Epigenetic modification of SOCS-1 differentially regulates STAT3 activation in response to interleukin-6 receptor and epidermal growth factor receptor signaling through JAK and/or MEK in head and neck squamous cell carcinomas

Tin Lap Lee, Jason Yeh, Carter Van Waes, and Zhong Chen

Tumor Biology Section, Head and Neck Surgery Branch, National Institute on Deafness and Other Communication Disorders, NIH, Bethesda, Maryland

Abstract

Signal transducer and activator of transcription 3 (STAT3) has been reported to be activated by interleukin-6 receptor (IL-6R) or epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas (HNSCC), which may have important implications for responsiveness to therapeutics targeted at EGFR, IL-6R, or intermediary kinases. Suppressor of cytokine signaling-1 (SOCS-1) has been implicated recently in the negative regulation of IL-6R/Janus-activated kinase (JAK)–mediated activation of STAT3, suggesting that SOCS-1 could affect alternative activation of STAT3 by EGFR, IL-6R, and associated kinases. We investigated whether epigenetic modification of SOCS-1 affects STAT3 activation in response to IL-6R, EGFR, JAK-, or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)–mediated signal activation. STAT3 was predominantly activated by IL-6R via Jak1/Jak2 in HNSCC lines UMSCC-9 and UMSCC-38 in association with transcriptional silencing of SOCS-1 by hypermethylation. In UMSCC-11A cells with unmethylated SOCS-1, STAT3 activation was regulated by both EGFR and IL-6R via a JAK-independent pathway involving MEK. Pharmacologic inhibitors of JAK and MEK and expression of SOCS-1 following demethylation or transfection inhibited STAT3 activation and cell proliferation and induced cell apoptosis in corresponding cell lines. Hypermethylation of SOCS-1 was found in about one-third of human HNSCC tissues, making it a potentially relevant marker for STAT-targeted therapy in HNSCC patients. We conclude that SOCS-1 methylation status can differentially affect STAT3 activation by IL-6R and EGFR through JAK or MEK in different HNSCC and response to pharmacologic antagonists. Identifying the potential factors and the regulatory pathways in STAT3 activation has important implications for the development and selection of molecularly targeted therapy in HNSCC.

Introduction

Signal transducers and activators of transcription (STAT) are a family of transcription factors that regulate fundamental cellular functions, such as proliferation, differentiation, cytokine production, and cell death (1). Constitutive activation of STATs, especially STAT3, has been reported to contribute to oncogenesis by promoting proliferation and inhibiting apoptosis of tumor cells in head and neck squamous cell carcinomas (HNSCC; refs. 2–8) as well as in a variety of other human malignancies (1, 9). Activation of STAT3 occurs in response to cytokine signals, such as interleukin-6 (IL-6), through IL-6 receptor (IL-6R; refs. 10–12), or to growth factors through tyrosine kinase growth factor receptors, such as epidermal growth factor receptor (EGFR; refs. 2, 4). Activated STATs can dimerize and translocate to the nucleus and function as transcription factors to activate a broad range of genes (13).

Constitutively activated STAT3 in HNSCC is an important proliferative and prosurvival signal in vitro and in vivo (2–8). EGFR and its ligand, transforming growth factor-α (TGF-α), which are overexpressed in most HNSCC, were first shown to contribute to activation of STAT3 in HNSCC (2–4, 14, 15). We showed that IL-6, another potential activator of STAT3, is also often expressed in tumor and serum of patients with HNSCC when compared with the normal subjects (16). IL-6 protein expressed in HNSCC culture supernatants was found to induce phosphorylation of STAT3 by IL-6R signaling and proliferation (17). In a subsequent study, Sriuranpong et al. reported that only 3 of 10 HNSCC cell lines exhibited moderate to strong activation of STAT3 by EGFR, and constitutive STAT3 activation was regulated by IL-6R in the majority of the lines independent of the EGFR pathway.
Results of a recent clinical trial with EGFR tyrosine kinase inhibitor are consistent with an EGFR-independent mechanism of signal activation of STAT3 in patients with HNSCC. Patients receiving gefitinib (Iressa, ZD1839) showed inhibition of EGFR and mitogen-activated protein kinase signal phosphorylation but not STAT3 phosphorylation (19). Together, these data suggest that STAT3 activation may be mediated by IL-6 and/or EGFR in HNSCC. These alternative mechanisms of activation could have important implications for effectiveness and design of molecularly targeted therapy.

The basis for the apparent differences in the role of IL-6R and EGFR observed in these studies is poorly understood. We hypothesized that both IL-6R and EGFR are important in signal activation of STAT3; however, different intermediate signal pathways or negative regulators may determine the different responses in heterogeneous HNSCC cells. Activation of STAT3 following IL-6R gp130 phosphorylation has been shown to involve recruitment and phosphorylation of Janus-activated kinases (JAK; refs. 10, 11), whereas EGFR has been reported to activate STAT3 either directly or through Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)-mediated mechanisms (20, 21). Conversely, STAT3 may be negatively regulated by a recently discovered novel gene family named suppressors of cytokine signaling (SOCS). Silencing of SOCS-1 expression by methylation has been observed in hepatocellular carcinoma, resulting in the increased sensitivity of JAK activation in response to cytokines, constitutive activation of STATs, and abnormal growth (22).

In the present study, we examined HNSCC lines expressing both IL-6 and TGF-α for possible differences in signal activation by IL-6R gp130, EGFR, JAK, and mitogen-activated protein kinase and for expression of SOCS-1. Using receptor ligands and pharmacologic antagonists, we showed that EGFR and IL-6R induce STAT3 activation through JAK or MEK pathways in a cell-specific manner. The specific pattern of signal activation was determined by the status of SOCS-1 expression. Silencing of SOCS-1 expression in two HNSCC cell lines was found to be due to promoter hypermethylation, and suppression of IL-6-mediated STAT3 activation could be restored by demethylation or transient expression of SOCS-1. Hypermethylation of SOCS-1 was also detected in about one-third of tumors in situ. Our results show that IL-6 and EGFR make different contributions to the activation of JAK/STAT pathways in different HNSCC. Methylation and loss of SOCS-1 expression may be one factor contributing to the preferential activation of STAT3 by the IL-6R/JAK pathway and can be detected in tumor as a potential marker for use in selection of therapy.

Materials and Methods
Reagents, Antibodies, and Plasmids
Chemical inhibitor U0126 (MEK inhibitor) was purchased from Promega (Madison, WI). Piceatannol (Jak1 inhibitor) and AG490 (Jak2 inhibitor) were from Calbiochem-Novabiochem Corp. (San Diego, CA). EGFR tyrosine kinase inhibitor PD153035 was purchased from Tocris (Ballwin, MO). Human recombinant IL-6 and TGF-α were purchased from R&D Systems (Minneapolis, MN). Phosphospecific and non-phosphospecific antibodies and control cell extract for STAT3 and ERK1/2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-IL-6R monoclonal antibody was obtained from Biosource International (Camarillo, CA). Anti-EGFR C225 monoclonal antibody was from Calbiochem-Novabiochem. Anti-Jak1 monoclonal antibody was from Transduction Laboratories (Lexington, KY). Goat polyclonal anti-EGFR antibody, goat polyclonal anti-phosphorylated gp130, rabbit polyclonal anti-gp130 antibody, rabbit polyclonal anti-Jak2 antibody, mouse monoclonal anti-STAT3 antibody, mouse monoclonal anti-phosphotyrosine antibody, and protein G Plus agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). The Bicinchoninic Acid Protein Assay and Supersignal West Pico Chemiluminescent Detection kits were obtained from Pierce (Rockford, IL). STAT3 luciferase reporter construct (pSTAT3-TA-Luc) was purchased from Clontech (Palo Alto, CA). The plasmid containing human SOCS-1 cDNA (pORF5-sSOCS-1) and control plasmid (pORF5) were obtained from InvivoGen (San Diego, CA). The STAT3Δi dominant negative (pIRES-STAT3Δi) and backbone plasmid (pIRES-EGFP) encoding enhanced green fluorescence protein were kindly provided from Drs. James Turkson and Richard Jove (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, FL). The construction and characterization of pIRES-STAT3 has been described previously (23). The constitutively activated MEK mutant (pcDNA III-MEK EE) and MEK dominant negative (pcDNA III-MEK AA) were provided by Dr. Silvio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). The pCMV-LacZ construct was made in our laboratory and consists of a LacZ gene inserted between the cytomegalovirus promoter and BGH polyadenylate signal sequence in pcDNA3 (Invitrogen, Carlsbad, CA).

Western Blot and Immunoprecipitation
Whole-cell lysates (20 μg) were mixed with Laemml loading buffer (containing β-mercaptoethanol) and heated at 100°C for 5 minutes. The samples were loaded onto 10% Tris-glycine precast gels and electrophoresed at 140 V for 90 minutes. The proteins were transferred to 0.45-μm nitrocellulose membranes (Invitrogen) for 2 hours at 20 V at room temperature using the Invitrogen Gel Blot Module. Immunoblotting for EGFR, gp130, phosphorylated gp130, and STAT3 was conducted according to the manufacturer’s specifications. Primary antibodies were diluted in 5% nonfat powdered milk prepared from TBS-Tween 20 as follows: 1:500 for phosphorylated gp130, Jak1, Jak2, and STAT3 and 1:1,000 for phosphorylated and nonphosphorylated STAT3, ERK1/2, EGFR, and gp130. For immunoprecipitation experiments, total EGFR, Jak1, and Jak2 were
immunoprecipitated from 500 μg whole-cell lysates according to the manufacturer’s protocol. Each blot was incubated with SuperSignal West Pico substrate and exposed to Kodak X-OMAT film.

**Tissue Array, H&E Staining, and Immunohistochemistry**

Formalin-fixed and paraffin-embedded HNSCC tissue array was obtained from Cybordi, Inc. (Gaithersburg, MD). The array contains HNSCC tumor tissues from 20 individuals and spotted in triplicates plus normal mucosa tissues from 6 normal subjects and spotted in duplicates. Each array spot was 1.5 mm in diameter and selected by the certified pathologist with histologic H&E and immunohistochemical pan-cytokeratin stainings. The tissues were sectioned 5 μm in thickness. H&E staining and immunohistochemistry were done according to the manufacturer’s protocol.

**STAT3-Binding Assay**

The STAT3-binding activity was evaluated using a TransAM STAT family transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. The absorbance was measured at wavelength of 655 nm by a microplate reader (Biotek, Winooski, VT).

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay**

A total of 5 × 10³ UMSCC-9, UMSCC-11A, or UMSCC-38 cells were plated in each well of a 96-well microtiter plate and incubated overnight. The cells were then washed twice with PBS and exposed to inhibitors in medium at the concentrations indicated. Cell density was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (Boehringer Mannheim, Indianapolis, IN). Colorimetric absorbance was measured at 570 nm by a microplate reader (Bio-Tek, Winooski, VT). This procedure was repeated at days 1, 3, and 5 after treatment.

**Flow Cytometry**

UMSCC cells treated with different inhibitors were incubated for 48 hours before harvesting. Both live monolayer cells and dead nonadhesive cells were collected and counted by hemocytometer with trypan blue solution (Invitrogen). The cells were stained with propidium iodide and counted by hemocytometer with trypan blue solution.

**Real-time and Regular Reverse Transcription-PCR**

The RNA expression was detected by real-time quantitative PCR using the Assays-on-Demand Gene Expression assay. Total RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA synthesis was done by using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) to synthesize single-stranded cDNA according to the manufacturer’s protocol. PCR was done together with endogenous control using eukaryotic 18S rRNA. Relative quantitation of the expression was done by normalizing the target gene signals with the 18S endogenous control.

To detect SOCS-1 gene expression, cDNA synthesis was done by using 3 μg total RNA reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim). PCR was carried out using 1 μg cDNA template in a total volume of 50 μL using 2 units Taq polymerase (Boehringer Mannheim) on a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems). The forward and reverse primers for SOCS-1 and glyceraldehyde-3-phosphate dehydrogenase are as follows: SOCS-1 5’-CAGCGACTTCCGACACCTCC-3’ and 5’-TCCAGGCTCAGGGA-3’ and glyceraldehyde-3-phosphate dehydrogenase 5’-ATCACCACCTTTCAGGAG-3’ and 5’-GCACCTCCAGTCTT-3’. The PCR for both genes was cycled at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. After 30 cycles, 10 μL aliquots were run on 1.5% agarose gels containing ethidium bromide and photographed.

**DNA Modification and Methylation-Specific PCR**

DNA from UMSCC cells was extracted by using DNA extraction kit (Boehringer Mannheim). Bisulfite modification of DNA (1 μg) was carried out by the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the manufacturer’s suggestion. The bisulfite-modified DNA samples were amplified by primers specific for both methylated and unmethylated sequences. The primer sequences for detecting methylated for SOCS-1 were 5’TTCGCGTTCGTTTATGTTTTCAGG-3’ (forward) and 5’-ACCCCCACCAGCCCGCATCT-3’ (reverse), whereas 5’TTCGCGTTCGTTTATGTTTTCAGG-3’ (forward) and 5’-ACCCCCACCAGCCCGCATCT-3’ (reverse) were used for unmethylated sequence detection (24). PCR products were analyzed in 2% agarose gels stained with ethidium bromide. The PCR products were also sequenced for confirmation.

**Transient Transfection and Luciferase Reporter Assays**

UMSCC-9, UMSCC-11A, and UMSCC-38 cells were seeded at 5 × 10³ per well in 24-well culture plates. On the following day, the cells were cotransfected with pSTAT3-TA-Luc at 0.1 μg/well, corresponding plasmids at 0.3 μg/well, and internal control plasmid pCMV-LacZ at 0.02 μg/well. The transfection was carried using Lipofectamine 2000 (Invitrogen). Cell density was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (Boehringer Mannheim). Each transfection was carried out in quadruplicate fashion. Three hours after transfection, the cells were stimulated with 10 ng/mL IL-6 or 5 ng/mL TGF-α for 24 hours. The cell lysates were collected and the luciferase activity was measured using Dual-Light reporter gene assay (Tropix, Bedford, MA) on Monolight 2010 luminometer according to the manufacturer’s instructions (Analytical Luminescence Laboratory, San Diego, CA). β-Galactosidase activity was determined to verify the reproducibility of each transfection experiment and the relative STAT3 luciferase activity was calculated by dividing the STAT3 luciferase activity with β-galactosidase activity in each transfection experiment.
Results

STAT3 Activation Can Be Differentially Regulated by Signals through IL-6R or EGFR in Different UMSCC Lines

To explore the potential differences in IL-6R or EGFR signaling in STAT3 activation among HNSCC, we compared the effects of specific IL-6R and EGFR inhibitory antibodies and the corresponding ligands recombinant IL-6 and TGF-α on phosphorylated and total STAT3 expression in a panel of UMSCC lines that were characterized in previous studies for IL-6R and EGFR (17, 25). Figure 1A shows a Western blot of phosphorylated and total STAT3 expression in HNSCC lines UMSCC-9, UMSCC-11A, and UMSCC-38. Constitutive phosphorylation and expression of STAT3 is detectable in all cell lines.

Figure 1. IL-6R and EGFR signals contributed differently to STAT3 activation in UMSCC cells. A, UMSCC-9, UMSCC-11A, and UMSCC-38 cells at 80% to 90% confluence were serum starved for 48 h and pretreated with anti-IL-6R antibody (1 μg/mL) and anti-EGFR antibody C225 (2 μg/mL) alone or in combination for 1 h at 37°C. The cells were then treated with or without IL-6 (10 ng/mL) for 30 min. Whole-cell lysates were harvested for Western blot analysis using anti-phosphorylated STAT3 (P-STAT3) and anti-STAT3 antibodies. B, UMSCC-9, UMSCC-11A, and UMSCC-38 and human normal keratinocytes (HNKC) in log growth phase were serum starved for 48 h followed by stimulation with (+) or without (-) IL-6 (10 ng/mL) for 30 min. Whole-cell lysates (20 μg) were used for Western blot analysis and detected with anti-phosphorylated STAT3 and anti-STAT3 antibodies or anti-phosphorylated gp130 (P-gp130) and anti-gp130 antibodies. EGFR immunoprecipitation was done using anti-EGFR antibody followed by immunoblotting with anti-phosphotyrosine antibody and Western blot using anti-EGFR antibody. The blot was probed by anti-β-tubulin antibody for the loading control. C, UMSCC-11A and UMSCC-38 were preincubated with 5 μmol/L PD153035 for 24 h and stimulated with or without 5 ng/mL TGF-α for 30 min. Whole-cell lysates were harvested for immunoprecipitation and Western blot analysis. D, quantitative real-time reverse transcription-PCR (RT-PCR) and ELISA for detection of IL-6 expression (top) and TGF-α (bottom) in cell lysates from the cell lines. E, selected examples of immunohistochemical staining by phosphorylated STAT3 antibodies on human head and neck cancer tissue array under high magnification (×400). Four HNSCC tumor samples (top and middle) and two normal mucosa samples from tongue and gingiva (bottom).
Both anti-IL-6R and anti-EGFR antibodies had inhibitory effects on STAT3 phosphorylation, but differences were observed among the cell lines. Anti-IL-6R antibody was found to inhibit constitutive STAT3 activation in all three UMSCC cells, and this inhibition was more complete in UMSCC-9 and UMSCC-38 relative to UMSCC-11A. In contrast, treatment with anti-EGFR antibody, C225, partially blocked STAT3 phosphorylation in UMSCC-11A cells but had no significant effect on STAT3 activity in UMSCC-9 and UMSCC-38. The strongest inhibition of constitutive STAT3 phosphorylation in UMSCC-11A cells was achieved when cells were treated with both antibodies simultaneously, suggesting that signals from both receptors contribute to STAT3 activation in UMSCC-11A cells. Because anti-IL-6R antibody inhibited STAT3 phosphorylation in all cell lines, the response of the cells to recombinant IL-6 was assessed. Recombinant IL-6-induced STAT3 phosphorylation in all cell lines and similar differences in the pattern of inhibition were observed when recombinant IL-6 ligand was added in the presence of the inhibitory antibodies. The results indicate that the contribution of IL-6R and EGFR to STAT3 activation can vary in different HNSCC cells.

Differences in Activation of STAT3 by IL-6R and EGFR among the UMSCC Lines Are Not Due to Absence of Receptor Activation or Ligand Expression

We next examined whether the differences observed could be attributed to lack of expression or phosphorylation of IL-6R or EGFR or expression of ligands IL-6 and TGF-α. All UMSCC cells, but not human normal keratinocytes, exhibited constitutive phosphorylation of STAT3, IL-6R subunit gp130, and EGFR (Fig. 1B). UMSCC-11A exhibited a higher level of phosphorylated STAT3 relative to the other cells, and this was associated with a higher level of both phosphorylated IL-6R gp130 and EGFR (Fig. 1B). All three UMSCC cell lines exhibited total STAT3, IL-6R gp130, and EGFR at similar levels to that detected in human normal keratinocytes, indicating that the differences in constitutive activation of STAT3 between UMSCC and human normal keratinocytes and among the UMSCC lines could not be attributed to simple variation in receptor expression. When cells were stimulated by exogenous recombinant IL-6, an increase in gp130 and STAT3 phosphorylation, but not on EGFR activation, was observed in all UMSCC cells and human normal keratinocytes, indicating that the IL-6R/STAT3 pathway is functionally intact and activated independently of EGFR (Fig. 1B). Two bands were detected by gp130 antibody on the Western blot, consistent with unmodified and glycosylated forms reported previously (26). The gp80 component of the IL-6R was also detected in UMSCC cell culture supernatants but not by Western blot analysis (data not shown). We further investigated the effect of EGFR signaling on STAT3 phosphorylation (Fig. 1C). TGF-α stimulated EGFR phosphorylation in both UMSCC-11A and UMSCC-38 cells but only induced STAT3 phosphorylation in UMSCC-11A cells (Fig. 1C). The EGFR tyrosine kinase inhibitor, PD153035, inhibited phosphorylation of EGFR in both cells but only affected STAT3 phosphorylation in UMSCC-11A cells (Fig. 1C). Thus, the differences in activation of STAT3 attributable to IL-6R and EGFR in the different cell lines were not related to lack of phosphorylation or expression of IL-6R and EGFR.

Expression of IL-6, TGF-α, and Phosphorylated STAT3 in HNSCC Cell Lines and Tissues

Expression of IL-6 and TGF-α mRNA and protein was characterized in the panel of UMSCC cells by quantitative real-time reverse transcription-PCR and ELISA (Fig. 1D). Different levels of IL-6 (top) and TGF-α (bottom) expression were detected among UMSCC cell lines and consistent between mRNA and protein detection. UMSCC-11A cells expressed both genes and proteins at higher levels relative to the other cell lines (Fig. 1D), which was consistent with higher level of phosphorylation of STAT3, gp130, and EGFR observed (Fig. 1A–C).

To verify whether constitutive STAT3 activation is detected in tumor tissue as well as in cell lines, the presence of phosphorylated STAT3 was examined in HNSCC tissue array by immunohistochemistry. Seventy-five percent (15 of 20) of HNSCC specimens showed increased phosphorylated STAT3 when compared with normal mucosa, as shown in representative specimen (Fig. 1E), consistent with the common detection of STAT3 activation in HNSCC cell lines and tissues (2–4, 17, 18; this study).

Activation of JAK or MEK Pathways by IL-6R and EGFR

We further examined the phosphorylation of kinases downstream of IL-6R and EGFR and response to pharmacologic inhibitors to identify possible mechanism(s) for the differential activation of STAT3 by IL-6R and EGFR in different HNSCC. Because regulation of STAT3 activation by IL-6 through JAK pathways has been established previously in lymphoid cells (27, 28), we examined the JAK pathway involvement in IL-6 and EGFR signaling in the UMSCC cells. All three cell lines exhibited constitutive phosphorylation of Jak1 and Jak2, and UMSCC-11A showed the highest levels, which is consistent with the strong IL-6 expression and IL-6R phosphorylation observed. Although IL-6 induced STAT3 activation in all three cell lines, IL-6 induced phosphorylation of Jak1 and Jak2 only in UMSCC-9 and UMSCC-38 cells. Using the chemical inhibitor piceatannol for Jak1 or AG490 for Jak2, we showed that inhibitor alone only partially blocks STAT3 phosphorylation in UMSCC-9 and UMSCC-38 cells (Fig. 2A and B), whereas a combination of the two inhibitors achieved complete inhibition of STAT3 activation (Fig. 2C). Surprisingly, in UMSCC-11A cells, IL-6 increased STAT3 activation without affecting Jak1 and Jak2 phosphorylation. The JAK inhibitors only suppressed constitutive and IL-6-induced phosphorylation of Jak1 and Jak2 but not STAT3, indicating the potential existence of a JAK-independent pathway regulating STAT3 activation in UMSCC-11A cells.

Because STAT3 activation in UMSCC-11A was dependent on both IL-6R and EGFR but independent from the JAK pathway, we hypothesized that the signal might be transduced through alternative pathways, such as the MEK or phosphatidylinositol 3-kinase pathway, which...
are activated by EGFR in HNSCC as we have shown previously (25). To test this hypothesis, total and phosphorylated forms of MEK or phosphatidylinositol 3-kinase substrates ERK and AKT were quantified by Western blot (Fig. 2D). UMSCC-11A cells exhibited the highest level of constitutive ERK phosphorylation, which was further induced by IL-6 (Fig. 2D). IL-6 did not affect ERK phosphorylation in UMSCC-9 and UMSCC-38 cells (Fig. 2D). U0126, a chemical inhibitor for MEK, which significantly suppressed ERK phosphorylation in all cells, completely blocked constitutive and IL-6-induced STAT3 phosphorylation in UMSCC-11A but not in UMSCC-9 and UMSCC-38 (Fig. 2D). This observation was also supported by comparing STAT3 luciferase reporter activity on transfection with MEK activator (MEK EE) and MEK dominant-negative (MEK AA) plasmids with or without IL-6 treatment (Fig. 2E). In UMSCC-11A cells, overexpression of MEK (MEK EE) enhanced STAT3 reporter activity, and MEK dominant negative (MEK AA) or U0126 suppressed the activity (Fig. 2E, top). In contrast, manipulation

Figure 2. JAK- and MEK-mediated STAT3 activation in UMSCC cells. Serum-starved UMSCC-9, UMSCC-11A, and UMSCC-38 cells were pretreated with (A) Jak1 inhibitor piceatannol (PIC; 50 μmol/L), (B) Jak2 inhibitor AG490 (5 μmol/L), or (C) both inhibitors in combination for 24 h at 37°C. Cells were then treated with IL-6 (10 ng/mL) for 15 min, and the cell lysates were harvested for Western blot analysis using anti-phosphorylated STAT3 and anti-STAT3 antibodies. The corresponding JAK protein in each experiment was immunoprecipitated using the corresponding JAK antibody followed by blotting with anti-phosphotyrosine antibody. The blot was stripped and reprobed for corresponding total JAK protein as control. (D) serum-starved UMSCC-9, UMSCC-11A, and UMSCC-38 cells were pretreated with MEK inhibitor U0126 (10 μmol/L) for 24 h at 37°C. Cells were then treated with IL-6 (10 ng/mL) for 15 min and cell lysates were harvested for Western blot analysis using anti-phosphorylated ERK, anti-ERK, anti-phosphorylated STAT3, or anti-STAT3 antibodies. (E) UMSCC-11A and UMSCC-38 were either transiently cotransfected with STAT3 luciferase reporter and pCMV-LacZ constructs with control vector pRES; wild-type MEK expression vector (MEK EE), or MEK dominant-negative plasmid (MEK AA) or pretreated with U0126 (10 μmol/L) for 24 h in the presence or absence of IL-6. Cell lysates were harvested and analyzed for luciferase activity using Dual-Light reporter assay system. Columns, mean luciferase activity relative to the β-galactosidase control calculated from quadruplicate transfections; bars, SD. (F) UMSCC-11A and UMSCC-38 were preincubated with 5 μmol/L PD153035 for 24 h and stimulated with or without 5 ng/mL TGF-α for 30 min. Whole-cell lysates were harvested for immunoprecipitation and Western blot analysis described in Materials and Methods.
Alteration of SOCS-1 and STAT3 Activation via JAK or MEK

of MEK had no effect on STAT3 activation in UMSCC-38 cells (Fig. 1E, bottom). LY-294002, a chemical inhibitor for phosphatidylinositol 3-kinase, showed no significant effect on STAT3 activation in any of the cell lines (data no shown). In addition, the effects of EGFR signaling on downstream pathways were evaluated. Figure 2F shows that ERK phosphorylation was induced by TGF-α or inhibited by PD153035 in both UMSCC-11A and UMSCC-38 cells, but the modulation of STAT3 phosphorylation was only observed in UMSCC-11A cells. EGFR signaling had no effect on Jak1 and Jak2 phosphorylation. These data clearly show that IL-6- and TGF-α-mediated STAT3 activation is mainly regulated through MEK pathway in UMSCC-11A cells.

Effects of JAK and MEK Inhibitors on STAT3-Binding Activity

Having shown the effects of JAK and MEK inhibitors on STAT3 phosphorylation, we next extended the study to how these inhibitors affect STAT3 DNA-binding activity (Fig. 3). UMSCC-11A exhibited the strongest constitutive STAT3-binding activity followed by UMSCC-9 and UMSCC-38, which is consistent with the results of STAT3 phosphorylation described previously. When cells were preincubated with a single inhibitor for 24 hours, STAT3-binding activity in UMSCC-9 and UMSCC-38 was hindered in the presence of JAK inhibitor piceatannol or AG490 but not by MEK inhibitor UO126. STAT3 activity was further deceased in UMSCC-9 and UMSCC-38 when treated with both JAK inhibitors. In contrast, STAT3 binding in UMSCC-11A was only inhibited by UO126 but not by JAK inhibitors. Combination of JAK inhibitor AG490 and MEK inhibitor UO126 did not potentiate the decrease of STAT3 activity significantly. Taken together, these findings further show that the JAK and MEK pathways are differentially involved in regulation of STAT3 activation and binding activity in different HNSCC, which is consistent with the results above.

Effect of JAK or MEK Inhibitors on Cell Proliferation and Viability

STAT3 activation has been associated with increased proliferation and inhibition of apoptosis. To study the relationship between inhibition of STAT3 and proliferation of UMCC by MEK and JAK inhibitors, a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted with various concentrations of the inhibitors (Fig. 4A). The chemical inhibitors of JAK or MEK showed a dose- and time-dependent growth inhibition in all cells. UMSCC-9 and UMSCC-38 cells were selectively more sensitive to the JAK inhibitors. On day 3, at the doses that completely blocked Jak1 and Jak2 phosphorylation, piceatannol (50 μmol/L) or AG490 (5 μmol/L) alone suppressed the growth of UMSCC-9 and UMSCC-38 cells >50% but suppressed the growth of UMSCC-11A cells only ∼30%. Higher doses of AG490 (10 μmol/L) completely suppressed cell growth in all cell lines in association with visible cytotoxic effects (data not shown). Although 10 μmol/L UO126 completely inhibited ERK and STAT3 phosphorylation, 10 and 30 μmol/L UO126 inhibited cell growth by only ∼30% in UMSCC-11A cells. In addition, when we tested cell apoptosis induced by the chemical inhibitors, MEK inhibitor UO126 significantly induced UMSCC-11A cell apoptosis, whereas JAK inhibitors had a greater proapoptotic effect on UMSCC-9 and UMSCC-38 (Fig. 4B). Taken together, these results showed that different UMSCC cells exhibit differences in sensitivity to growth inhibition and apoptosis by inhibitors specific for JAK or MEK.

Transcriptional Silencing of SOCS-1 by Aberrant Methylation Augments Constitutive and IL-6-Induced STAT3 Activation in UMSCC-9 and UMSCC-38 Cells

Having shown that STAT3 activation is differentially regulated through JAK or MEK pathway in different UMSCC lines, we further explored the possible upstream control mechanism for such regulation. SOCS-1 has been discovered recently as a novel negative regulator of IL-6 signaling through the suppression of JAK activity (22, 29, 30). We hypothesized that SOCS-1 might be involved in the differential regulation of STAT3 activation in UMSCC cells. Therefore, we determined SOCS-1 expression in UMSCC cells by reverse transcription-PCR. SOCS-1 was weakly expressed and induced by IL-6 stimulation in UMSCC-11A, but no SOCS-1 expression was observed in either unstimulated or IL-6 stimulated UMSCC-9 and UMSCC-38 cells (Fig. 5A). This suggested that the SOCS-1 gene in UMSCC-9 and UMSCC-38 cells was either deleted or silenced by epigenetic mechanisms, such as aberrant gene methylation (22, 31–33). When we examined the potential promoter hypermethylation of SOCS-1 in these cells, methylated PCR product was found in UMSCC-9 and UMSCC-38 but not in UMSCC-11A, which is consistent with epigenetic silencing of SOCS-1 in UMSCC-9 and UMSCC-38 (Fig. 5B). Aberrant
Figure 4. Effect of JAK and MEK inhibitors on the proliferation and apoptosis of UMSCC-9, UMSCC-11A, and UMSCC-38 cells. A, proliferation of UMSCC-9, UMSCC-11A, and UMSCC-38 cells was studied by a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay treated with U0126 (1, 10, and 30 μmol/L), piceatannol (10, 50, and 100 μmol/L), or AG490 (1, 5, and 10 μmol/L). Cell growth was evaluated by the relative increase in the absorbance at 570 nm compared with the initial value in each experiment. Points, mean of quadruplicate cultures; bars, SD. B, effects of JAK and MEK inhibitors on apoptosis of UMSCC cells. UMSCC-9, UMSCC-11A, and UMSCC-38 cells were treated with piceatannol (50 μmol/L), AG490 (5 μmol/L), and U0126 (10 μmol/L) for 48 h. The DNA content of 5 × 10^5 cells stained with propidium iodide was analyzed by FACScan flow cytometry. Data were collected by CellQuest software and the percentages of apoptotic cells were analyzed by ModFit LT software.
methylation of SOCS-1 was also occurred in two of five microdissected tumor tissues from HNSCC patients (Fig. 5B). To further confirm the results, demethylation was done in UMSCC-9 and UMSCC-38 cells by culture with 5-azacytidine, a drug known to remove methyl group from the nucleotides (Fig. 5C). 5-Azacytidine (5–10 μmol/L) completely demethylated SOCS-1 in UMSCC-9 and UMSCC-38 cells (Fig. 5C), and both constitutive and IL-6-induced STAT3 phosphorylation was inhibited in UMSCC-9 and UMSCC-38 cells after demethylation (Fig. 5D). Therefore, the epigenetic status of SOCS-1 is one of the important factors that govern IL-6 responsiveness and STAT3 activity in UMSCC-9 and UMSCC-38 cells.

**SOCS-1 Expression Regulates STAT3 Promoter Reporter Activity**

We further investigated the functional importance of SOCS-1 in regulating STAT3 activation. SOCS-1 expression plasmid was transiently cotransfected with a promoter construct containing four STAT3-binding sites upstream of a luciferase reporter. Constitutive reporter activity of STAT3 was observed in all UMSCC cells, and the constitutive reporter activity in UMSCC-11A cells was highest (Fig. 6A). IL-6 significantly induced the STAT3 reporter activity in all cells. In UMSCC-9 and UMSCC-38 cells, cotransfection of SOCS-1-expressing plasmid led to a >60% reduction in constitutive STAT3 reporter activity and reduced IL-6-inducible activation by >50%. In contrast, no significant inhibition was observed in UMSCC-11A in either condition. However, a broad suppression of STAT3 reporter activity was observed in all cell lines under both constitutive and inducible conditions when we cotransfected the dominant-negative STAT3 construct (Fig. 6B). These data further confirmed that SOCS-1 can specifically suppress STAT3 activation by the JAK pathway in UMSCC-9 and UMSCC-38 but not in UMSCC-11A cells.

**Discussion**

Emerging evidence from different studies have suggested the critical role of activated STAT3 in the progression of various human malignancies, including HNSCC (1–9). Grandis et al. showed that constitutively activated STAT3 is present in HNSCC specimens and the adjacent mucosa when compared with unaffected subjects, which is associated with activated EGFR (3). However, the activation of STAT3 may not be limited to the EGFR pathway in HNSCC. The classic JAK/STAT pathway, which is under IL-6R signal control, is also well known to activate STAT3 (1). We reported previously that HNSCC cell lines constitutively produce various levels of IL-6 in their culture supernatants, and up-regulated IL-6 protein level was detected in HNSCC primary cultures, patient’s tumor specimens, and sera (16). Furthermore, we provided evidence for the importance of IL-6 in STAT3 activation and cell growth in HNSCC (16, 17). Recently, we expanded the study of serum IL-6 level in a relatively larger group of HNSCC patients (n = 29) and showed that the patients with significantly decreased serum IL-6 level post-treatment had a better prognosis (34). The findings suggested that serum IL-6 level in HNSCC was associated with aggressiveness of disease and was an important indicator for the clinical response to treatments. In support of the importance of IL-6-mediated STAT3 activation, Suriyanpong et al. showed that only 3 of 10 HNSCC cell lines expressed moderate to strong activation of EGFR, and constitutive STAT3 activation was regulated by IL-6R independent of the EGFR pathway in HNSCC cell lines with low or no EGFR expression (18). The evidence from both clinical and basic science studies support the hypothesis that IL-6/IL-6R as well as EGFR-mediated STAT3 signal transduction pathway may contribute to the molecular pathogenesis of HNSCC.

In the present study, we showed that in UMSCC-9 and UMSCC-38 cells, where IL-6R and EGFR and their ligands were expressed and activated (Fig. 1), SOCS-1 methylation and inactivation could preferentially favor IL-6- and JAK-mediated signaling and activation of STAT3 (Figs. 2 and 5). In UMSCC-38 cells, the EGFR stimulator TGF-α and the inhibitor PD153035 were able to alter the phosphorylation of EGFR and ERK but not STAT3 (Figs. 1C and 2F), and genetic manipulation of MEK did not affect STAT3 reporter activity (Fig. 2E, bottom). All the evidence above supports the hypothesis that STAT3 activation is mainly through IL-6R/JAK pathway in UMSCC-38. In contrast, both EGFR and IL-6R pathways contributed to activation of STAT3 via MEK and ERK, independent of JAK (Figs. 1 and 2), in SOCS-1-expressing UMSCC-11A cells (Fig. 5). IL-6 or TGF-α was able to induce ERK and STAT3 activation through IL-6R or EGFR phosphorylation (Figs. 1 and 2), and chemical inhibitors of JAK and genetic alteration of MEK significantly affected STAT3 activation (Fig. 2). Such different dominant receptor/pathway combinations for STAT3 activation may have important clinical implications. Recently, several clinical trials of gefitinib, a EGFR tyrosine kinase inhibitor, have been concluded in cancer patients, including lung, head and neck, and colorectal cancers, and the clinical efficacy was limited (35–37). Although gefitinib significantly suppressed EGFR phosphorylation and mitogen-activated protein kinase activation, the STAT3 phosphorylation level remained high in skin specimens of the patients (19). Because the single-agent targeting at EGFR alone did not reach the expected clinical efficacy in these recent trials, it has been speculated that the activation of STAT3 or other downstream pathways independent from EGFR activation may have contributed to the resistance and clinical outcomes. Our results show that STAT3 activation is mediated by IL-6R or EGFR, through either JAK (UMSCC-9 and UMSCC-38) or MEK (UMSCC-11A), providing evidence that multiple pathways should be selectively targeted in patients with HNSCC to reach the maximal clinical efficacy.

Although several studies showed previously that the activation of the JAK pathway is critical for the constitutive and IL-6-induced STAT3 activation in HNSCC, breast cancer, and myeloma (11, 18, 21, 27, 28), the role of JAK components and negative regulators in JAK-STAT3 activation in cancer cells have not been clearly defined.
Our study suggests that both Jak1 and Jak2 are required for the constitutive and IL-6-induced STAT3 activation in UMSCC-9 and UMSCC-38 cells. We provided evidence that the dominant contribution of the IL-6/JAK pathway to STAT3 activation in UMSCC-9 and UMSCC-38 was due to the loss of SOCS-1 expression by epigenetic hypermethylation of the SOCS-1 promoter (Fig. 5). Restoration of STAT3 inhibition by demethylating agent or transient expression of SOCS-1 inhibited JAK-mediated STAT3 activation in UMSCC-9 and UMSCC-38 cells but not for MEK-mediated STAT3 activation in UMSCC-11A cells (Figs. 5 and 6), which is consistent with the findings by Rottapel et al. in v-Src-transformed cells (30). In our study of UMSCC-9 and UMSCC-38 cells, where the constitutive and IL-6-induced STAT3 activation was mediated by both Jak1 and Jak2, SOCS-1 could directly inhibit JAK activity and lead to subsequent STAT3 inhibition (38–40). In addition, our data clearly showed that EGFR activation did not affect JAK activation in all cell lines (Fig. 2F). Whereas the JAK-mediated STAT3 activation has been well studied, the role of MEK-mediated STAT3 activation seems unclear. Some studies indicate that MEK may promote the phosphorylation of a specific serine residue at 727 within the transcriptional activation domain of STAT3, which either contributes to the maximal transcription activity or induces the negative regulation of STAT3 activity, depending on the specific cell lines or stimuli (41, 42). A more detailed study on MEK regulation on STAT3 activation is needed in UMSCC-11A and other HNSCC cells in vitro and in vivo.

Apart from JAK and MEK, further studies are needed to examine the possible regulatory effects of other members of SOCS family. Recent studies on mouse hepatocytes or macrophages suggest that SOCS-3 may also be involved in the negative regulation of IL-6 in vivo. SOCS-3−/− mice showed elevated IL-6 and activation of STAT1 and STAT3, whereas SOCS-1−/− mice showed enhanced STAT1 phosphorylation in response to IFN-γ (43). These findings provided further evidence that the SOCS family is important in STAT regulation and the role of SOCS family members may vary in different cell types. Development of HNSCC cell lines expressing constitutive JAK or SOCS family members, or specifically knocking out the genes will be helpful in determining the relative importance of these and other members of the JAK and SOCS families.

It has been proposed that SOCS-1 may act as a tumor suppressor gene, as its expression has been shown to have antitumor effects through attenuation of signal by IL-6R (44). Ablent DNA methylation of CpG islands is usually associated with the silencing of tumor suppressor genes in human malignancies (45, 46), and this phenomenon seems to be nonrandom and occurs in certain tumors (47). Ablent methylation of other genes related to growth regulation, cell cycle, and DNA repair has been shown in HNSCC (48, 49). In the case of SOCS-1, aberrant methylation has been observed in other tumors that actively express IL-6, such as hepatocellular carcinoma (22, 31, 33), multiple myeloma (32), gastric carcinoma (50), and colorectal cancer (51). The frequency of aberrant methylation of SOCS-1 was 65% and 63% in hepatocellular carcinoma and in multiple myeloma patients, respectively. We observed
SOCS-1 hypermethylation in 2 of 8 (25%) UMSCC cell lines (Fig. 5; data not shown) and 2 of 5 (40%) of microdissected HNSCC tissues (Fig. 5), indicating that the prevalence may be lower than observed in hepatocellular carcinoma. Other than epithelial alteration, it has been reported that viruses, such as hepatitis C virus, could activate STAT3 through JAK (52). Therefore, it may also be interesting to examine the potential effects of viruses frequently associated with HNSCC, such as EBV and human papillomavirus, on STAT and SOCS-1 regulation.

Our study suggests several strategies for molecularly targeted therapy aimed at the STAT3 signal transduction pathway. First, inhibition of both IL-6R and EGFR, JAK and MEK, or STAT3 further downstream may be required for targeted therapy aimed at the STAT3 signal transduction pathway. Our study suggests several strategies for molecularly targeted therapy aimed at the dominant signal transduction pathway. A thorough understanding of the molecular mechanisms in each cancer type needs to be achieved before successful therapy may be developed.

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References

1. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474 – 88.
2. Grandis JR, Drenning SD, Chakraborty A, et al. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth in vivo. J Clin Invest 1998;102:1385 – 92.
3. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinoma in vivo. Proc Natl Acad Sci U S A 2000;97:4227 – 32.
4. Grandis JR, Zeng Q, Drenning SD. Epidermal growth factor receptor-mediated Stat3 signaling blocks apoptosis in head and neck cancer. Laryngoscope 2000;110:766 – 74.
5. Song Ji, Grandis JR. STAT signaling in head and neck cancer. Oncogene 2000;19:2489 – 95.
6. Masuda M, Suzuki M, Yasumatsu R, et al. Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. Cancer Res 2002;62:3351 – 5.
7. Kijima T, Niwa H, Steinman RA, et al. STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth in vivo. Cell Growth Different 2002;13:355 – 62.
8. Leong PL, Andrews GA, Johnson DE, et al. Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci U S A 2003;100:4138 – 43.
9. Turkson J, Jove R. STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 2000;19:6613 – 26.
10. Hirano T, Ishihara K, Hibi M. Roles of STAT3 in mediating the cell differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 2000;19:2548 – 56.
11. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Gravele L. Interleukin-6-type cytokine signaling through the gp130/Jak/STAT pathway. Biochem J 1998;334:297 – 314.
12. Taka T, Gp130, a shared signal transducing receptor component for hematopoietic and neuroepoietic cytokines. J Neurochem 1998;67:1 – 10.
13. Bromberg J, Darnell JE, Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000;19:2468 – 73.
14. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor α and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53:3579 – 84.
15. Grandis JR, Melhem MF, Barnes EL, Tweardy DJ. Quantitative immunohistochemical analysis of transforming growth factor-α and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. Cancer 1996;78:1284 – 92.
16. Chen Z, Malhotra PS, Thomas GR, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. Clin Cancer Res 1999;5:1369 – 79.
17. Hong SH, Ondrey FG, Avis IM, et al. Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation? FASEB J 2000;14:1499 – 507.
18. Sriuranpong V, Park Ji, Amorphimoltham P, Patel V, Nelkin BD.
Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res 2003;63:2948 – 56.

19. Albanell J, Rojo F, Averbuch S, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. J Clin Oncol 2002;20:110 – 24.

20. Zushi S, Shinomura Y, Kiyohara T, et al. STAT3 mediates the survival signal in oncogenic ras-transfected intestinal epithelial cells. Int J Cancer 1998;78:326 – 30.

21. Amorino GP, Hamilton VM, Valerie K, Dent P, Lammersing G, Schmidt-Ullrich RK. Epidermal growth factor receptor dependence of radiation-induced transcription factor activation in human breast carcinoma cells. Mol Biol Cell 2002;13:2233 – 44.

22. Yoshikawa H, Matsubara K, Qian GS, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 2001;28:29 – 35.

23. Catlett-Falcone R, Lansdownski VS, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999;10:105 – 15.

24. Miyoshi H, Fujie H, Moriya K, et al. Methylation status of suppressor of cytokine signaling-1 gene in hepatocellular carcinoma. J Gastroenterol 2004;39:563 – 9.

25. Bancroft CC, Chen Z, Yeh J, et al. Effects of pharmacologic antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-κB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous cell carcinoma lines. Int J Cancer 2002;99:538 – 48.

26. Vermes C, Jacobs JJ, Zhang J, Firneisz G, Roebuck KA, Glant TT. Shedding of the interleukin-6 (IL-6) receptor (gp80) determines the ability of IL-6 to induce gp130-transfected human osteoblasts. J Biol Chem 2002;277:16879 – 87.

27. O’Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002;109 Suppl:S121 – 31.

28. Aaronson DS, Horvath CM. A road map for those who know JAK-STAT. Science 2002;296:1653 – 5.

29. Nicholson SE, Hilton DJ. The SOCS proteins: a new family of negative regulators of signal transduction. J Leukoc Biol 1998;63:665 – 8.

30. Rottapel R, Ilangumaran S, Neale C, et al. The tumor suppressor activity of SOCS-1. Oncogene 2002;21:4351 – 62.

31. Nagai H, Kim YS, Konishi N, et al. Combined hypermethylation and chromosome loss associated with inactivation of SSI-1/SOCS-1/JAB gene in human hepatocellular carcinomas. Cancer Lett 2002;186:59 – 65.

32. Galm O, Yoshikawa H, Esteller M, Osieka R, Herman JG. SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma. Blood 2003;101:2784 – 8.

33. Nagai H, Naka T, Terada Y, et al. Hypermethylation associated with inactivation of the SOCS-1 gene, a JAK/STAT inhibitor, in human hepatoblastomas. J Hum Genet 2003;48:65 – 9.

34. Druzgal CH, Chen Z, Yeh NT, et al. A pilot study of longitudinal serum cytokine and angiogenesis factor levels as markers of therapeutic response and survival in patients with head and neck squamous cell carcinoma. Head Neck 2005;27:771 – 84.

35. Cohen EE, Rosen F, Stadler WM, et al. Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol 2003;21:1980 – 7.

36. Douglass EC. Development of ZD1839 in colorectal cancer. Semin Oncol 2003;30:17 – 22.

37. Ransohoff M, ZD1839 (Iressa): for more than just non-small cell lung cancer. Oncologist 2002;7 Suppl 4:16 – 24.

38. Starr R, Wilson TA, Viney EM, et al. A family of cytokine-inducible inhibitors of signalling. Nature 1997;387:917 – 21.

39. Naka T, Narazaki M, Hirata M, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924 – 9.

40. Endo TA, Masuhara M, Yokouchi M, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921 – 4.

41. Wen Z, Zhong Z, Darnell JE, Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241 – 50.

42. Chung J, Uchida E, Timothy C, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and-independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997;17:6508 – 16.

43. Crocker BA, Krebs DL, Zhang J-G, et al. SOCS3 negatively regulates IL-6 signaling in vivo. Nat Immunol 2003;4:540 – 5.

44. Nicola NA, Greenhalgh CJ. The suppressors of cytokine signaling (SOCS) proteins: important feedback inhibitors of cytokine action. Exp Hematol 2000;28:1105 – 12.

45. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of HML1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A 1998;95:6870 – 5.

46. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687 – 92.

47. Cooney RN. Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock 2002;17:83 – 90.

48. Sanchez-Cespedes M, Esteller M, Wu L, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 2000;60:892 – 5.

49. Hasegawa M, Nelson HH, Peters E, Ringstrom E, Posner M, Kelsey KT. Patterns of gene promoter methylation in squamous cell cancer of the head and neck. Oncogene 2002;21:4231 – 6.

50. Oshimo Y, Kuraoka K, Nakayama H, et al. Epigenetic inactivation of SOCS1 by CpG island hypermethylation in human gastric carcinoma. Int J Cancer 2004;112:1003 – 9.

51. Bai AH, Tong JH, To KF, et al. Promoter methylation of tumor-related genes in the progression of colorectal neoplasia. Int J Cancer 2004;112:846 – 53.

52. Sarcar B, Ghosh AK, Steele R, Ray R, Ray RB. Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. Virology 2004;322:51 – 60.