44/42 MAPK signaling, unlike AdSOCS1 (Fig. 4b).

Next, we examined the association between FAK/p44/42 MAPK signaling and ESCC cell proliferation using FAK-siRNA. Cell proliferation was suppressed by inhibition of FAK activation in three ESCC cell lines, and p44/42 MAPK phosphorylation was inhibited by FAK-siRNA in TE8 and TE14 cells, similar to the effects observed in response to AdSOCS1.

Figure 3. Evaluation of the mechanism through which AdSOCS1 inhibited proliferation in ESCC cell lines. (a) Levels of SOCS1, p-STAT3, and STAT3 at 24 h after infection with AdSOCS1 or AdLacZ at 10–160 MOI. (b) Levels of p-FAK/FAK and p-p44/42 MAPK/p44/42 MAPK at 48 h after infection with AdSOCS1 or AdLacZ at 10–160 MOI. (c) Caspase-3/7 activity was determined using luminescence assays 48 h after infection with AdSOCS1 or AdLacZ at 10–160 MOI. (d) Expression of cleaved-caspase3 48 h after infection with AdSOCS1 or AdLacZ at 10–160 MOI. Statistical analyses were performed using Student’s t-tests (*p < 0.05, **p < 0.01).
Figure 4. Evaluation of the effects of JAK/STAT and FAK/p44/42 MAPK signaling inhibition on proliferation in ESCC cell lines. (a) Cell proliferation was determined by WST-8 assays at 72 h after exposure of JAK inhibitor I (0.125–2.0 μM). Each value is the means ±SDs. (b) Levels of p-STAT3/STAT3, p-FAK/FAK, and p-p44/42 MAPK/p44/42 MAPK 48 h after exposure to JAK inhibitor I (0.125–2.0 μM). (c) Cell proliferation was determined by WST-8 assays at 72 h after transfection with FAK-siRNA or nonspecific siRNA as a control. Each value is the means ±SDs. (d) Levels of pFAK/FAK, p-p44/42 MAPK/p44/42 MAPK, and p-STAT3/STAT3 72 h after transfection with FAK-siRNA or nonspecific siRNA as a control. (e) Cell proliferation was determined by WST-8 assays at 72 h after exposure to a combination of JAK inhibitor I and FAK-siRNA or nonspecific siRNA as a control. Each value is the means ±SDs. Statistical analyses were performed using Student’s t-tests (*p < 0.05, **p < 0.01).
AdSOCS1 (Figs. 4c and 4d). In addition, we examined the combined effects of JAK inhibitor I and FAK-siRNA. As shown in Figure 4e, combined treatment was more effective for inhibition of cell proliferation in three ESCC cell lines. In particular, TE14 cells showed reduced growth inhibition in response to JAK inhibitor I, but increased growth inhibition when JAK inhibitor I was combined with inhibition of FAK/p44/42 MAPK signaling. Thus, these results suggested that both JAK/STAT and FAK/p44/42 MAPK signaling played crucial roles in the proliferation of ESCC cells.

**Antitumor effects of SOCS-1 in ESCC xenograft models**

Finally, we evaluated the therapeutic effects of AdSOCS1 infection on ESCC in vivo. For this purpose, we established two xenograft models; the first was ICR nu/nu mice in which TE14 cells were subcutaneously implanted, whereas the second was NOD-SCID mice in which human ESCC tissues were subcutaneously implanted. When the tumor volume reached approximately 100 mm$^3$, $1 \times 10^8$ plaque-forming units (pfu) AdSOCS1 or AdLacZ was injected intratumorally in a volume of 50 μL seven times, twice per week. Tumor volumes were determined weekly by measuring the length, width, and height, and tumor weights were also evaluated (Figs. 5a and 5d). Intratumoral injection of AdSOCS1 ($1 \times 10^8$ pfu/50 μL) significantly suppressed tumor growth, including tumor volume and tumor weight, compared with that in the control AdLacZ-injected groups in both xenograft mouse models (Figs. 5b, 5c, 5e and 5f). In particular, PDX mouse model treated with AdSOCS1 showed no increase in tumor volume (Figs. 5e and 5f). We also evaluated the CAR expression of ESCC xenograft tumors by immunohistochemical analysis. Tumors in the PDX model showed markedly stronger expression of CAR than that in the TE14 xenograft model (Fig. 5g).

We next examined the therapeutic effects of AdSOCS1 injection in vivo by examination of pathological changes in tumor tissues. Immunohistochemical analysis confirmed the overexpression of SOCS1 in mice treated with AdSOCS1, but not in mice treated with AdLacZ (Fig. 6a). The subcutaneous tumors were also harvested and analyzed by western blotting. In AdSOCS1-injected tumor, we confirmed the expression of SOCS1 and the inhibition of p-STAT3 (Tyr705). In addition, FAK/p44/42 MAPK signaling was also downregulated, similar to the in vitro findings (Fig. 6b). The expression of Ki-67 was significantly suppressed in AdSOCS1-injected tumors as compared with that in AdLacZ-injected tumors. Furthermore, few Ki-67-positive nuclei were detected by immunohistochemical analysis in AdSOCS1-injected tumors compared with that in AdLacZ-injected tumors, and strong expression of SOCS1 was associated with decreased expression of Ki-67 in subcutaneous tumors (Fig. 6c). The Ki-67 index in AdSOCS1 group was significantly decreased compared with that in AdLacZ group (Fig. 6d). These results indicated that proliferating cells were decreased by overexpression of SOCS1. TUNEL staining showed that SOCS1 gene therapy induced apoptosis in vivo (Fig. 6e).

**CAR expression in resected tissue specimens from ESCC patients**

We evaluated the expression of CAR in resected tissue specimens from 34 ESCC patients who were underwent esophageal resection in our hospital. We confirmed the CAR expression in all ESCC specimens except one, and 74% of ESCC specimens showed a stronger expression of CAR than that of normal esophageal tissue (Fig. 1f).

**Discussion**

We have previously reported that overexpression of SOCS protein in cells using an adenovirus vector causes potent antiproliferative effects, mainly by targeting JAK/STAT signaling in various cancers.27–31 In addition, SOCS gene therapy has been shown to have antiproliferative mechanisms other than the inhibition of JAK/STAT signaling in various types of cancer; for example, SOCS gene therapy causes inhibition of FAK, p44/42 MAPK, p38 MAPK, and EGFR and stabilization of p53.27–31 In ESCC, SOCS1 gene therapy induced apoptosis via the inhibition of JAK/STAT signaling, similar to other cancers.27–31 On the other hand, we demonstrated the unique inhibitory effect of SOCS1 on FAK signaling in ESCC. Although previous reports for other cancers showed that some cell lines showed less efficiency by AdSOCS1, all ESCC cell lines demonstrated suppressed proliferation in our reports. These findings suggest that ESCC is a good candidate for clinical application. We also previously reported that SOCS1 activates p53 via a direct interaction between the SH2 domain of SOCS1 and the N-terminal transactivation domain of p53,38 and overexpression of SOCS1 using an adenovirus vector has been shown to increase the phosphorylation and expression of p53. In the carcinogenic process of ESCC, integration of dysplastic lesions and p53 gene mutations has been observed in esophageal epithelium,39 and p53 gene mutations have been reported in about 80% of ESCC specimens.40 Most of ESCC with p53 gene mutation showed high expression of p53 at baseline by accumulation of abnormal p53 which had longer half-life. Therefore, antitumor effects associated with SOCS1-dependent stabilization of p53 might not be observed (Supporting Information Fig. S1), in contrast to these prior reports.

In the analysis of methylation status, we detected hypermethylation of SOCS1 in both human ESCC tissues and ESCC cell lines, similar to that observed in other cancers.25,26,28 In addition, because ESCC cell lines showing SOCS1 hypermethylation exhibited reduced induction of SOCS1 after stimulation with INF-γ, activation of STAT3 was not suppressed in ESCC. Therefore, the antitumor effects of SOCS1 overexpression in ESCC were similar to those in other cancers, as expected.
Figure 5. AdSOCS1 had antitumor effects in xenograft mouse model. (a) ESCC cell line (TE14) xenograft mice model; female ICR nu/nu mice (6–8 weeks of age) were injected with $2 \times 10^6$ TE14 cells. When the tumor volume reached approximately 100 mm$^3$, AdSOCS1 or AdLacZ was injected intratumorally a total of seven times twice per week. (b) Tumor volumes were determined twice per week. (c) Twenty-eight days after tumor cell inoculation, tumor volumes were calculated. (d) Female NOD-SCID mice (8 weeks of age) were transplanted with surgical resected samples from patients with ESCC who did not receive any preoperative radiation or chemotherapy. When the tumor volume reached approximately 100 mm$^3$, AdSOCS1 or AdLacZ was injected intratumorally a total of seven times twice per week. (e) Tumor volumes were determined twice per week. (f) Twenty-eight days after tumor cell inoculation, tumor volumes were calculated. (g) The evaluation of CAR expression of TE14 xenograft mice and PDX mice tumor by immunohistochemical analysis. Data are the mean volumes ± SEMs of four tumors in each group and were analyzed by Student’s t-tests (*p < 0.05, **p < 0.01).
In our analyses in other types of cancer, we confirmed the antitumor effects of SOCS1 in some cell lines, but not in others. In a previous study of gastric cancer cell lines by Natatsuka et al., the antitumor effects of SOCS1 were found in approximately half of the gastric cancer cell lines tested. In particular, based on the mechanism of SOCS function, cells

![Image of immunohistochemical analysis and Western blot analysis](image-url)

Figure 6. (a) Immunohistochemical analysis of SOCS1 in TE14 cell xenograft mice and PDX mice-derived tissue from animals injected with AdSOCS1 or AdLacZ. (b) Western blot analysis of SOCS1, p-STAT3/STAT3, pFAK/FAK, and p-p44/42 MAPK/p44/42 MAPK in TE14 cell-derived tissues from AdSOCS1- or AdLacZ-injected animals. (c) Immunohistochemical analysis of Ki-67 in TE14 cell and PDX-derived tissues from animals injected with AdSOCS1 or AdLacZ. (d) Ki-67 staining was recorded as the ratio of positively stained cells to all tumor cells in five fields (200× magnification). Statistical analyses were performed using Student’s t-tests (*p < 0.05, **p < 0.01). Values shown represent the means ± SDs. (e) Analysis of apoptosis by TUNEL staining (blue fluorescence, DAPI staining for nuclei; cyan fluorescence, TUNEL-positive staining) in TE14 cell and PDX-derived tissues from animals treated with AdSOCS1 or AdLacZ. (f) Thirty-four ESCC patients were studied. The normal esophageal mucosa basal layer was used as internal control for evaluation of CAR expression intensity. ESCC tissue specimens were analyzed for the degree of CAR expression by scoring from 0 to 3 on a categorical scale. The intensity of membrane staining was grouped into four categories: no staining/background of negative controls (0), weak staining less than internal control (1), moderate staining as same as internal control (2), and intense staining than internal control (3). Each section was scored independently by two individuals (T. S. and T. T.). Scale bar = White 100 μm, Black 25 μm.
exhibiting JAK/STAT3 phosphorylation were more likely to be sensitive to overexpression of SOCS. Similar to previous reports in other cancers, the effects of SOCS1 gene therapy were observed in ESCC cell lines with high levels of p-STAT3 in the present study. However, the effects of overexpressed SOCS1 were also observed in cell lines with low levels of p-STAT3, suggesting the involvement of pathways other than the JAK/STAT pathway.

FAK is a ubiquitously expressed nonreceptor protein tyrosine kinase that has emerged as a crucial molecule involved in the integration of signals from integrin and receptor tyrosine kinases in processes such as cell survival, proliferation, and motility. Additionally, previous studies have indicated that FAK contributes to tumor cell proliferation, survival, and metastasis. SOCS1 recruits the elongin BC-containing E3 ubiquitin-ligase complex through the conserved SOCS box to promote the degradation of target proteins, such as FAK. SOCS1 was found to inhibit FAK-dependent signaling events by suppressing FAK-associated kinase activity and tyrosine phosphorylation and total protein of FAK by promoting polyubiquitination and degradation of FAK in a SOCS box. In this study, in TE14 cells, which showed low levels of p-STAT3, the introduction of SOCS1 inhibited FAK phosphorylation. In addition, inhibition of p44/42 MAPK phosphorylation, a signaling pathway associated with FAK, was also observed. In TE14 cells, although JAK inhibitor I did not cause antiproliferative effects, inhibition of FAK suppressed p44/42 MAPK phosphorylation, suggesting an association with FAK/p44/42 MAPK pathway inhibition. Moreover, by inhibiting FAK and JAK using a pseudo-SOCS1 treatment model, the same antiproliferative effects observed in TE4 and TE8 cells with high levels of p-STAT3 were also observed in TE14 cells. From this result, the contribution of FAK/p44/42 MAPK cascade to the therapeutic effect was elucidated. Through these complex mechanisms, SOCS1 gene therapy may have anticancer effects in various esophageal cancer cell lines, suggesting that SOCS1 may be an effective therapeutic target.

To evaluate the efficacy of new anticancer drugs, more clinically predictive models of human cancers are clearly needed. Accordingly, in this study, we established two in vivo xenograft mouse models subcutaneously implanted with ESCC cell line or human ESCC tissues. Tumor cell lines are widely used for preclinical studies; however, they often do not reflect the original structural and molecular characteristics (e.g., heterogeneity) of real tumors. On the other hands, an important advantage of the PDX mouse model using human tumor tissues is that this model retains key characteristics of patients’ tumors. The histological characteristics, genomic signatures and heterogeneity of patient cancer cells are highly preserved in PDX tumors. Thus, PDX mouse models are the most clinically relevant cancer models developed to date and represent a highly predictive drug response platform that recapitulates the therapeutic outcome in human patients. To accomplish this, we subcutaneously implanted surgical resected fresh tissues from patients with ESCC who had not received any preoperative radiation or chemotherapy in NOD-SCID mice. Injection with AdSOCS1 significantly suppressed tumor growth; in particular, in the PDX mouse model, tumor volumes were not increased by injection of AdSOCS1.

For successful cellular entry, efficient attachment of the adenovirus capsid proteins to cell surface proteins, the expression of CAR is essential. All the adenoviral therapies rely on the ability of the virus to gain entry efficiently into the malignant target cells. Therefore, the level of CAR on the cell surface is an important factor in the efficacy of adenoviral therapy. Here, we evaluated the CAR expression in ESCC cell lines, ESCC xenograft mice, and resected tissue specimens from ESCC patients. Since tumors in the PDX model showed markedly stronger expression of CAR than that in the TE14 xenograft model (Fig. 5g), the PDX model might demonstrate a good therapeutic effect of AdSOCS1 owing to the degree of CAR expression. Although previous studies have demonstrated a loss of CAR expression in several cancers, Schrupp et al. suggested the high CAR expression in ESCC. In addition, our results of the CAR expression in resected ESCC specimens from ESCC patients revealed that 74% of ESCC tumor tissue showed a stronger expression of CAR than that of normal esophageal tissue (Fig. 1f). Thus, AdSOCS1 therapy can be expected to have a good therapeutic effect in ESCC patients in the clinical setting.

These findings provide the first evidence of the antitumor effects of SOCS1 gene therapy in PDX mice implanted with heterogeneous human cancer tissue. Based on these results, SOCS1 gene therapy may represent a new treatment for ESCC by inducing a variety of antitumor mechanisms. For locally advanced ESCC that has invaded adjacent indispensable organs, although chemoradiotherapy is reported to be the first treatment choice, local disease control may be difficult to achieve in some cases. Our findings demonstrate that SOCS1 gene therapy might be facilitated by administering in multiple sites of the tumor using endoscopy. The survival signal of STAT3 activation can result in resistance to chemoradiotherapy, SOCS1 gene therapy, which mainly targets JAK/STAT signaling, may be an effective novel therapy for local cancer after radiation therapy. In addition, because this therapeutic agent can be administered by endoscopic local injection, this approach may provide maximum therapeutic effects with fewer systemic side effects and may be easily applied in the clinical setting. However, since we used replication-defective recombinant adenovirus vector that has been modified not prevent intracellular growth in except for 293 cells, AdSOCS1 therapy had limited therapeutic effects only in the injected area of the tumor. Therefore, we consider that the therapeutic effects of this approach for lymph node metastasis and distant metastasis might be poor. To improve the therapeutic efficacy of this agent, a better drug delivery system may be needed.
In conclusion, SOCS1 gene therapy using an adenovirus vector suppressed the proliferation of all ESCC cell lines and induced apoptosis via inhibition of JAK/STAT signaling. In addition, we also discovered the contribution of FAK/p44/42 MAPK cascade to the therapeutic effect. Previous reports of other cancer showed that several cell lines were not suppressed the proliferation by AdSOCS1, therefore ESCC might have the potential to be a better target of SOCS1 gene therapy. Additionally, we demonstrated the antitumor effects of AdSOCS1 in two ESCC xenograft mouse models. In particular, this result in PDX mouse model which was implanted with heterogenous human cancer tissue is a first report of SOCS1 gene therapy, and the effectiveness for ESCC patients can be more expected. We hope that these findings may lead to the successful clinical application of SOCS1 gene therapy for ESCC.

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