SOCS1 Gene Therapy Improves Radiosensitivity and Enhances Irradiation-Induced DNA Damage in Esophageal Squamous Cell Carcinoma

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Abstract

STAT3 has been implicated recently in radioresistance in cancer. In this study, we investigated the association between STAT3 and radioresistance in esophageal squamous cell carcinoma (ESCC). Strong expression of activated phospho-STAT3 (p-STAT3) was observed in 16/22 ESCC patients with preoperative chemoradiotherapy (CRT) compared with 9 of 24 patients with surgery alone, where the prognosis of those with CRT was poor. Expression of p-STAT3 and the antiapoptotic proteins Mcl-1 and survivin was strongly induced in ESCC cells by irradiation. Ectopic STAT3 expression increased radioresistance, whereas expression of the STAT3 negative regulator SOCS1 via an adenovector improved radioresponse. Inhibiting the STAT3–Mcl-1 axis by SOCS1 enhanced DNA damage after irradiation and induced apoptosis. Combining SOCS1 with radiotherapy enhanced anti-tumor responses in a murine xenograft model compared with the individual therapies. Tumor repopulation occurred transiently after treatment by irradiation but not the combination SOCS1/radiotherapy. Tumors subjected to this combination expressed high levels of γH2AX and low levels of Ki-67, which was maintained after cessation of treatment. Overall, we demonstrated that inhibiting the STAT3–Mcl-1 signaling axis by ectopic SOCS1 improved radiosensitivity by inducing apoptosis and enhancing DNA damage after radiotherapy, offering a mechanistic rationale for a new ESCC treatment.

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related mortality in the world (1). In Asia, esophageal squamous cell carcinoma (ESCC) is the predominant histologic form of esophageal cancer. Although multimodal treatments, including surgery, radiotherapy, and chemotherapy are currently used, patients with ESCC still face with poor prognosis (2, 3). Definitive chemoradiotherapy (CRT) is a standard treatment option for locally advanced or unresectable ESCC (4, 5). However, loco-regional recurrence after CRT is observed in 40% to 60% of patients (6, 7). In addition, salvage surgery after CRT is associated with a high complication rate (50%–77%) and high mortality (approximately 15%; refs. 7–9). Therefore, outcomes for these patients are presently not satisfactory. To improve prognosis, the development of new multimodal treatments for the initial treatment of advanced ESCC is necessary.

A recent report suggested that members of the signal transducers and activators of transcription (STAT) family of proteins, and most prominently STAT3, are promising target candidates for combined CRT treatment (10). STAT3 is a DNA-binding factor that selectively binds the IL6-responsive element of promoters of acute-phase genes in IL-6–stimulated hepatocytes (11). STAT3 acts as an important transcriptional mediator of proinflammatory cytokine signaling pathways and contributes to oncogenesis by preventing apoptosis and enhancing cell proliferation (12, 13). Recently, a number of reports showed that irradiation (IR) activates STAT3, as indicated by increased phosphorylation (14), and that STAT3 mediates CRT resistance; thus, inhibition of STAT3 holds great promise for future multimodal treatment concepts in oncology (10).

Suppressor of cytokine signaling (SOCS) is a negative regulator of inflammatory cytokine signaling, including the JAK/STAT pathway (15). Among the SOCS family, which is characterized by a central src homology 2 (SH2) domains and a conserved C-terminal SOCS box, SOCS1 is the most potent negative regulator of proinflammatory cytokine signaling (16). SOCS1 interacts with phosphotyrosine residues to interfere with activation of STAT or other signaling intermediates (17, 18). We previously reported that SOCS1 overexpression using an adenovirus vector (AdSOCS1) results in antitumor effects by targeting the JAK/STAT and other signaling pathways.
The expression of phospho-STAT3 (p-STAT3) was examined by IHC staining using formalin-fixed and paraffin-embedded ESCC tissue sections using an anti-p-STAT3 (Tyr705) antibody (#9145, Cell Signaling Technology). Formalin-fixed paraffin-embedded sections (5-μm-thick) were collected for IHC experiments. Briefly, after deparaffinization, antigen retrieval using HistoVT One (Nacalai Tesque) – xenograft mouse model by targeting the JAK/STAT and FAK/ERK signaling pathway in ESCC (23).

Thus, we investigated the association between STAT3 activation and IR-resistance, and further examined the antitumor effects and associated mechanisms of radiotherapy combined with SOCS1 gene therapy for ESCC. In addition, we evaluated the expression of activated STAT3 in ESCC patients who underwent preoperative CRT, and examined changes in expression as it relates to prognosis.

Patients and Methods

Patients

Forty-six patients with thoracic advanced ESCC (surgery alone, N = 24; preoperative CRT, N = 22) who underwent curative resection between 2005 and 2012 at Osaka University Hospital were eligible for this retrospective study. This study was conducted in accordance with Declaration of Helsinki and was approved by an Institutional Review Board at Osaka University Hospital (No. 08226-6). CRT consisted of two courses of 5-fluorouracil (5-FU), cisplatin (CDDP), and adriamycin (ADM) and radiation (50.4 Gy). The data regarding patient characteristics, histologic examination, and survival were reviewed from medical reports. Follow-up was performed through routine visits for clinical assessment, and this information was obtained from outpatient records. Patient status was assessed at the time of the last follow-up, and survival was evaluated. We obtained informed written consent from all patients.

IHC

IHC staining was evaluated by two independent bodies, as described previously. Terminal dUTP nick-end labeling (TUNEL) assays (with DAPI nuclear counterstaining) were performed using an improved in vitro ligation method, as described previously (25). An adenoviral vector expressing the LacZ gene (AdLaCZ) was constructed using similar methods. After incubating ESCC cells for 24 hours, they were infected with adenoviral vectors at a multiplicity of infection (MOI).

Clonogenic assays

Cell lines and irradiation

Three human ESCC cell lines, namely, TE4 (RCB2097), TE8 (RCB2098), and TE14 (RCB2101), were obtained from the RIKEN BRC cell bank (Tsukuba, Japan). The identity of each cell line was confirmed by DNA fingerprinting via short tandem repeat profiling on Jan 21, 2014. All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS (HyClone Laboratories), 100 U/mL penicillin, and 100 μg/mL streptomycin (PS; Nacalai Tesque, Japan) at 37°C, in a humidified atmosphere of 5% CO2. The cells and mice were irradiated using the MBR-1520R-3 system (Hitachi Medico Technology). We generated ESCC cells stably expressing high levels of STAT3 using the pEB-Multi-constitutive-STAT3 (c-STAT3) vector. TE8 and TE14 cells were seeded at 5 × 104 cells per well in 6-well plates and transfected with pEB-Multiconstitutive-STAT3 (c-STAT3). G418 sulfate was used at a concentration of 200 μg/mL.

Western blotting analysis

ESC cell lines were harvested and lysed as described previously (23). The following antibodies were used: anti-phospho-STAT3 (Tyr705) (#9145, 1:1,000 dilution), anti- Stat3 (c-STAT3) #5435, 1:1,000 dilution), anti-survivin (#2808, 1:1,000 dilution), anti-cleaved caspase-3 (#9664, 1:500 dilution), anti-H2AX (#9718, 1:1,000 dilution), all from Cell Signaling Technology, as well as anti-STAT3 (sc-482, 1:1,000 dilution) and anti-GAPDH (sc-32233, 1:2,000 dilution), both from Santa Cruz Biotechnology, and anti-SOCS1 (#18381, 1:1,000 dilution; IBL).

Adenoviral vectors

A replication-defective recombinant adenoviral vector expressing the mouse SOCS1 gene (AdSOCS1) was provided by Dr. Hiroyuki Mizuguchi (Osaka University) and was constructed using an improved in vitro ligation method, as described previously (25). An adenoviral vector expressing the LacZ gene (AdLaCZ) was constructed using similar methods. After incubating ESCC cells for 24 hours, they were infected with adenoviral vectors at a multiplicity of infection (MOI).

Cell proliferation assays

ESCC cell lines were plated in 96-well plates at a density of 2 × 103 cells per well and incubated for 24 hours. Cell proliferation was evaluated using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assays (Cell Counting Kit-SF; Nacalai Tesque) at the indicated times after treatment. The absorbance 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 of treated cells versus that of control cells. Experiments were performed using six replicate wells for each sample, and the data are presented as averages.
Caspase-3/7 activity assays
ESCC cell lines were seeded in 96-well white plates at a density of 2 × 10^3 cells per well. Irradiation (IR) was performed 24 hours after infection with AdLacZ or AdSOCS1. Caspase-3 and -7 activities in cell culture were detected 24 hours after IR using Caspase Glo 3/7 Assays (Promega), according to the manufacturer’s instructions. Luminometer readings were recorded 1 hour after reagent addition using a Spectra Max Gemini EM Microplate Reader (Molecular Devices).

Small-interfering RNA transfection
The following siRNA ON-TARGET Plus SMART pools were purchased from Thermo Scientific Dharmacon: nontargeting siRNA (D-001810-10-20) and human MCL1 siRNA (L-004501-00). ESCC cells were seeded in antibiotic-free medium. The next day, cells were transfected with nontargeting or MCL1 siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer’s instructions.

ESCC cell xenograft mouse models
All animal experiments were conducted according to the institutional ethical guidelines for animal experimentation of the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). Female ICR nu/nu mice (6–8-weeks of age) were obtained from Charles River Laboratories, Japan. For cell inoculation, 2.5 × 10^6 TE14 cells in 100 μL of 1:1 (v/v) PBS/Matrigel (Becton Dickinson) were injected subcutaneously into the flanks of these mice. Animals were monitored several times per week for tumor growth. To evaluate changes in tumor volume after each therapeutic regimen, we continued to measure tumor volume twice per week until day 32 from the start of therapy. Tumor volumes were determined by measuring the tumor length and width; volume = (width^2 × length)/2

Statistical analysis
Overall survival (OS) was evaluated using the Kaplan–Meier method, and assessed by the log-rank test. For in vitro experiments, data are shown as means ± standard deviations (SD) based on the indicated number of experiments. For xenograft mouse models, data are shown as means ± standard errors of the means (SEM). To test for statistically significant differences between two groups, unpaired Student t tests were performed. Two-sided P values less than 0.05 were considered significant. These analyses were performed using JMP version 12.0 (SAS Institute).

Results
Strong expression of p-STAT3 is confirmed in ESCC patients after CRT
We examined the expression of p-STAT3 in advanced ESCC patients who underwent surgery alone or preoperative CRT by immunohistochemistry. p-STAT3 positivity was noted in the nuclei. Of 46 ESCC patient samples analyzed in this study, 25 (54%) specimens strongly expressed this marker in ESCC tissue, but not in normal tissue (Fig. 1A). Significantly stronger expression of p-STAT3 was observed in samples from ESCC patients who underwent preoperative CRT (N = 16, 64%) compared with expression in those from patients undergoing surgery alone (N = 9, 36%; P = 0.015). There were no significant differences between age, gender, pT, or pStage (UICC 7th edition) and STAT3 expression in those from patients undergoing surgery alone (Table 1). The total 5-year overall survival (5-year OS) rate of ESCC patients undergoing preoperative CRT was 32.7%. For ESCC patients who underwent preoperative CRT, poorer OS was associated with those exhibiting strong p-STAT3 expression compared with those with weak expression (5-year OS rate, 13.4% vs. 66.7%; log rank P = 0.0362) and this difference was significant (Fig. 1B).

STAT3 activation is induced by IR and involved in IR-resistance
We confirmed that p-STAT3 is strongly induced by IR using three ESCC cell lines (TE4, TE8, and TE14). In addition to the activation of STAT3, anti-apoptotic proteins such as Mcl-1 and survivin were also induced by IR (Fig. 2A). To evaluate the association between STAT3 activation and IR-resistance, we generated ESCC cells stably expressing high levels of STAT3 using the pEB-multiconstitutive-STAT3 (c-STAT3) vector, as previously described (Fig. 2B; ref. 26). ESCC cells (TE8 and TE14) with c-STAT3 showed no significant differences in cell proliferation compared with that in parental and mock-transfected cells (Fig. 2C). However, ESCC cells with c-STAT3 showed significantly increased colony forming ability after IR compared with that in parental and mock-transfected cells (Fig. 2D).

Figure 1.
Immunohistochemical staining for p-STAT3 in ESCC patient samples. A, Typical weak and strong p-STAT3 IHC staining. Scale bars, white, 100 μm; black, 25 μm. B, Survival curves based on p-STAT3 expression levels in ESCC patients treated with preoperative CRT (N = 22).
Combining radiotherapy with AdSOCS1 enhances the therapeutic effect of IR

We next examined the therapeutic effect of combining radiotherapy with AdSOCS1, as compared with each monotherapy, in ESCC cell lines. Combining radiotherapy with AdSOCS1 resulted in a remarkable decrease in colony forming ability compared with that in untreated ESCC cells and those with AdLacZ, in TE4, TE8, and TE14 cells (Fig. 3A). In terms of proliferation, although IR monotherapy resulted in a growth inhibitory effect of approximately 70% to 80%, combining radiotherapy with AdSOCS1 significantly enhanced this antitumor effect at a low AdSOCS1 dose (10 MOI in TE4 and 10 and 20 MOI in TE8 cells; Fig. 3B). Figure 3C shows the antitumor mechanisms after combining radiotherapy with AdSOCS1 in TE14 cells, as assessed by Western blotting. The expression of p-STAT3 was markedly inhibited in response to SOCS1 overexpression after infection with AdSOCS1. Although the expression of Mcl-1 and survivin were enhanced in ESCC cell lines treated with IR compared with that in untreated cells, combining radiotherapy with AdSOCS1 suppressed this effect, and cells expressed markedly high levels of cleaved-caspase-3 (Fig. 3C). To evaluate the induction of apoptosis after combining radiotherapy with AdSOCS1, we measured caspase-3/7 activity in TE4, TE8, and TE14 cells using luminescence-based assays. Although there were no significant differences between non-IR- and IR-treated groups for both uninfected (Non-Ad) and combined radiotherapy with AdLacZ groups, caspase-3/7 activity was significantly higher upon combining radiotherapy with AdSOCS1 compared with that with IR or AdLacZ monotherapy (Fig. 3D). In TE14 cells, caspase-3/7 activity increased according to IR-dose when radiotherapy was combined with AdSOCS1 (Fig. 3E).

AdSOCS1 inhibits Mcl-1, enhances IR-induced DNA damage, and induces apoptosis

To evaluate IR-induced DNA damage during combining radiotherapy with AdSOCS1, we evaluated γH2AX as an indicator of double-strand breaks (DSB) after IR. Although there was undetectable expression of γH2AX without IR in TE14 cells, the expression of this factor reached its maximum after 0.5 hours of IR (5 Gy and 10 Gy) treatment and gradually decreased (Fig. 4A). Combining radiotherapy with AdLacZ also resulted in the same tendency. Combining radiotherapy with AdSOCS1 resulted in decreased Mcl-1 expression and enhanced γH2AX expression at 1.5 and 4 hours post-IR, compared with that with other therapies (Fig. 4B). On the basis of the results in Figure 4B, we examined if Mcl-1 in ESCC is associated with DNA damage. We inhibited Mcl-1 expression in TE14 cells using siRNA. Mcl-1 knockdown in TE14 cells resulted in markedly enhanced γH2AX expression 4 hours post-IR compared to that in parental and control-siRNA cells. Mcl-1 knockdown had no effects on p-STAT3 levels (Fig. 4C). This result suggested that Mcl-1 inhibition might be involved in DNA repair.

Next, we evaluated the induction of apoptosis through Mcl-1 inhibition by AdSOCS1 because combining radiotherapy with AdSOCS1 resulted in Mcl-1 inhibition and the induction of cleaved-caspase-3 expression by SOCS1 overexpression (Fig. 3C). Mcl-1 knockdown in TE14 cells resulted in markedly enhanced cleaved-caspase-3 expression 24 hours post-IR compared with that in parental and control-siRNA cells. Increased γH2AX signals from apoptosis at later time points post-IR with AdSOCS1 were also observed in Mcl-1 knockdown cells (Fig. 4D). In addition, although there were no significant changes in caspase-3/7 activity after IR in parental and control-siRNA cells compared with that in non-IR cells, the induction of caspase-3/7 activity in TE14 cells was stronger in Mcl-1 knockdown cells compared to that in parental and control-siRNA cells (Fig. 4E). The proliferative activity of TE14 cells with Mcl-1 siRNA and IR significantly decreased compared with that with Mcl-1 siRNA and no treatment, but was unchanged in parental cells (Fig. 4F). Finally, ESCC cells with stable high c-STAT3 expression showed increased Mcl-1 expression and decreased γH2AX signals after IR compared with that in parental and mock-transfected cells (Fig. 4G).

Combining radiotherapy with AdSOCS1 results in therapeutic effects in ESCC xenograft mouse

We next evaluated the therapeutic effects of combining radiotherapy with AdSOCS1 in ESCC in vivo. For this, we established an ESCC xenograft mouse model with subcutaneously-implanted TE14 cells (2.5 × 10^6 cells) in ICR nu/nu mice (6–8-week-old, females). When tumor volume reached approximately 100 mm3, 1 × 10^6 plaque-forming units of AdSOCS1 or AdLacZ were injected intratumorally twice per week, six times. Before injection of the adenovirus vector, mice were irradiated with 2 Gy (6 fractions; total = 12 Gy) using an X-ray irradiator (Fig. 5A). The injection of AdSOCS1 significantly suppressed tumor growth compared with that in mice injected with AdLacZ. In addition, radiotherapy significantly suppressed tumor growth compared with that in untreated mice, resulting in a therapeutic effect similar to that with AdSOCS1 injection. On day 32, the tumors of irradiated mice exhibited marked repopulation compared with that in mice injected with AdSOCS1. In contrast, mice with this combination radiotherapy showed little increase and no repopulation in tumor volume after treatment (Fig. 5B). IHC confirmed the overexpression of SOCS1 in mice treated with AdSOCS1, but not in mice administered control therapy. The tumors of irradiated mice also exhibited increased expression of p-STAT3 compared with that in tumors of untreated mice. Tumors of AdSOCS1-injected mice had little expression of p-STAT3 (Fig. 5C).

Combining radiotherapy with AdSOCS1 enhances DNA damage and induces apoptosis in ESCC xenograft mouse

We then examined the therapeutic effects of combining radiotherapy with AdSOCS1 in vivo by examining pathological changes in tumor tissues. The expression of γH2AX significantly increased in tumor tissue from irradiated mice compared with that in

Table 1. Correlation between p-STAT3 and various clinicopathological parameters in advanced ESCC patients with preoperative CRT

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<th>Gender, Gender (%)</th>
<th>Age, years, median (range)</th>
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<th>pN, pN (%)</th>
<th>pStage, pStage (%)</th>
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<td>13 (81)</td>
<td>3 (17)</td>
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<tr>
<td>Female 2 (33)</td>
<td>70 (48–72)</td>
<td>12 (75)</td>
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<td>pStage, pStage (%)</td>
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<td>I/II 3 (50)</td>
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<td>II/IV 3 (50)</td>
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*a*UICC 7th edition.
tumors from untreated mice, and was highest in tissues from mice treated with combining radiotherapy with AdSOCS1. In addition, the expression of γH2AX in the tumors of irradiated mice decreased on day 32, whereas that in tumors from animals treated with combining radiotherapy with AdSOCS1 was sustained (Fig. 6A and B). We next examined the expression of Ki-67 to evaluate the proliferative index of xenograft mouse tumors. The expression of Ki-67 was significantly suppressed in tumors from irradiated mice and animals treated with AdSOCS1 compared with that in untreated mice and AdLacZ-injected mice, and the lowest expression was observed in mice treated with combining radiotherapy with AdSOCS1. In addition, the expression of Ki-67 in irradiated mouse tumors increased on day 32, whereas that in tumors from animals treated with combining radiotherapy with AdSOCS1 remained low (Fig. 6C and D). TUNEL staining showed that combining radiotherapy with AdSOCS1 induced the highest levels of apoptosis of all treatments in vivo (Fig. 6E).

**Discussion**

CRT has been established as an effective treatment modality for many human cancers such as breast, esophageal, and lung. However, treatment resistance represents a substantial and complex problem, as more and more patients are treated with CRT alone or in combination with other modalities. Increasing evidence has demonstrated that STAT3, which is an indicator of poor prognosis in various cancers (27, 28), plays a critical role in mediating resistance to CRT because of its diverse functions in controlling various (patho-) physiological cellular processes. STAT3 suppresses the transcription of proapoptotic genes (29)
Figure 3.
Antitumor effect of combining radiotherapy with AdSOCS1 in ESCC cell lines. A, Colony forming activity; IR: 0, 2, 4, 6, 8 Gy; AdLacZ and AdSOCS1: 5 MOI. B, Cell proliferative activity 48 hours after IR and 24 hours after AdSOCS1 or AdLacZ infection; IR: 10 Gy; AdLacZ and AdSOCS1: 10 MOI for TE4 and TE14 cells; 20 MOI for TE8 cells. C, Expression levels of SOCS1, p-STAT3, Mcl-1, survivin, and cleaved-caspase-3 24 hours after IR (0, 2, 5, and 10 Gy) of TE14 cells and 24 hours after AdSOCS1 or AdLacZ infection (TE4, TE14: 10 MOI; TE14: 20 MOI). D, Caspase-3/7 activity 24 hours after IR (5 Gy) of TE14 cells and 24 hours after AdSOCS1 or AdLacZ infection. E, Caspase-3/7 activity compared with that in Non-Ad-treated TE14 cells. Statistical analyses were performed using Student t tests (**, *P < 0.01).
and induces the expression of proliferative genes (30), in addition to stemness-promoting or stemness-preserving genes (31). Furthermore, stress response pathway components regulate STAT3 activity and are regulated by it, and several DNA repair genes are also targets of STAT3 (32). Spitzner and colleagues (10) reviewed that STAT3 is a promising therapeutic target that can augment the treatment of CRT-resistant tumors. In our study, the analysis of resected specimens from ESCC patients showed that the

Figure 4.
Evaluation of DNA damage after combining radiotherapy with AdSOCS1. A, The expression of γH2AX after IR (5 Gy and 10 Gy) in time-dependent manner (0, 0.5, 1.5, 4, 12, and 24 hours). B, Time-dependent expression of SOCS1, p-STAT3, Mcl-1, and γH2AX in TE14 cells and 36 hours after AdSOCS1 or AdLacZ infection and after IR (5 Gy). C, Expression of Mcl-1, γH2AX, and p-STAT3 4 hours after IR (5 Gy) of Mcl-1-knockdown TE14 cells. D, Expression of Mcl-1, cleaved-caspase-3, γH2AX, and p-STAT3 24 hours after IR (5 Gy) of Mcl-1-knockdown TE14 cells. E, Caspase-3/7 activity 24 hours after IR (5 Gy) of Mcl-1-knockdown TE14 cells. Mcl-1 expression was suppressed by siRNA. F, Expression of Mcl-1 and γH2AX 24 hours after IR (5 Gy) of parental, mock-transfected, and c-STAT3-expressing cells. Statistical analyses were performed using Student t tests (*, P < 0.05).
expression of p-STAT3 is significantly higher after CRT compared with expression in those without CRT. Moreover, among ESCC patients with preoperative CRT, prognosis was significantly poorer in those with strong expression of p-STAT3 compared with those with weak expression. We also demonstrated that STAT3 activation might be induced by IR \textit{in vitro} and \textit{in vivo} and that ESCC cells with constitutive STAT3 expression are associated with poor therapeutic outcome after IR. In contrast, there were no significant changes in p-STAT3 and Mcl-1 induction after exposure to chemotherapy (CDDP and 5-FU) \textit{in vitro} (Supplementary Fig. S1A–S1C).

From this, we considered that STAT3 activation is involved in therapeutic resistance to radiotherapy in ESCC, and thus the development of new modalities that combine radiotherapy with drugs that target STAT3 activation could improve the therapeutic efficacy of CRT against advanced ESCC.

Mcl-1 is an antiapoptotic member of the Bcl-2 family that was originally isolated from the ML-1 human myeloid leukemia cell line during phorbol ester-induced differentiation to the monocyte/macrophage lineage (33). Similar to other Bcl-2 family members, Mcl-1 localizes to mitochondria, interacts with and antagonizes proapoptotic Bcl-2 family members (34, 35), and inhibits apoptosis induced by a number of cytotoxic stimuli (36). A recent report showed that Mcl-1 inhibition, either alone or in combination with other anti-cancer drugs, is effective against several solid cancer-derived cell lines (37). In ESCC cell lines, the expression of antiapoptotic proteins such as Mcl-1 and survivin was strongly induced after IR, similar to the induction of STAT3 activation. We also demonstrated that Mcl-1 was strongly induced after IR in ESCC cells stably high expression levels of STAT3, which also conferred IR-resistance. Therefore, enhanced activation of the STAT3–Mcl-1 axis might be involved in IR-resistance and repopulation after IR. Apoptosis was not readily induced by IR monotherapy, and this might have been caused by enhanced activity of the STAT3–Mcl-1 axis after IR, which would have been mitigated by SOCS1 gene therapy.

We previously reported that the overexpression of SOCS1, which is a negative regulator of inflammatory cytokine signaling, including JAK/STAT signaling has potent antitumor effects in various cancers including ESCC (19–23). Tagami-Nagata and colleagues (22) demonstrated that the repression of antiapoptotic proteins such as Mcl-1 by AdSOCS1 was one mechanism of growth suppression. In this study, we showed that the expression of antiapoptotic proteins such as Mcl-1 and survivin was markedly

![Figure 5](https://example.com/figure5.png)
suppressed by SOCS1 expression in ESCC cell lines similar to that observed in a previous report (22). In addition, we also demonstrated that Mcl-1 knockdown strongly induces apoptosis and significantly suppresses cell proliferation after IR. From these results, we revealed that SOCS1 gene therapy for ESCC strongly induces apoptosis by suppressing the STAT3–Mcl-1 axis. Therefore, we hypothesize that this approach could be clinically useful in combination with radiotherapy. Indeed, we demonstrated that combining radiotherapy with AdSOCS1 improved IR-resistance by suppressing the STAT3–Mcl-1 axis, which was induced after IR, and had additional antitumor effects pertaining to the induction of apoptosis compared to that with radiation monotherapy. In a mouse model, combining radiotherapy with AdSOCS1 showed marked antitumor effects compared with that with each monotherapy. After each monotherapy, tumor volumes were repopulated, especially in the IR group. However, the therapeutic effect was maintained with combining radiotherapy with AdSOCS1, and repopulation was not observed. Chemotherapy is currently used for combination therapy to improve the efficacy of radiotherapy. However, regrowth and local recurrence after CRT is a common clinical problem for ESCC (6, 7). Therefore, the results of this our study are considered noteworthy.

In accordance with other mechanisms through which SOCS1 gene therapy improved the therapeutic effect of IR, we focused on the association between STAT3 activation and DNA damage after IR, in addition to the aforementioned effect on apoptosis. Several reports have shown that STAT3 modulates the DNA damage response pathway (32, 38, 39). Accordingly, inhibition of activated STAT3 decreases DNA repair activity after IR (40, 41). In this study, we demonstrated that the expression of γH2AX, which is an indicator of double-strand DNA breaks after IR, is enhanced in vitro and in vivo after combining radiotherapy with SOCS1 gene therapy, as compared with that with each monotherapy. In the mouse model, although tumors exposed to AdSOCS1

Figure 6. A, Immunohistochemical analysis of γH2AX in tumors from xenograft mice after each therapy on day 22 and day 32. B, γH2AX staining was recorded as the ratio of positively stained cells to the total number of tumor cells based on 5 fields (magnification, ×400). C, Immunohistochemical analysis of Ki-67 in xenograft mouse samples after each therapy on day 22 and day 32. D, Ki-67 staining was recorded as the ratio of positively stained cells to the total number of tumor cells based on five fields (magnification, ×400). Statistical analyses were performed using Student t tests (*, P < 0.05 and **, 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 xenograft mice after each therapy.
expression of Ki-67 (19–23). Therefore, the combining radiotherapy with AdSOCS1 modality might reduce tumor malignancy. We next examined the association between the STAT3-Mcl-1 axis and DNA damage in vitro. Mattoo and colleagues (42) revealed that MCL-1 depletion increases 53BP1 and RIF1 colocalization at DSBs, which in turn inhibits BRCA1 recruitment and sensitizes cells to IR-induced DSBs or stalled replication forks that require homologous recombination for DNA repair. In our study, enhanced expression of γH2AX was observed in ESCC cell lines with low Mcl-1 expression as a result of SOCS1 gene therapy or Mcl-1–siRNA. Thus, we revealed for the first time that suppression of the STAT3–Mcl-1 axis by SOCS1 gene therapy strongly enhances DNA damage. Calabrese and colleagues (43) showed the regulation of p53 signaling by SOCS1 in response to DNA damage. However, the p53 gene was mutated in most esophageal cancer specimens and the ESCC cell lines used in this study also harbored mutant p53 (44). Therefore, the p53-related response by IR and/or SOCS1 overexpression might not have been observed in our study (Supplementary Fig. S2A–S2E).

There are some clinical problems associated with current radiotherapy with respect to the treatment of ESCC. Although various radiotherapy methods have been devised to minimize the impact on nontumor tissues, the side effects of radiotherapy on normal organs and other adjacent tissue remain a clinical problem. The location of the esophagus, specifically, in a very narrow mediastinum, results in clinical complications after radiation exposure in adjacent organs such as the trachea, bronchi, lung, and aorta (45, 46). AdSOCS1, which we have used as a therapeutic agent, can be easily administered, in a tumor-specific manner, through local endoscopic injection, to treat ESCC. In addition, this gene therapy is expected to improve the SOCS1 expression area (i.e., cancer tissue), relative to the SOCS1 non-expressing area (i.e., normal tissue), based on our results, which showed that SOCS1 gene therapy has the potential to improve radiosensitivity. Therefore, combining radiotherapy with SOCS1 gene therapy has the potential to increase tumor specificity, which could decrease the required radiation dose and improve the local therapeutic effects in ESCC. Second, tumor relapse and repopulation after radiotherapy is also a problem (47–49). Options for local therapy in response to tumor relapse and repopulation after radiotherapy only include surgical resection. Considering the association between STAT3 activation and resistance to radiotherapy, which was clarified in this study, SOCS1 gene therapy might represent one effective option for salvage therapy in the future. Spitzner and colleagues reviewed that STAT3 is involved in CRT resistance in brain cancer, breast cancer, colon cancer, prostate cancer, bladder cancer, head and neck cancer, lung cancer and skin cancer, which are currently treated with CRT (11). Considering our results, we believe that SOCS1 gene therapy might lead to improved radiation resistance and mitigate some of the clinical problems associated with radiotherapy in various cancers.

However, there are some problems associated with applying SOCS1 gene therapy in a clinical setting. First, immune cell suppression might occur through the inhibition of the JAK/STAT signaling by SOCS1 overexpression. In addition, because the recombinant adenovirus vector is replication-defective and has been modified to prevent intracellular growth (except in 293 cells), the therapeutic efficacy is limited to the injection area of the tumor in this system. Therefore, a better drug delivery system to enhance tumor cell selectively might be needed to improve the therapeutic efficacy of this agent. However, local disease control in advanced ESCC is also important to improve prognosis, as is treatment of lymph node and distant metastases (48–50). Therefore, combining radiotherapy with SOCS1 gene therapy is expected to result in therapeutic efficacy at lower doses and reduce the side effects associated with each monotherapy. Regarding SOCS gene therapy, we are scheduled to begin human clinical trials, and the treatment efficacy and safety results are awaited. In conclusion, we demonstrated that adenovirus-based SOCS1 gene therapy enhances the therapeutic efficacy of radiotherapy for ESCC in vitro and in vivo. We revealed for the first time that the STAT3–Mcl-1 axis is associated with IR-resistance in ESCC and that inhibition of this axis through SOCS1 expression improved radiosensitivity by inducing apoptosis and enhancing DNA damage after IR. These results suggest that combining radiotherapy with SOCS1 gene therapy targeting STAT3 activation will become a promising therapeutic option for ESCC in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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