

Absence of STAT1 disturbs the anticancer effect induced by STAT3 inhibition in head and neck carcinoma cell lines

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Abstract. The family of signal transducers and activators of transcription (STAT) are transcription factors. Among them, STAT1 is associated with an apoptosis pathway, while STAT3 is associated with tumorigenicity in various cancer cells. In order to investigate the primary roles of STAT1 and STAT3 in head and neck squamous cell carcinoma (HNSCC), we blocked STAT3 with two JAK inhibitors: AG490 (JAK2-STAT3 pathway inhibitor) and JAK total inhibitor. When we inhibited STAT3 with AG490, significant cell death was observed. However, in the case of JAK kinase total inhibitor, no cell growth retardation was observed. We focused on the role of STAT1 in this phenomenon. Suppression of STAT1 by si-RNA resulted in increased cell survival. Furthermore, the growth inhibitory effect of AG490 was reduced by treatment with si-RNA of STAT1. These results reveal that STAT1 is required to promote the tumor killing effect of STAT3 inhibition in HNSCC.

Introduction

The family of signal transducers and activators of transcription (STAT) plays a central role in signaling by various cytokines and polypeptide growth factors. The binding of activator molecules causes receptor clustering and phosphorylation of critical residues on the receptor cytoplasmic domain by JAK kinase. Constitutive activation of STATs has been observed in a variety of tumor types. In particular,

STAT1, 3 and 5 were found to be overexpressed in head and neck cancer cell lines (1-4).

In head and neck squamous cell carcinoma (HNSCC), inhibition of the EGFR pathway or TGF- α reduced STAT3 DNA-binding activity, and inhibition of STAT3 led to growth inhibition of HNSCC (5-7). These findings indicate that STAT3 signaling is important in HNSCC oncogenesis.

On the other hand, current evidence suggests that STAT1 is predominantly growth inhibitory, which is consistent with the antiproliferative response to IFN- γ , which activates STAT1 (8). It has been demonstrated that c-myc expression is down-regulated by IFN- γ , which activates STAT1-deficient human U3A fibrosarcoma cells which are less susceptible to TNF- α -induced cell death than parental cells which contain STAT1 (9). Another report indicated that STAT1 negatively regulated angiogenesis, tumorigenesis and metastasis of tumor cells (10).

STAT1 and 3 may have different effects on cell growth and survival. For example, activated STAT3 antagonizes the proapoptotic effects of activated STAT1 in fibroblasts (11). Many studies on STAT proteins have suggested that STAT3 is an oncogene, in contrast to the tumor suppressor function of activated STAT1 (12-15). As already mentioned, Shen *et al* showed that STAT3 antagonizes the proapoptotic effects of activated STAT1 (11). Recently, however, one group reported that the therapeutic mechanisms of STAT3 inhibition using a decoy are independent of STAT1 activation (16).

Based on these observations, we investigated the actions of STAT1 and 3 simultaneously. Several cell lines originating from HNSCC expressed activated STAT1 and 3. STAT3 inhibitors demonstrated different killing effects depending on the presence or absence of STAT1. Therefore, we assumed the possibility that STAT1 might play a key role in the tumor killing effect of STAT3 inhibition. Moreover, we observed that STAT1 action was important for cell death induction by anti-cancer drugs. These findings were confirmed using si-RNA directed toward STAT1.

Materials and methods

Cell culture. HNSCC cell lines, SNU-1041, SNU-1076 and SNU-1066, were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). HOK 16B are

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Abbreviations: STAT, signal transducers and activators of transcription

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immortalized cells from pharyngeal mucosa (a gift from Dr Jeffrey N. Myers, M.D. Anderson Cancer Center, University of Texas). All cells were cultured in RPMI-1640 media supplemented with 10 mg/ml gentamicin and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified, 5% CO₂, 95% air atmosphere and routinely subcultured using trypsin-EDTA (0.25% w/v). All cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA).

Chemicals. AG490 (JAK2-STAT3 inhibitor), 2-cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide, and JAK total inhibitor, 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz(h)-imidaz(4,5-f)isoquinolin-7-one, were obtained from Calbiochem (La Jolla, CA). All chemicals were used according to the manufacturer's instructions.

Cell proliferation assay. Exponentially growing cells were trypsinized, seeded at a specific density (10⁴ cells/well in a flat-form 96-well plate) and grown for 24 h. After 24 h, fresh medium with various concentrations of chemicals was added. Following a 3-day incubation, an MTT assay (Promega, Madison, WI) was performed. Twenty microliters of MTT solution (2 mg/ml in deionized water) was added to each well and incubated for 4 h. After removal of the supernatant, 120 µl of DMSO was added, and the pellet was resuspended for 30 min with vigorous shaking. The optical density was determined at a wavelength of 564 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA).

Small-interfering RNA transfection. After the cells had grown for 24 h, 4 µl of Lipofectamin 2000 (Life Technologies, Gaithersburg, MD) and 200 nM/l small-interfering RNA solution were incubated at room temperature for 15 min with RPMI-1640 media. The cells were then treated with the transfection complexes for 24 h. After 24 h, the media were removed, and fresh media containing AG490 or JAK total inhibitor were added. The cells were incubated for 48 or 72 h.

Protein extraction and Western blot assay. Cultured cells were rinsed with PBS, suspended in lysis buffer (0.5% NP40, 50 mM Tris-Cl, 150 mM NaCl, 1 mM dithiothreitol, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 µM aprotinin and 1 µM pepstatin A) and incubated at 4°C for 30 min. The cell lysates were then centrifuged at 13,000 r.p.m. for 20 min at 4°C. An appropriate amount of each supernatant (determined by protein assay) was mixed with 4X sample loading buffer and denatured for 10 min at 70°C. The denatured protein samples were fractionated on 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA), transferred onto nitrocellulose membranes (Schleicher & Schuell, Dachen, Germany) and incubated with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat dry milk. The membranes were then incubated with anti-p-STAT3(Tyr), anti-p-STAT1(Tyr), anti-p-STAT1(Ser), anti-STAT1 total or anti-STAT3 total (all from Cell Signaling Technology, Danvers, MA) for 2 h at room temperature or overnight at 4°C. The membranes were then washed (from 4 to 15 min) with 1X TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford,

IL) for 1 h. Immunoreactive proteins were visualized by development with the Lumi-light Western Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany) and exposed to LAS-3000 film (Fuji Photo Film Co., Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis. Data shown in the graphs represent the mean value ± standard deviation (SD) of 3 independent experiments. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL).

Results

Differential effects on cell death induced by two JAK pathway inhibitors. We observed up-regulation of STAT3 by Western blot analysis in HNSCC cell lines (SNU-1041, SNU-1066 and SNU-1076) but not in HOK 16B, normal cells from pharyngeal mucosa (Fig. 1A). To evaluate the role of STAT3, we used two chemical inhibitors. First, AG490, an inhibitor of the JAK2-STAT3 pathway, was added to SNU-1041, SNU-1066 and SNU-1076 cells at different doses (1, 3, 10, 30 and 60 µM) for 72 h (Fig. 1B). Inhibition of STAT3 in all the tested cell lines demonstrated effective cell growth inhibition (Fig. 1B). Next, we attempted to identify the growth-inhibitory effect of another JAK inhibitor (total JAK inhibitor) in HNSCC cell lines since previous reports suggest that there were several pathways of JAK subtypes associated with the activation of STAT3 (1,2). Notably, blocking all the possible pathways of STAT3 activation via various JAK subtypes did not lead to effective inhibition of cell growth. Following treatment with various concentrations of JAK total inhibitor for 72 h, none of the tested cell lines showed growth inhibition as determined by the MTT assay (Fig. 1C).

Verification of differential expression of STAT1 and STAT3. We compared the effects of two JAK inhibitors (AG490 and JAK total kinase inhibitor) on the activities of STAT1 and 3 using a Western blot assay. When the cells were treated with AG490, p-STAT3 decreased in a dose-dependent manner, but the phosphorylated form of STAT1 was minimally affected (Fig. 1D and E). Owing to the low basal level of p-tyr-STAT1, we used IFN-γ to induce its expression (Fig. 1D). On the other hand, the JAK total inhibitor completely blocked both p-STAT1 and p-STAT3, and no significant cell growth inhibition was consistently observed (Fig. 1C-E). These findings suggest that STAT1 might play a modulatory role in cell death caused by JAK inhibition.

Cell viability was enhanced in the absence of STAT1. In order to confirm that the role of STAT1 is critical in cell death induced by JAK inhibition, we performed a small-interfering RNA (si-RNA)-based experiment. Fig. 2A and B illustrates the effective suppression of STAT1 expression at a dose of 200 nM/l si-RNA. As already shown, basal expression levels of p-tyr-STAT1 were undetectable. To confirm the action of si-STAT1 on p-tyr-STAT1, IFN-γ was used to induce p-tyr-STAT1 expression. Treatment with si-STAT1 completely blocked IFN-γ-induced expression of p-tyr-STAT1. We tested the effect of AG490 on cell growth under conditions in which STAT1 was suppressed by si-STAT1. As shown in Fig. 2C,

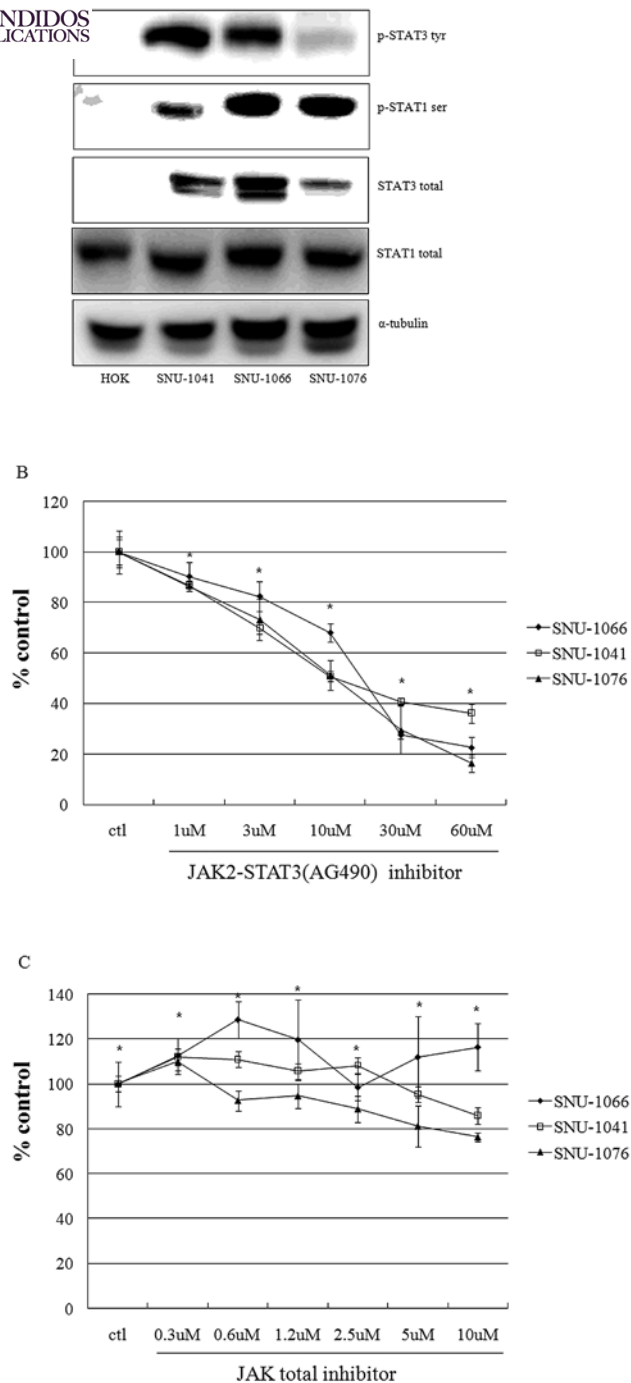
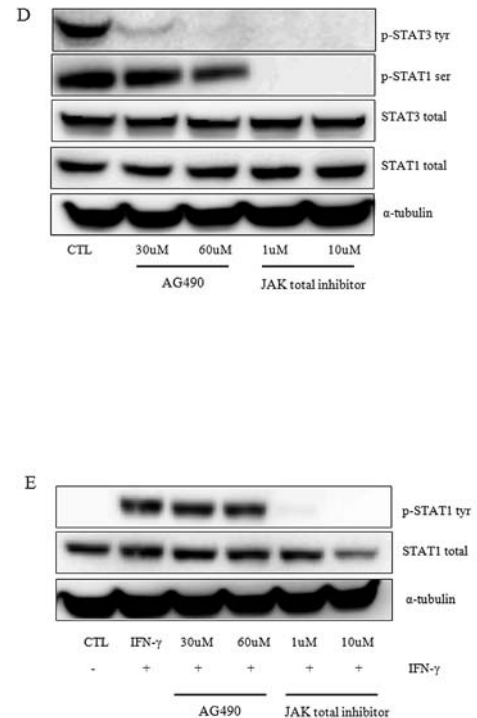
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Figure 1. Expression of STAT3 and the effect of cell growth by two types of JAK inhibitors in head and neck squamous carcinoma. (A) Expression of STAT3 in heads and neck squamous carcinoma cells (SNU-1041, SNU-1066 and SNU-1076) and HOK 16B (immortalized cells from pharyngeal mucosa). Cells were harvested, and total proteins were separated and transferred for Western blotting with an antibody against phospho-STAT3(tyr), p-STAT1(ser), STAT3 total and STAT1 total. STAT3 was detected in various head and neck cancer cell lines but not in HOK 16B. (B) Effect on cell growth by JAK2-STAT3 (AG490) inhibition. Head and neck cancer cell lines were treated with AG490 concentrations ranging from 1 to 60 μ M for 72 h. (C) Effect of cell growth by JAK total inhibition. Cells were treated with JAK total inhibitor for 72 h. Cell viability was measured by the MTT assay. The graph represents the mean $\pm$ SD of triplicate samples from 3 independent experiments. * $p=0.00$ (Kruskal-Wallis method). (D) SNU-1041 cells were treated with AG490 (JAK2-STAT3 inhibitor) or JAK total inhibitor for 8 h. AG490 completely blocked STAT3 expression but had only a minimal effect on STAT1. However, JAK total inhibitor blocked both STAT1 and 3. (E) Conditions were identical to (D) except that cells were treated with IFN- γ for 15 min prior to protein extraction. The blots are representative of 3 independent experiments showing similar trends.



treatment with si-STAT1 partially decreased the growth-inhibitory effect of AG490. The basal level of cell viability was much higher in the si-STAT1 group than in the si-control counterpart. For each dose of AG490, the viability of cells transfected with si-STAT1 was ~2-fold higher than that of si-control-treated cells. This suggests that, although AG490 effectively and dose-dependently induced cell growth arrest, the absence of STAT1 hindered cell growth inhibition by AG490.

Blocking STAT1 interferes with the effects of anti-cancer drugs. In order to confirm that STAT1 is important in various cell death pathways, we compared cell killing induced by the anti-cancer agents, paclitaxel, doxorubicin, 5FU, cisplatin and etoposide. When cells were treated with anti-cancer drugs for 6 h, we observed enhanced expression of p-ser-STAT1 in all cases (Fig. 3A). However p-tyr-STAT1 was not detected (Fig. 3A). This suggests that the cell death pathway of anti-cancer drugs is associated with the presence of STAT1.

Next, we investigated the effect of cell growth inhibition by anti-cancer drugs when STAT1 was absent (Fig. 3B). Small-interfering RNA targeting STAT1 was transfected for 24 h, and cell growth inhibition by the various anti-cancer drugs was assayed using the MTT assay. Fig. 3B indicates that when STAT1 expression was suppressed by si-STAT1, cell viability increased. In all cases, a 20-30% increase in cell growth was observed. These data indicate that cell death or growth-arrest signals induced by anti-cancer drugs are attenuated in the absence of STAT1.

We simulated a set of conditions that provoked more efficient tumor killing by blocking STAT3 with AG490 and/or activating STAT1 with anti-cancer drugs (Fig. 4). We observed a synergistic effect in the SNU-1041 head and neck cancer cell line by combining AG490 with cisplatin (Fig. 4A, middle) and an additive effect following treatment with both

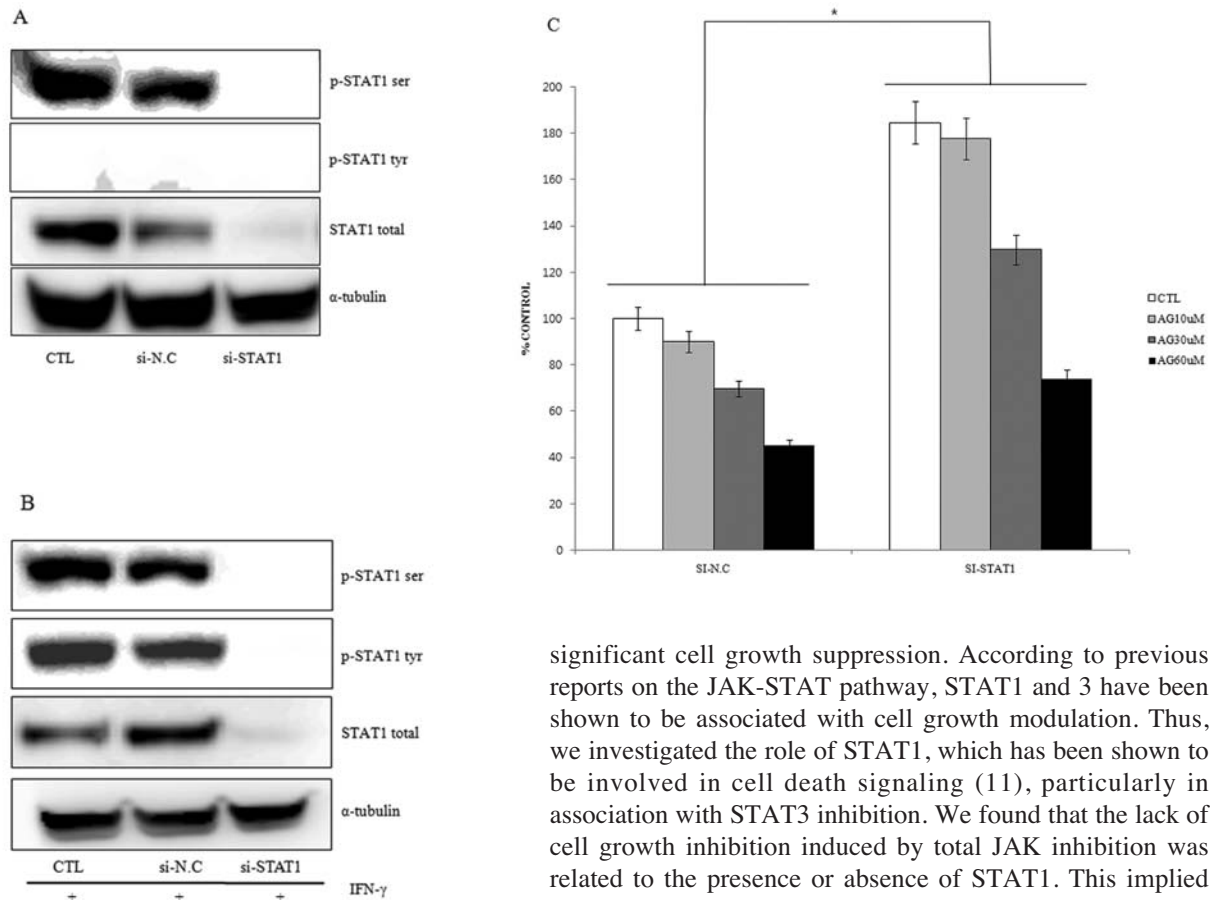


Figure 2. Cell growth inhibition by AG490 in the presence or absence of STAT1. (A and B) Small-interfering RNA suppressed STAT1 completely. Owing to the lack of basal levels of phosphotyrosine-STAT1, we used IFN- γ to induce its expression. Small-interfering RNAs were transfected for 24 h. SNU-1041 cell extracts of total protein were used for Western blotting using p-STAT1 antibody recognizing either the tyrosine or serine form. (+) IFN- γ , an inducer of STAT1, was added, and cells were incubated for 15 min before harvesting. Blots are representative of 3 independent experiments showing similar trends. (C) Comparing each dose of AG490, much higher cell viability was observed in si-STAT1-treated cells. SNU-1041 cells were treated with AG490 for 48 h after being transfected with si-control (si-N.C) or with si-STAT1 for 24 h. Cell viability was measured using the MTT assay. The graph represents the mean $\pm$ SD of triplicate samples from 3 independent experiments. * $p=0.00$ (Kruskal-Wallis method).

5FU and doxorubicin (Fig. 4A, left and right arms). We found similar effects in two other HNSCC cell lines, SNU-1076 and SNU-1066 (Fig. 4B and C). Whereas a tumor killing effect was demonstrated in both the si-control and si-STAT1-treated cells following application of the drugs, an enhancement of cell viability similar to the results shown in Fig. 2C was noted in the si-STAT1 groups relative to the si-control groups. From these observations, cell death induced by STAT3 inhibition and/or anti-cancer agent treatment was impaired when STAT1 was absent.

Discussion

We compared the effects of two types of chemical inhibitors blocking the JAK-STAT pathway on inhibition of cell growth. Total JAK inhibitor had no effect on cell growth inhibition, whereas AG490, a JAK-STAT3 pathway inhibitor, showed

significant cell growth suppression. According to previous reports on the JAK-STAT pathway, STAT1 and 3 have been shown to be associated with cell growth modulation. Thus, we investigated the role of STAT1, which has been shown to be involved in cell death signaling (11), particularly in association with STAT3 inhibition. We found that the lack of cell growth inhibition induced by total JAK inhibition was related to the presence or absence of STAT1. This implied that STAT1 may have a modulatory role in cell death signaling when tumor cell growth is blocked by STAT3 inhibition.

In contrast, Lui *et al* reported that the therapeutic mechanisms of STAT3 inhibition using a decoy in HNSCC are independent of STAT1 activation (16). However, they employed IFN- γ to induce STAT1, which may have triggered many other downstream signals. Thus, we excluded the effect of IFN- γ signaling on STAT1 and other downstream pathways by using resting cells. Instead, we observed p-ser-STAT1, which was constitutively detected in a resting state without induction. Our results support the observation of Shen *et al*, who reported that STAT3 antagonizes the proapoptotic effect of activated STAT1 (11).

However, it is not yet certain which phosphorylation site of STAT1 is essential for killing cancer cells. The activation of STAT proteins involves two phosphorylation sites: tyrosine and serine. Serine phosphorylation in the C-terminal transcriptional activation domain of some STATs has been shown to enhance their transcriptional activity (17-19). Members of the MAPK family (20), extracellular signal-regulated kinase (ERK) (21,22), JNK (23) and p38 (24,25) have been reported as strong candidate kinases which participate in the serine phosphorylation of STAT1 and 3.

In our study, a significant difference in the basal level expression of p-tyr-STAT1 and p-ser-STAT1 was observed. Basal expression of p-ser-STAT1 was detected, but p-tyr-STAT1 was not observed in the resting state (Fig. 1D and E). Thus, the differential expression of STAT1 leads to the question of which type of STAT1 (phosphorylated or unphosphorylated, tyrosine or serine phosphorylated) is important to the survival of cancer cells. Previous observations postulated that tyrosine phosphorylation of STAT1 induced

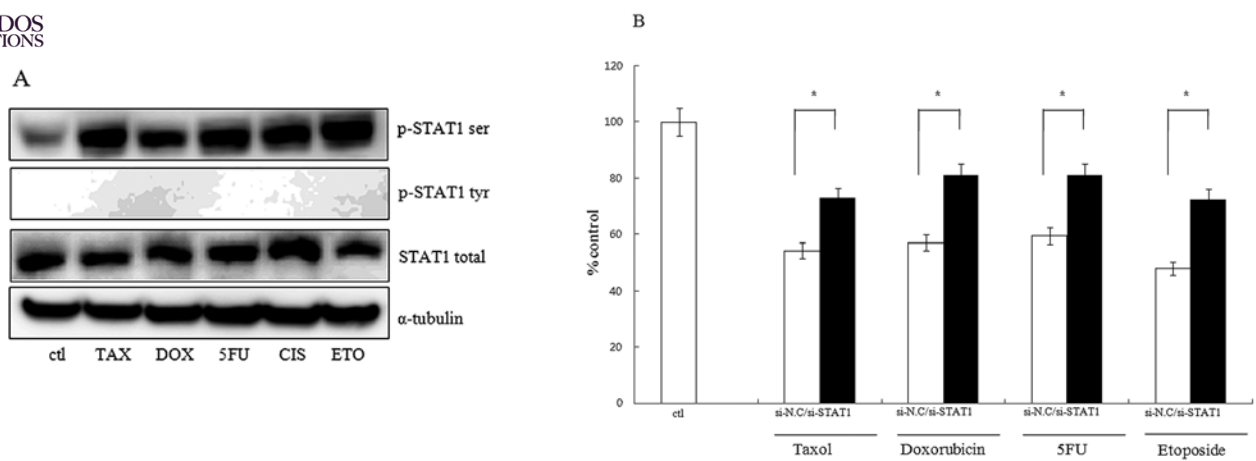


Figure 3. Activation of p-STAT1(ser) by anti-cancer drugs and the effect of anti-cancer drugs on cell growth inhibition in the presence or absence of STAT1. (A) When cells were treated with anti-cancer agents, increased expression of STAT1 was observed. In the case of tyrosine-phosphorylation, no p-STAT1 (tyr) was detected. SNU-1041 was treated with anti-cancer drugs for 8 h. Cell extracts were isolated and analyzed by Western blotting using a specific antibody against p-STAT1 (ser). [ctl, no treatment; TAX, paclitaxel (12 μ g/ml); DOX, doxorubicin (4 μ g/ml); 5FU (10 μ g/ml); CIS, cisplatin (0.5 μ g/ml); ETO, etoposide (20 μ g/ml)]. Blots are representative of 3 independent experiments showing similar trends. (B) Comparing si-control and si-STAT1-treated cells, higher cell viability was observed in si-STAT1-treated cells. After small-interfering RNA transfection for 24 h, SNU-1041 was treated with anti-cancer drugs for 48 h [taxol, paclitaxel (6 μ g/ml); doxorubicin (2 μ g/ml); 5FU (5 μ g/ml) and etoposide (10 μ g/ml)]. The graph represents the mean $\pm$ SD of triplicate samples from 3 independent experiments. *p=0.00 (Kruskal-Wallis method).

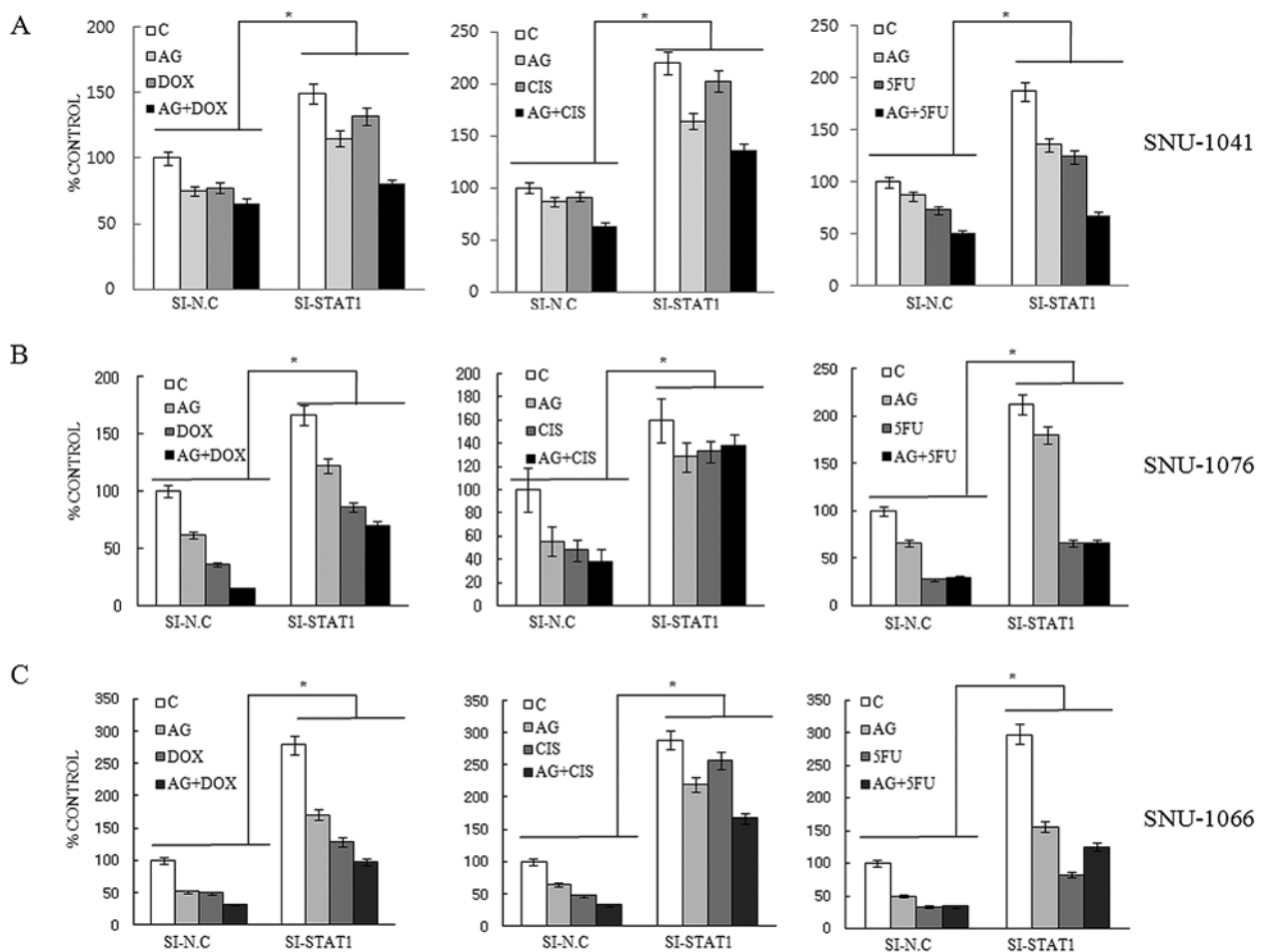


Figure 4. The effect of a combination of AG490 and an anti-cancer drug on cell growth in the presence or absence of STAT1. Tumor killing effects were observed in both the si-control and si-STAT1-treated cells following application of the drugs, however, a greater enhancement of cell viability was shown in the si-STAT1-treated cells. We tested 3 different head and neck cancer cell lines: (A) SNU-1041, (B) SNU-1076 and (C) SNU-1066. Cells were transfected with small-interfering RNA for 24 h followed by treatment with 10 μ M of AG490 (AG) plus 2 μ g/ml of doxorubicin (DOX, left graph in each row), 0.25 μ g/ml of cisplatin (CIS, middle graphs) or 5 μ g/ml of 5FU (graphs at the right) for 48 h. The graph represents the mean $\pm$ SD of triplicate samples from 3 independent experiments. *p=0.00 (Kruskal-Wallis method).

by IFN- γ constitutes an early event in the activation of transcription factors, which are required for their dimerization and DNA-binding activities (26). In addition, phosphorylation of a serine residue in the C-terminal transcriptional activation domain, which corresponds to ser-727 in both STAT1 and 3, enhances the transcriptional activity (19). Tyrosine phosphorylation is required for STAT1 dimerization, nuclear translocation and DNA binding in response to IFN- γ ; however, full transcriptional activity of the homodimer is regulated only when the serine in the transcriptional activation domain is also phosphorylated (26). Janjua *et al* suggest the importance of p-ser-STAT1 in mediating an apoptotic pathway (27).

We observed that the basal level of cell growth was significantly higher in cells treated with si-STAT1 than with a si-control. When STAT3 was blocked in the absence of STAT1, the efficiency of cell death decreased compared to that in the presence of STAT1. Furthermore, when we inhibited STAT3 with AG490 following STAT1 activation using IFN- γ , which induces phosphotyrosine-STAT1, the patterns of cell growth inhibition (data not shown) were similar to those shown in Fig. 2C. This suggests that the presence of STAT1 itself, regardless of the type of phosphorylation, might have a role in regulating cell death. These data are consistent with the research of Ramana *et al* (8).

In conclusion, this study confirmed that the tumor killing effect induced by STAT3 inhibition is associated with STAT1 action. To improve the tumor killing effect of the JAK pathway inhibition in HNSCC, we should consider the simultaneous activation of STAT1 and inactivation of STAT3.

Acknowledgements

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