

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Constitutive Activation of JAK3/STAT3 in Colon Carcinoma Tumors and Cell Lines

Inhibition of JAK3/STAT3 Signaling Induces Apoptosis and Cell Cycle Arrest of Colon Carcinoma Cells

Quan Lin,* Raymond Lai,[†] Lucian R. Chirieac,* Changping Li,* Vilmos A. Thomazy,* Ioannis Grammatikakis,* George Z. Rassidakis,* Wei Zhang,* Yasushi Fujio,[‡] Keita Kunisada,[‡] Stanley R. Hamilton,* and Hesham M. Amin*

From the Division of Pathology and Laboratory Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas; the Department of Laboratory Medicine and Pathology,[†] University of Alberta, Edmonton, Alberta, Canada; and the Department of Molecular Medicine,[‡] Graduate School of Medicine, Osaka, Japan*

Signal transducer and activator of transcription 3 (STAT3) has oncogenic potential. The biological effects of STAT3 have not been studied extensively in the pathogenesis of colon cancer, nor has the role of Janus kinase 3 (JAK3), the physiological activator of STAT3, been evaluated. Here, we demonstrate that activated STAT3 (pSTAT3) and activated JAK3 (pJAK3) are expressed constitutively in two colon cancer cell lines, SW480 and HT29. To evaluate the significance of JAK3/STAT3 signaling, we inhibited JAK3 with AG490 and STAT3 with a dominant-negative construct. Inhibition of JAK3 down-regulated pSTAT3. The blockade of JAK3/STAT3 signaling significantly decreased viability of colon cancer cells due to apoptosis and cell-cycle arrest through down-regulation of Bcl-2, Bcl-X_L, Mcl-1, and cyclin D2 and up-regulation of p21^{waf1/cip1} and p27^{kip1}. We also examined histological sections from 22 tumors from patients with stage II or stage IV colon cancer and found STAT3, JAK3, and their activated forms to be frequently expressed. Furthermore, quantitative reverse transcriptase-polymerase chain reaction identified JAK3 mRNA in colon cancer cell lines and primary tumors. Our findings illustrate the biological importance of JAK3/STAT3 activation in the oncogenesis of colon cancer and provide novel evidence that JAK3 is expressed and contributes to STAT3 activation in this malignant neoplasm. (*Am J Pathol* 2005, 167:969–980)

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor with known oncogenic potential.¹ Activated STAT3 has been proposed recently to be a novel molecular target for therapeutic intervention in malignant neoplasms.² STAT3 normally resides in the cytoplasm. After its activation via phosphorylation of the tyrosine⁷⁰⁵ residue, STAT3 dimerizes and translocates to the nucleus where it controls the transcription of several apoptosis- and cell cycle-regulatory proteins. The net effect of STAT3-mediated transcription directs the cells into cell survival and cell-cycle progression.³ Three major mechanisms are implicated in the activation of STAT3: cytokine stimulation of membrane receptors with innate kinase activity, such as epidermal growth factor and platelet-derived growth factor receptors; cytoplasmic kinases such as Src and Abl; and a family of receptor-associated tyrosine kinases known as Janus kinases (JAKs).^{4–10}

Colorectal cancer is one of the leading causes of morbidity and death in the world and is the third leading cause of cancer-related deaths in North America.¹¹ Approximately 147,500 new colon cancer cases were detected in the United States in 2003 and an estimated 57,100 patients died from this disease in the same year.¹¹ Recent studies have shown that mechanisms involving STAT3 pathway may play a role in colon carcinogenesis. For example, a recent study showed that Src kinases play a major role in the pathogenesis of colon cancer,¹² at least in part, via activation of

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Address reprint requests to Hesham M. Amin, M.D., M.Sc., Division of Pathology and Laboratory Medicine, Box 72, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. E-mail: hamin@mdanderson.org.

STAT3.¹³ In addition, Fer was found to phosphorylate and activate STAT3 in colon cancer cells.¹⁴ However, the role of STAT3 in the pathogenesis of colon cancer has not been extensively evaluated. In addition, it is unclear as to the role of JAK3, the physiological activator of STAT3, in stimulating STAT3 in colon carcinoma cells.

In the present study, we demonstrated that STAT3 and JAK3 are constitutively activated in two human colon carcinoma cell lines, SW480 and HT29. To investigate the biological significance of JAK3/STAT3 activation in these cells, we used AG490, a pharmacological JAK3 inhibitor, as well as an adenoviral vector harboring the dominant-negative construct of STAT3 (AdSTAT3DN). We investigated changes in cell viability, occurrence of apoptotic cell death, and alterations in cell cycle progression. In addition, we evaluated changes in the protein expression level of several downstream targets of JAK3/STAT3 signaling pathway that are known to be directly related to apoptotic cell death (Bcl-2, Bcl-X_L, Mcl-1, and survivin) and cell cycle regulation (cyclin D2, p21^{waf1/cip1}, and p27^{kip1}). We also studied the expression of STAT3 and JAK3 in a number of primary colon cancer tumors from patients.

Materials and Methods

Colon Cancer Cell Lines, Cell Culture, and Antibodies

The human colon carcinoma cell lines used in this study SW480 and HT29 were a gift from Dr. Gary Gallick (The University of Texas MD Anderson Cancer Center). The human embryonic kidney cell line 293 was purchased from American Type Culture Collection (Manassas, VA) and the anaplastic large cell lymphoma cell line Karpas 299 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cell lines were cultured in Dulbecco's modified Eagle's medium/F-12 or RPMI (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (56°C for 30 minutes), 100 IU/ml penicillin, and 10 µg/ml streptomycin, and maintained under an atmosphere of 95% oxygen and 5% carbon dioxide in 98% humidity at 37°C. Excluding Bcl-X_L (Zymed Laboratories Inc., South San Francisco, CA), common γ chain (γc; R&D Systems, Minneapolis, MN), FLAG (Abcam, Inc., Cambridge, MA), and β-actin (Sigma, St. Louis, MO), all antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies used for the detection of STAT3 were catalogue number sc-8019; lot B0703 for both Western blot and immunohistochemistry, pSTAT3^{Tyr705}: sc-8059; lot H142 for Western blot and sc-7993-R; lot G1503 for immunohistochemistry, JAK3: sc-513; lot C3004 for both Western blot and immunohistochemistry, pJAK3^{Tyr980}: sc-16567; lot S0702 for Western blot and sc-16567-R; lot J0702 for immunohistochemistry, and γc: BAF 284 for Western blot.

Treatment of Human Colon Carcinoma Cells with AG490

AG490 (tyrphostin; Alexis Biochemicals, San Diego, CA) was reconstituted in dimethyl sulfoxide and stored at -20°C until used. It was added in the dark at 16 to 24 hours after the cells were seeded. Dimethyl sulfoxide at a concentration equivalent to the highest concentration of the inhibitor was used as a negative control. Cell viability, determined by exclusion of staining with trypan blue dye, was greater than 90% at the beginning of all experiments.

Infecting the Human Colon Carcinoma Cells with STAT3 Dominant-Negative Adenoviral Vector (AdSTAT3DN)

The characteristics of AdSTAT3DN have been previously detailed.¹⁵ Two days after seeding, the cells were infected with AdSTAT3DN suspended in Dulbecco's modified Eagle's medium at different multiplicity of infection (MOI) and incubated for 2 hours. The viral suspension was removed and the colon cancer cells were cultured. GFP adenoviral vector (AdGFP) was used as a negative control.

Cell Viability by Exclusion of Staining with Trypan Blue Dye

Control and treated colon cancer cells were stained with trypan blue dye. Briefly, the cells were seeded for 18 hours and then the medium was removed and fresh medium was added with the inhibitor. At 24 hours after treatment, the cells were stained with trypan blue dye and examined using light microscopy and a hemocytometer.

Detection of Apoptosis

Morphological Changes Using Giemsa and Hoechst 33258 Staining

Cytospin slides were stained with Giemsa stain using an automatic stainer and examined using a light microscope. In addition, cell suspensions (100 µl at a concentration of 1.0×10^6 cells/ml) were incubated with 1 µl of Hoechst 33258 (1 mg/ml in distilled water) for 10 minutes at room temperature. Subsequently, cytospin slides were prepared. After air-drying, cells were examined under a fluorescence microscope. Cells were considered to be apoptotic when the nuclei showed chromatin condensation and/or fragmentation.

Detection of Annexin V Binding to the Cell Membrane by Flow Cytometry

Detection of apoptosis was studied after staining of the cells with annexin V-fluorescein isothiocyanate and propidium iodide in accordance with the manufacturer's protocol (BD Pharmingen, San Diego, CA). Briefly, 5.0×10^5 cells were washed in ice-cold phosphate-buffered saline

(PBS) without Ca^{2+} or Mg^{2+} (Life Technologies). The cells were then suspended in 100 μl of binding buffer and incubated with 2.0 μl of annexin V-fluorescein isothiocyanate and 5.0 μl of propidium iodide for 15 minutes in the dark at room temperature. After adding 400 μl of the binding buffer, analysis was immediately performed using a flow cytometer (FACScan flow cytometer; BD, San Jose, CA).

Caspase 3 Activity Assay

The activity of caspase 3 was detected by using a commercially available kit (BD Biosciences, Palo Alto, CA). Briefly, the cells were washed in PBS free of Ca^{2+} and Mg^{2+} and lysed in cell lysis buffer. The cell lysate was centrifuged at 12,000 rpm for 3 minutes at 4°C to precipitate the cellular debris. Reaction buffer containing dithiothreitol and caspase 3/CPP32 substrate (DEVD-pNA) were added to the supernatant. The samples were analyzed in a 96-well-plate at 405 nm. Both the treated samples and negative controls were run simultaneously in triplicate.

Cell Cycle Analysis

Cell-cycle analysis was performed using a bromodeoxyuridine (BrdU) kit (BD Biosciences) and analyzed using a flow cytometer. Briefly, cells were incubated with 10 μM BrdU in cell culture medium for 1 hour at 37°C, fixed with 100 μl of Cytotix/Cytoperm buffer for 15 to 30 minutes on ice, and then treated with DNase for 1 hour at 37°C. Fluorescein isothiocyanate-labeled anti-BrdU antibody was incubated with the cells for 20 minutes at room temperature. After washing, the cells were suspended with 7-AAD and analyzed by flow cytometry.

Relative Quantification of JAK3 Gene Expression by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) in Colon Cancer Cell Lines

Total RNA was isolated from the cell lines using the RNeasy kit (Qiagen, Valencia, CA). A commercially available kit (Optimum FFPE Isolation; Ambion, Austin, TX) was used for the extraction of total RNA from formalin-fixed, paraffin-embedded tissue sections from some of the patients. DNA was removed by digestion with DNase I (Qiagen). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in a 20 μl reaction according to the manufacturer's instructions. PCR was performed in a MicroAmp Optical 96-well reaction plate (Applied Biosystems, Foster City, CA) using 1.0 μl of cDNA template, 12.5 μl of 2 \times TaqMan Master Mix (Applied Biosystems), 1.25 μl of 20 \times Assay-on-Demand gene expression product (assay ID Hs00169663_m1 for JAK3 target gene, product number 4319413E for human 18s rRNA as endogenous control, Applied Biosystems). The RT reaction was performed in triplicate wells for 2 minutes at 50°C, followed by hot-start PCR (10 minutes at

95°C) and 40 cycles of 15 seconds of denaturation at 95°C and 1 minute annealing/extension at 60°C using an ABI 7900 sequence detection system (Applied Biosystems). The relative quantification of JAK3 gene expression was calculated according to the following formula: $2^{-\Delta C_T}$ ($\Delta C_T = C_T$ of JAK3 - C_T of 18s rRNA); where C_T is the cycle threshold. As a control of specificity, the PCR products were electrophoresed on 8% polyacrylamide gel.

Western Blot and Immunoprecipitation Studies

Immunoprecipitation was used to identify possible changes in the tyrosine phosphorylation of STAT3 and JAK3 proteins after treatment with JAK3/STAT3 inhibitor. The cell lysates were incubated with primary antibody overnight at 4°C. Agarose beads conjugated with protein A/C were then added and incubated for 2 hours at 4°C. The immunocomplexes were spun down, washed five times with cold PBS and once with lysis buffer, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed using standard techniques. Briefly, the cells were lysed in lysis buffer and centrifuged at 14,000 $\times g$ for 10 minutes at 4°C. The supernatant was collected and 50 to 80 μg of protein were electrophoresed on a 6 to 12% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to nitrocellulose and were probed with various specific primary antibodies and then with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL).

Patients, Tissue Microarray, Immunohistochemical Staining, and Relative Quantitative RT-PCR of JAK3

Archival tissue material from 22 patients with stage II or IV colon cancer was included in the study after approval of the institutional ethical research committee. Tissue microarray was prepared using standard techniques from formalin-fixed, paraffin-embedded colonic samples obtained from these patients by surgical resections. The tissue microarray included normal colonic epithelium, adenoma (if available), superficial carcinoma, deep carcinoma, and metastatic lesions (stage IV) to the liver and/or the lymph nodes from each of the patients.

Histological sections from the tissue microarray as well as from cellblocks prepared from SW480 and HT29 cells lines were used for immunohistochemical staining. The sections were first deparaffinized in xylene and rehydrated using a graded series of ethanol. A three-step streptavidin-biotin-horseradish peroxidase method was used after heat-induced epitope retrieval. Briefly, endogenous peroxidase activity was blocked for 30 minutes in 3% hydrogen peroxide and subsequently, the slides were incubated with Protein Blocking Solution (DakoCy-

tomation, Carpinteria, CA) for 15 minutes. Thereafter, the slides were incubated overnight with the primary antibodies diluted in 0.1% bovine serum albumin, 50 mM Tris-HCl buffer, pH 7.6. The dilutions were 1:50 for STAT3, 1:200 for pSTAT3^{Tyr705}, and 1:500 for both JAK3 and pJAK3^{Tyr980}. Detection of the immunoreaction was achieved using the LSAB+ kit (DakoCytomation) that contains the secondary biotinylated antibody (incubation time, 20 minutes) and the streptavidin/horseradish peroxidase complex (incubation time, 20 minutes). 3,3'-Diaminobenzidine/H₂O₂ (DakoCytomation) was used as the chromogen and hematoxylin (DakoCytomation) as the counterstain. Sections prepared from formalin-fixed, paraffin-embedded Karpas 299 cells were used as positive controls. Application of mouse IgG₁ antibody (DakoCytomation) was used as a negative control to exclude unspecific cross-reactions of the primary antibodies in all experiments. Immunohistochemical staining of paraffin-embedded sections was also performed after depletion of pSTAT3 and pJAK3 antibodies with antigen-specific peptides (Santa Cruz Biotechnology). The peptide was mixed with the specific antibody at a ratio of 5:1 (weight), and incubated for 2 hours at room temperature. Thereafter, immunohistochemical staining was performed as summarized above.

In addition, we studied the relative expression of JAK3 mRNA in frozen primary colon cancer tumors from eight patients and paraffin-embedded tissue sections from two patients using relative quantitative RT-PCR as explained previously. Corresponding benign colonic tissues were also studied when available.

Results

Detection of pSTAT3, JAK3, pJAK3, and γ c in Colon Cancer Cell Lines

Immunohistochemical staining demonstrated the expression of pSTAT3 and pJAK3 in the two colon carcinoma cell lines, SW480 and HT29 (Figure 1A). The expression of pSTAT3 was primarily nuclear and pJAK3 was cytoplasmic and membranous. To confirm the specificity of the antibodies, immunohistochemical staining of the cell lines was also performed after depletion by antigen-specific peptides (Figure 1A, insets). Figure 1B shows PCR product for JAK3 using nondenaturing polyacrylamide gel electrophoresis after RT-PCR in SW480 and HT29 colon cancer cell lines. Signal bands are detected in the colon cancer cell lines as well as in Karpas 299 anaplastic large cell lymphoma cells that express high levels of JAK3. Figure 1C demonstrates quantitative RT-PCR of JAK3 mRNA in SW480 and HT29 cells. The cell lines 293 and Karpas 299 cells were used as negative and positive controls, respectively. Human 18s rRNA was used as endogenous control. The expression of JAK3 was ~3.4 times higher in Karpas 299 cells compared with HT29 and SW480 cells. Our Western blot studies also showed the expression of γ c, which is required for JAK3 signal-

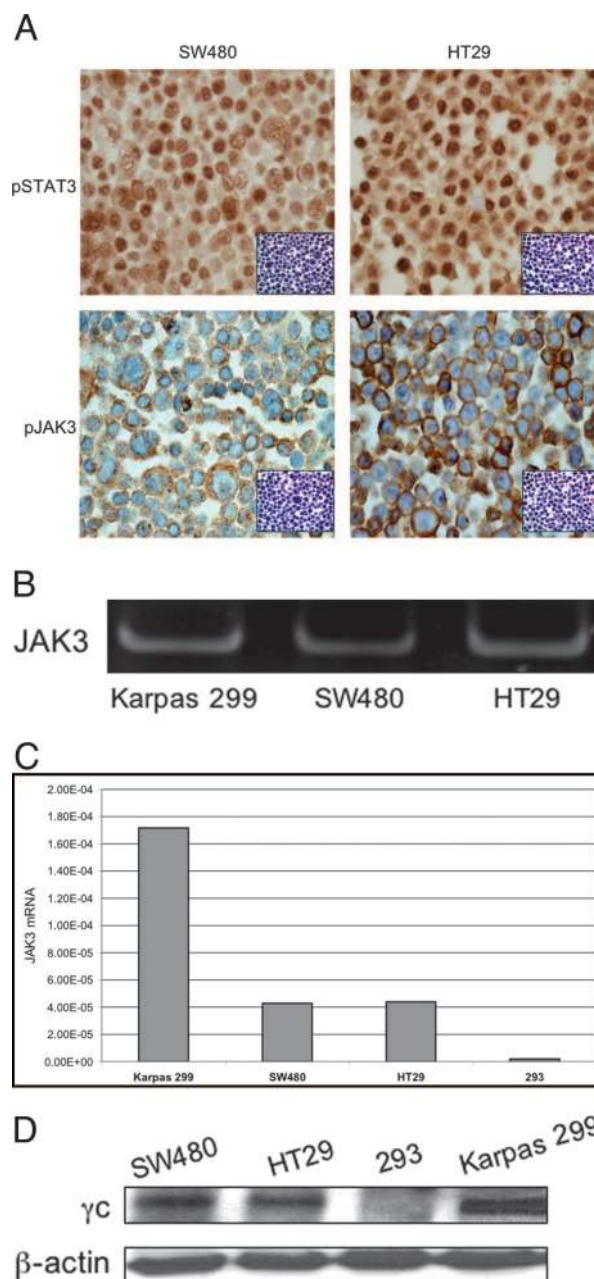


Figure 1. A: The colon cancer cell lines SW480 and HT29 are positive for pSTAT3 (predominantly nuclear staining) and pJAK3 (cytoplasmic and membranous staining) (immunohistochemical staining). To confirm the specificity of the antibodies, immunohistochemical staining using pSTAT3 and pJAK3 antibodies was also performed after depletion with antigen-specific peptides as shown in the insets. **B:** PCR product for JAK3 using nondenaturing polyacrylamide gel electrophoresis after RT-PCR in HT29 and SW480 colon cancer cell lines as well as the anaplastic large cell lymphoma cell line, Karpas 299, as positive control. **C:** Quantitative RT-PCR of JAK3 mRNA in SW480 and HT29 cells. The 293 and Karpas 299 cell lines were used as negative and positive controls, respectively. Human 18s rRNA was used as endogenous control. **D:** Western blot studies show that γ c chain, which is required for JAK3-mediated signaling, is expressed in the colon cancer cells SW480 and HT29. The cell lines 293 and Karpas 299 were used as negative and positive controls, respectively. β -Actin shows equal loading of the protein. Original magnifications, $\times 400$ (A).

ing, in SW480 and HT29 cells (Figure 1D). The cell lines 293 and Karpas 299 were used as negative and positive controls, respectively.

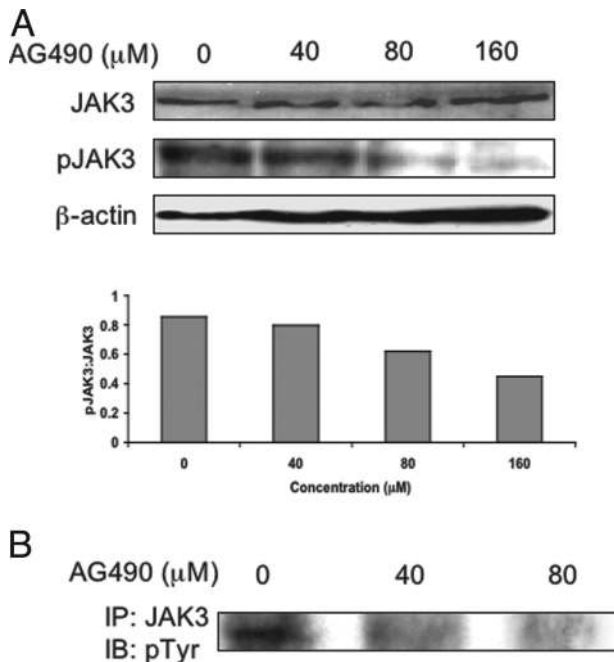


Figure 2. A, top: AG490 induced a concentration-dependent decrease in pJAK3 in SW480 cells as shown by Western blotting. pJAK3 was almost undetectable at a concentration of 160 $\mu\text{mol/L}$. There was no change in JAK3. The experiment was performed at 24 hours after treatment. β -Actin shows equal loading of the protein. **A, bottom:** Densitometric analysis of the Western blot bands showing a concentration-dependent decrease in pJAK3:JAK3 ratio. **B:** Immunoprecipitation studies confirmed that AG490 induces a concentration-dependent decrease in tyrosine-phosphorylated JAK3 in SW480 cells at 24 hours after treatment.

AG490-Induced Down-Regulation of pJAK3

Western blot studies showed a concentration-dependent decrease in the level of pJAK3 at 24 hours after treating SW480 cells with AG490 (Figure 2A, top). pJAK3 was almost undetectable at a concentration of 160 $\mu\text{mol/L}$ of AG490. No detectable changes in the JAK3 level were seen. Figure 2A (bottom) also shows densitometric analysis of the Western blot bands. There was a gradual decrease in pJAK3:JAK3 ratio with increasing the concentration of AG490. The results were confirmed by immunoprecipitation, in which JAK3 protein was pulled down with an anti-JAK3 antibody and the blots were stained with an anti-phosphorylated tyrosine antibody (Figure 2B).

AG490- or AdSTAT3DN-Induced Down-Regulation of pSTAT3

Similar to the changes in the pJAK3 level, AG490 induced concentration-dependent decreases in the pSTAT3 level in SW480 cells at 24 hours, as shown by Western blots (Figure 3A, top). AG490 (80 μM) induced nearly complete absence of pSTAT3. No changes in the STAT3 level were seen. Using densitometry, there was gradual decrease in pSTAT3:STAT3 ratio with increasing the concentration of AG490 (Figure 3A, bottom). The findings were confirmed using immunoprecipitation techniques, in which STAT3 protein was pulled down with an

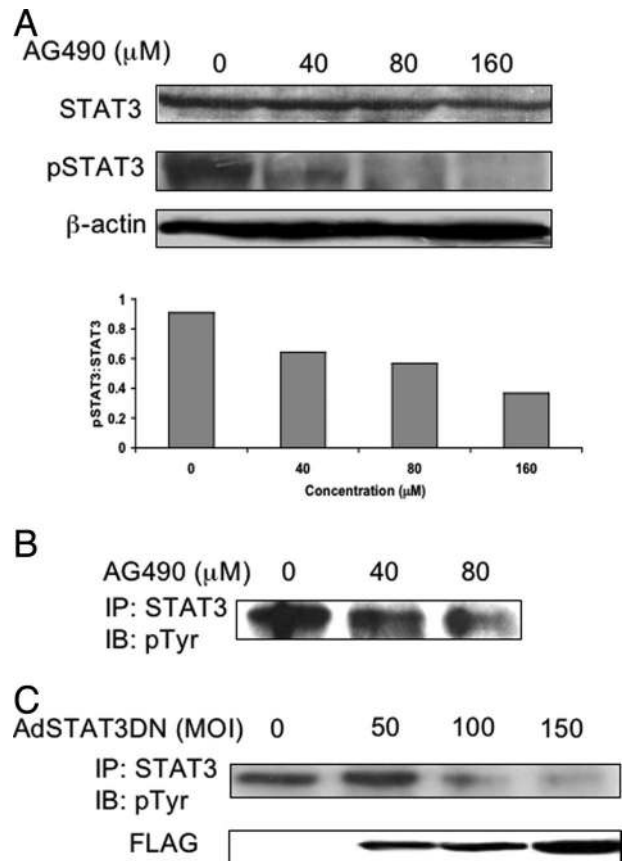


Figure 3. A, top: Western blot study showing that AG490 induces concentration-dependent down-regulation in pSTAT3, without significant changes in STAT3 levels in SW480 cells. pSTAT3 was almost undetectable at 160 μM of AG490. β -Actin shows equal loading of the protein. The experiments were performed at 24 hours after treating the cells with AG490. **A, bottom:** Densitometry of the Western blot bands demonstrating gradual decrease in pSTAT3:STAT3 with increasing the concentration of AG490. **B:** Immunoprecipitation studies confirmed that AG490 induces a concentration-dependent decrease in pSTAT3 in SW480 cells. The experiment was performed at 24 hours after treatment. **C:** AdSTAT3DN induced a concentration-dependent decrease in tyrosine-phosphorylated STAT3 as shown in SW480 cells by immunoprecipitation studies. The immunoprecipitation studies were performed at 24 hours after treating the cells with AdSTAT3DN. Infecting the cells with AdSTAT3DN was associated with a gradual increase in FLAG as shown by Western blotting.

anti-STAT3 antibody and the blots were stained with an anti-phosphorylated tyrosine antibody (Figure 3B). Furthermore, AdSTAT3DN induced concentration-dependent decreases in pSTAT3 levels in SW480 cells as shown in the immunoprecipitation studies illustrated in Figure 3C. The level of pSTAT3 was minimal at an MOI of 150. Immunoblots stained with anti-FLAG antibody revealed the presence of FLAG only in infected cells but not the negative control. The staining intensity of FLAG increased with higher MOI.

Inhibition of JAK3/STAT3 Signaling by AG490 or AdSTAT3DN Induced a Significant Decrease in the Viability of Colon Carcinoma Cells

Figure 4A shows that, after 24 hours of treatment, AG490 induced a concentration-dependent decrease in the number of viable SW480 and HT29 cells as detected by

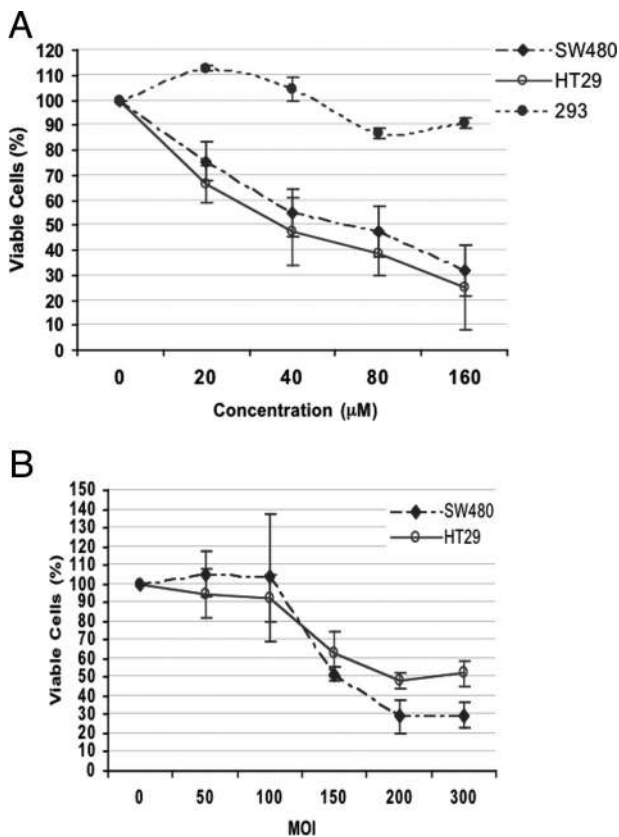


Figure 4. Treating SW480 and HT29 cells with AG490 (**top**) and AdSTAT3DN (**bottom**) induced a concentration-dependent decrease in the number of viable cells as demonstrated by the exclusion of staining with trypan blue dye. The IC₅₀ for AG490 was 40 μM in HT29 and 80 μM in SW480 cells. AG490 did not induce a significant decrease in the viability of 293 cells that lack the expression of JAK3 (**top**). The IC₅₀ for AdSTAT3DN was 150 MOI for SW480 and 200 MOI for HT29. The results represent mean ± SE of three experiments.

exclusion of staining with trypan blue dye. The IC₅₀ (ie, inhibitory concentration to induce 50% reduction of viable cells) of AG490 was 40 μM for HT29 and 80 μM for SW480 cells. In contrast, a significant decrease in cell viability was not detected in the 293 cells that are lacking JAK3 (Dr. R. Kirken, personal communication). Similarly, AdSTAT3DN induced concentration-dependent decrease in the viability of SW480 and HT29 at 48 hours with IC₅₀ of 150 and 200 MOI, respectively (Figure 4B).

Inhibition of the JAK3/STAT3 Signaling Induced Caspase 3-Dependent Apoptotic Cell Death in Colon Carcinoma Cells

To evaluate whether the decrease in cell viability might have occurred due to apoptotic cell death, we evaluated cell morphology after staining SW480 and HT29 cells with Giemsa after treatment with AdSTAT3DN at 100 MOI for 48 hours (Figure 5A, top). Cell shrinkage and nuclear condensation and fragmentation were evident in the two cell lines after treatment. AdGFP at an MOI equivalent to AdSTAT3DN did not induce significant apoptotic cell death as shown in Figure 5A (top). Figure 5A (bottom) shows efficient infection of the cells with AdGFP. Simi-

larly, staining SW480 cells with Hoechst 33258 showed typical morphological features of apoptosis including nuclear condensation and/or fragmentation at 72 hours after treatment with 80 μM of AG490 (Figure 5B).

To quantify apoptotic cell death, we performed flow cytometric studies to evaluate annexin V binding to the cell surface after treating the cells with increasing concentrations of AG490. At 24 hours after treatment, annexin binding to the surface of SW480 cells increased with increasing the concentration of AG490 (Figure 6A). Our studies show that there was a 3.5-fold increase in apoptotic SW480 cells at 80 μM of AG490. Similarly, there was a 4.5-fold increase in apoptotic HT29 cells at 80 μM of AG490 after 24 hours of treatment (data not shown). AdSTAT3DN induced similar effects. At 48 hours after treating HT29 cells there was 2.3- and 3.7-fold increase in apoptotic cell death at a MOI of 100 and 200, respectively (Figure 6B). AdGFP at a MOI equivalent to AdSTAT3DN did not induce significant apoptotic cell death in HT29 at 48 hours after treatment as shown in Figure 6C.

Increased caspase 3 activation in cells treated with increasing MOI of AdSTAT3DN was shown by enzymatic assay based on the cleavage of DEVD-*p*-nitroanilide (DEVD-pNA) peptide substrate. Figure 7A shows a gradual increase in caspase 3 activity in HT29 cells with increasing MOI of AdSTAT3DN at 16 hours after treatment. In addition, Western blot analysis demonstrated that AdSTAT3DN at an MOI of 150 induced peptide cleavage of caspase 3 in HT29 cells at 24 hours after infecting the cells (Figure 7B).

Inhibition of JAK3/STAT3 Signaling Induced G₁ Cell Cycle Arrest in Colon Carcinoma Cell Lines

Inhibition of JAK3/STAT3 signaling induced G₁ cell cycle arrest as demonstrated by the marked decrease in SW480 cells in the S phase from 25 to 7% at 6 hours after treatment with AG490 (Figure 8).

Effect of Inhibition of JAK3/STAT3 Signaling by AG490 or AdSTAT3DN on Downstream Targets

We also examined the changes in the protein levels of various apoptosis- and cell cycle-regulatory proteins that are known to be downstream targets of the JAK3/STAT3 signaling pathway. At 18 hours, increased concentrations of AG490 induced down-regulation of cyclin D2 and Bcl-2 that was simultaneously associated with up-regulation of p21^{waf1/cip1} and p27^{kip1} in SW480 cells (Figure 9A). No detectable changes at the protein level of survivin were seen (data not shown). In addition, treatment of SW480 cells with AdSTAT3DN induced concentration-dependent decreases in three major anti-apoptotic proteins, Bcl-2, Bcl-X_L, and Mcl-1, which were detected at 24 hours after infection of the cells (Figure 9B).

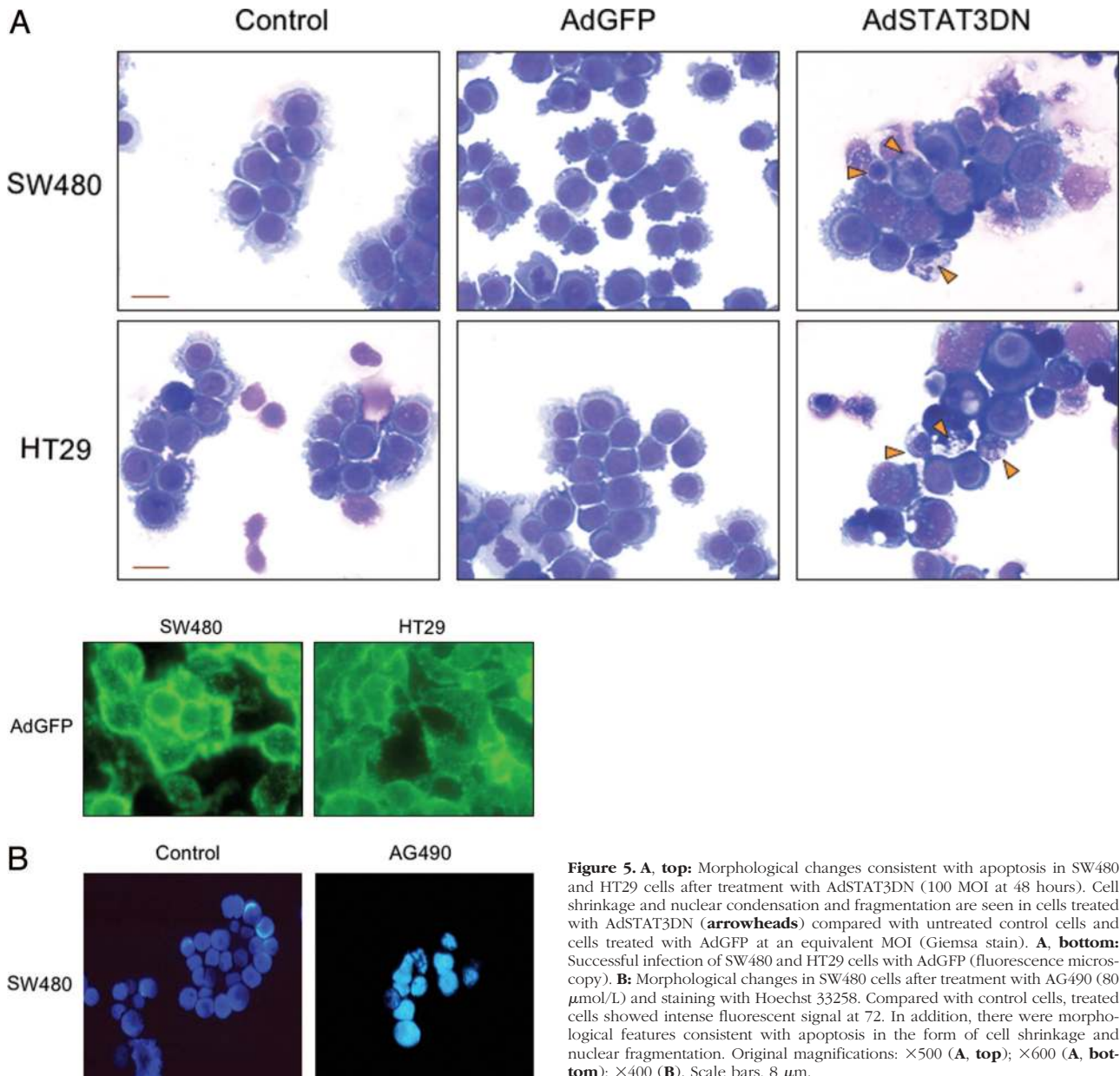


Figure 5. A, top: Morphological changes consistent with apoptosis in SW480 and HT29 cells after treatment with AdSTAT3DN (100 MOI at 48 hours). Cell shrinkage and nuclear condensation and fragmentation are seen in cells treated with AdSTAT3DN (**arrowheads**) compared with untreated control cells and cells treated with AdGFP at an equivalent MOI (Giemsa stain). **A, bottom:** Successful infection of SW480 and HT29 cells with AdGFP (fluorescence microscopy). **B:** Morphological changes in SW480 cells after treatment with AG490 (80 μ mol/L) and staining with Hoechst 33258. Compared with control cells, treated cells showed intense fluorescent signal at 72. In addition, there were morphological features consistent with apoptosis in the form of cell shrinkage and nuclear fragmentation. Original magnifications: $\times 500$ (**A, top**); $\times 600$ (**A, bottom**); $\times 400$ (**B**). Scale bars, 8 μ m.

Expression of STAT3, JAK3, and Their Activated Forms in Colon Cancer Primary Tumors

Characterizations of the patients included in the study are shown in Table 1. Eleven patients were in stage II and 11 in stage IV of the disease. The two groups were age- and sex-matched. Each of the two groups included six males and five females. The age of the patients in stage II group ranged between 54 and 74 years, with a mean \pm SE of 63.0 ± 1.8 years, whereas the age of the patients included in stage IV group ranged between 48 and 79 years, with a mean \pm SE of 64.2 ± 2.6 years.

We used standard immunohistochemical staining to explore the expression of JAK3, STAT3, and their activated forms in colon carcinoma cell lines and normal epithelium, adenomas (when available), primary tumors, and metastatic lesions (stage IV patients) from each of

the patients. Table 2 shows the frequency of expression of STAT3, pSTAT3, JAK3, and pJAK3 in colon carcinoma patients. STAT3 was the only protein present in normal colonic epithelium (80% of the samples), whereas all other proteins were undetectable. In addition, STAT3 was present in 62.5% of adenomas, 90.9% of stages II and IV, and 100% of metastatic colon carcinoma lesions. pSTAT3 was frequently expressed; 12.5% of adenomas, 72.7% of stage II, 63.6% of stage IV, and 77.7% of the metastatic lesions. JAK3 protein was detected in a significant number of the patients; 50% of adenomas, 81.8% of stage II, and 100% of stage IV and metastatic lesions. pJAK3 was present in 25% of adenomas, 81.8% of stage II, 90.9% of stage IV, and 100% of metastatic lesions. Figure 10A demonstrates examples of the patterns of expression of pSTAT3 and STAT3 (top panel) and pJAK3 and JAK3 (bottom panel). The sections are from the same

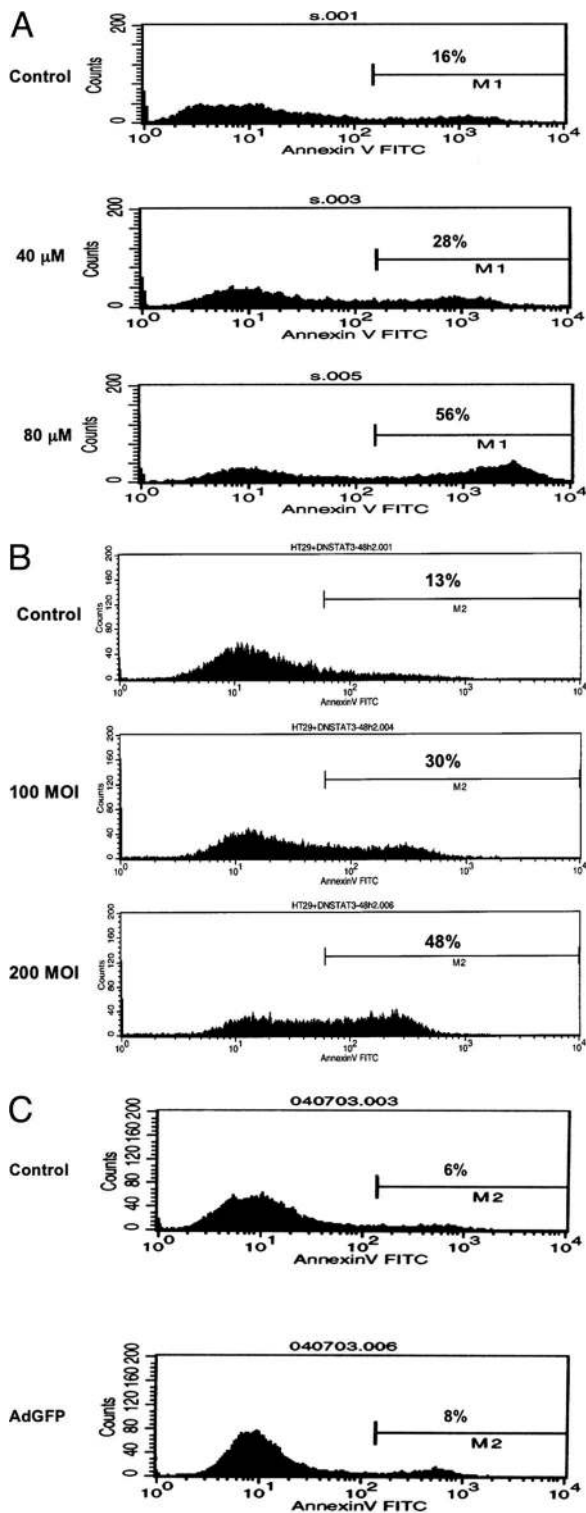


Figure 6. A: Flow cytometric analysis of SW480 cells showing that AG490 induces a concentration-dependent increase in annexin V-prestained apoptotic cells at 24 hours after treatment. Apoptotic cells increased from 16% in control cells to 28% and 56% at 40 μ M and 80 μ M respectively. The results are representative of three consistent experiments. **B:** AdSTAT3DN induced a concentration-dependent increase in apoptotic cell death in HT29 cells at 48 hours after treatment. AdSTAT3DN increased the apoptotic cells to 30% at 100 MOI and 48% at 200 MOI, compared with 13% in the untreated cells. The results are similar to two other experiments. **C:** Compared with control untreated HT29 cells, AdGFP, at a MOI equivalent to AdSTAT3DN, did not induce a significant increase in apoptotic cell death after 48 hours of treatment.

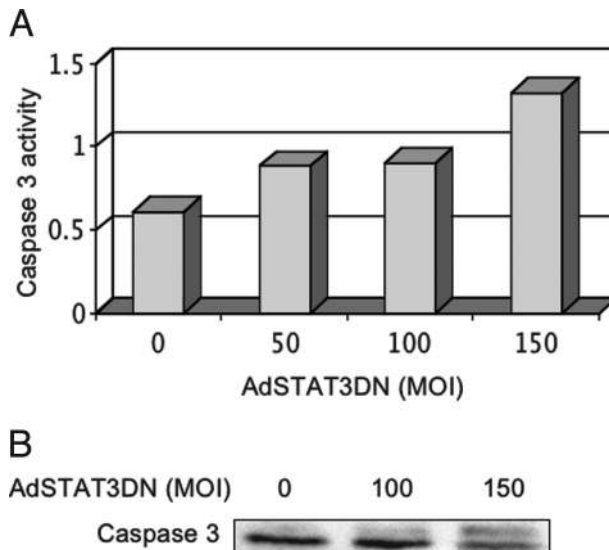


Figure 7. A: A gradual increase in caspase 3 activity in HT29 cells was detected with increasing the MOI of AdSTAT3DN. Two consistent experiments were performed at 16 hours after treating the cells. **B:** Western blot studies show cleavage of caspase 3 at 24 hours after infecting HT29 cells with AdSTAT3DN. The cleavage occurred at an MOI of 150.

patient and demonstrate normal colonic mucosa that was negative for pSTAT3 and pJAK3 compared with adenoma and colon carcinoma lesions that were positive for both pSTAT3 and pJAK3. STAT3, JAK3, and pJAK3 showed cytoplasmic staining, whereas pSTAT3 showed predominantly nuclear localization (Figure 10A).

Furthermore, we performed quantitative RT-PCR on archival tissues available from some of the patients. The studies included frozen tumors and available corresponding benign tissues from eight patients as well as from paraffin-embedded tissues from two patients after microdissection. Figure 10B shows the ratio of JAK3 mRNA in the tumors relative to its levels in the benign colonic tissues. JAK3 mRNA was detected in all of the colon cancer tumors. In five patients, the level of JAK3 mRNA was higher in the tumors compared with benign tissues, whereas in three patients JAK3 mRNA was detectable in the tumors albeit at lower levels than the benign tissues. In addition, JAK3 mRNA was present in

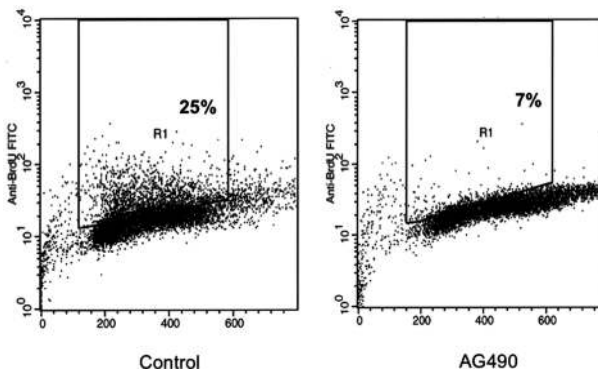


Figure 8. Treating SW480 cells with 80 μ M of AG490 induced G_1 cell-cycle arrest as demonstrated by the decrease in the cells in the S phase from 25% to only 7% at 6 hours after treatment. The experiment was performed twice with consistent findings.

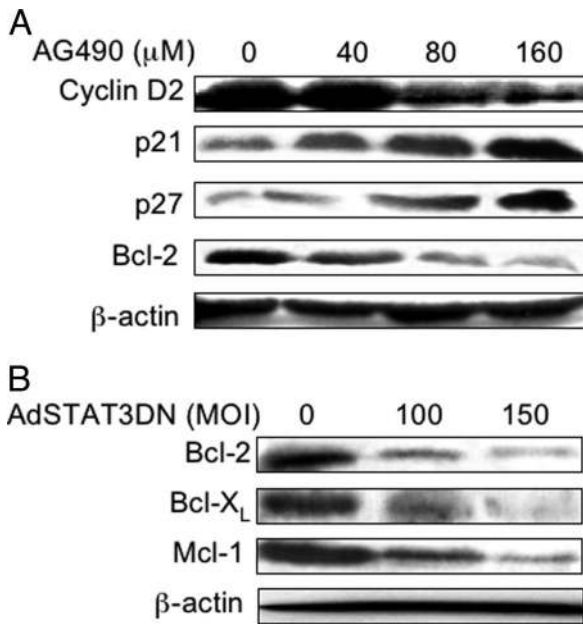


Figure 9. A: AG490 induced concentration-dependent alterations in downstream targets of JAK3/STAT3 signaling in SW480 cells at 18 hours after treatment. Western blot studies showed down-regulation of cyclin D2 and Bcl-2. Simultaneously, there was gradual increase in the cyclin-dependent kinase inhibitors p21^{waf1/cip1} and p27^{kip1}. **B:** Western blot studies showing that AdSTAT3DN induced significant down-regulation of three major anti-apoptotic proteins in HT29 cells, Bcl-2, Bcl-X_L, and Mcl-1, at 24 hours after infecting the cells.

the tumor tissue from one patient and undetectable in the corresponding benign mucosa. JAK3 mRNA was detected in another colon carcinoma tumor; however, corresponding archival benign tissue was not available from this patient.

Discussion

Whereas the pathogenetic role of JAK/STAT signaling pathway has been documented recently in several malignant neoplasms, such as carcinomas of the breast,¹⁶ prostate,¹⁷ and head and neck,¹⁸ as well as in hematopoietic neoplasms,^{19,20} the possible oncogenic role of JAK/STAT signaling pathway has not been extensively investigated in colon cancer. Recent studies have proposed that the STAT3 signaling pathway may be involved in colon carcinogenesis because the tyrosine kinases Src and Fer appear to induce their oncogenic effect in colon carcinoma cells, at least in part, via phosphorylation of STAT3.^{13,14} However, the possible role of JAK3, the physiological activator of STAT3, has not been previously investigated in human colon carcinoma cells.

Our study shows that both STAT3 and JAK3 are constitutively phosphorylated and activated in two colon cancer cell lines, SW480 and HT29. Controversy has surrounded the expression of JAK3 in epithelial tumors including colon carcinoma. Under physiological conditions, the expression of JAK3 appears to be restricted to hematopoietic and endothelial cells. Previous studies, however, showed the presence of JAK3 in lung carcinoma cell lines.^{21,22} Whereas some previous studies concluded that JAK3 is not expressed in HT29 cells,^{23,24} Lai and colleagues²⁵ demonstrated the presence of JAK3 and two spliced variants in SW480 cells using Northern blotting and RT-PCR. We demonstrated herein the presence of JAK3 and pJAK3 in HT29 and SW480 cells using four different techniques including quantitative RT-PCR, Western blotting, immunoprecipitation, and immunohistochemical staining. Furthermore, we demonstrated the expression of the common γ c that is required for JAK3 signaling, in the two cell lines, SW480 and HT29, using

Table 1. Characterization of Colon Carcinoma Patients Included in the Study

	Age	Sex	Anatomical site	Histology	Stage	Metastatic site
1	55	M	Ascending colon	AC/MD	II	
2	67	F	Colon, NOS	AC/MD	II	
3	65	M	Rectum	AC/MD	II	
4	62	M	Cecum	AC/MD	II	
5	54	M	Sigmoid colon	AC/MD	II	
6	65	F	Cecum	AC/MD	II	
7	59	M	Rectosigmoid	AC/MD	II	
8	69	M	Rectum	AC/MD	II	
9	58	F	Cecum	AC/MD	II	
10	65	F	Colon, NOS	AC/MD	II	
11	74	F	Right transverse colon	AC/MD	II	
12	58	M	Cecum	AC/MD	IV	Liver, LNs
13	59	F	Splenic flexure	AC/MD	IV	LNs
14	70	M	Sigmoid colon	AC/MD	IV	Liver
15	62	M	Cecum	AC/PD/MU	IV	LNs
16	64	M	Splenic flexure	AC/MD	IV	Gerota's fascia
17	64	F	Cecum	AC/MD	IV	LNs
18	58	M	Rectum	AC/MD	IV	LNs
19	72	M	Rectum	AC/MD	IV	LNs
20	48	F	Ascending colon	AC/MD	IV	LNs
21	72	F	Right transverse colon	AC/MD	IV	LNs
22	79	F	Cecum	AC/MD	IV	LNs

AC, adenocarcinoma; MD, moderately differentiated; NOS, not otherwise specified; LNs, lymph nodes; PD, poorly differentiated; MU, mucinous.

Table 2. Frequency of Expression of JAK3, STAT3, and Their Activated Forms

	Normal colonic epithelium	Adenoma	Stage II colon carcinoma	Stage IV colon carcinoma	Metastasis
STAT3	16/20 (80%)	5/8 (62.5%)	10/11 (90.9%)	10/11 (90.9%)	9/9 (100%)
pSTAT3	0/21 (0%)	1/8 (12.5%)	8/11 (72.7%)	7/11 (63.6%)	7/9 (77.7%)
JAK3	0/21 (0%)	4/8 (50%)	9/11 (81.8%)	11/11 (100%)	9/9 (100%)
pJAK3	0/21 (0%)	2/8 (25%)	9/11 (81.8%)	10/11 (90.9%)	9/9 (100%)

Western blotting. The presence of JAK3 and pJAK3 in colon carcinoma cells is further confirmed in our study by immunohistochemical staining of primary tumors from patients with colon carcinoma. Importantly, quantitative RT-PCR studies showed the presence of JAK3 mRNA in primary colon cancer tumors. It is of note that we did not detect JAK3 protein in benign colonic epithelium by immunohistochemistry, whereas JAK3 mRNA was detected by using quantitative RT-PCR in some of the benign tissues. This finding implicates that most likely the expression of JAK3 protein in colon carcinoma cells is inducible and associates the neoplastic transformation, as has been illustrated in previous *in vitro* studies.²⁶ The

cytokines known to induce the expression and subsequent activation of JAK3 are limited to those interacting with receptors that contain γ_c , including interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15.^{26,27} Previous studies have demonstrated the importance of some of these cytokines, particularly IL-4 and IL-15, in the pathogenesis of colon carcinoma.^{23,24,26,28,29} The frequent expression of JAK3 and pJAK3 in colon cancer tumors and metastatic lesions signifies an important role in mediating the effect of these cytokines and the initiation of colon cancer tumorigenesis. Despite that a majority of colon cancer tumors in our study demonstrated expression of pJAK3, the expression of pSTAT3 was less frequent. This finding implicates that

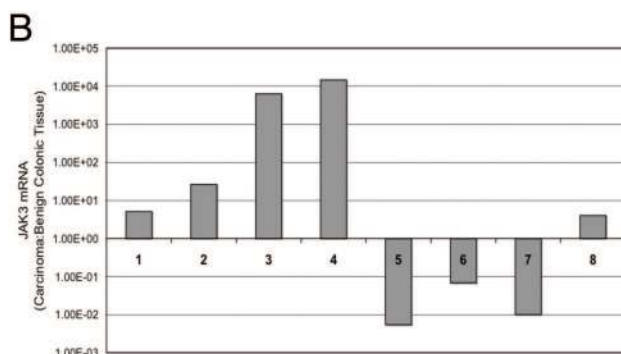
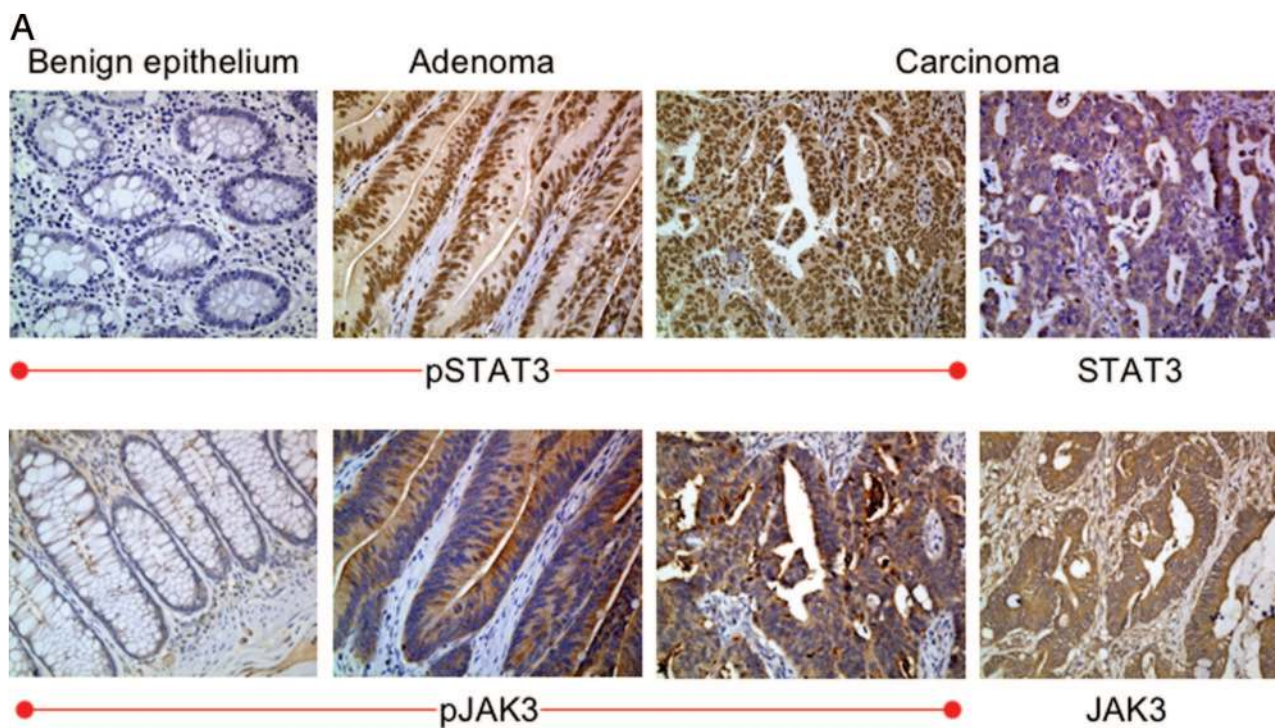


Figure 10. A: Immunohistochemical staining of formalin-fixed tissue samples from one representative patient included in the tissue microarray. pSTAT3 and pJAK3 are negative in benign colonic epithelium, whereas tubular adenoma and invasive carcinoma lesions are positive for pSTAT3 (nuclear staining) and pJAK3 (cytoplasmic and membranous staining). The inactive forms, STAT3 and JAK3, show cytoplasmic expression in the same tumor (immunohistochemical staining). **B:** The ratio of JAK3 mRNA, as detected by quantitative RT-PCR, in primary colon cancer tumors versus benign colonic tissues (logarithmic scale). All of the eight tumors demonstrated the presence of JAK3 mRNA, with five showing higher levels in the tumors compared with benign tissues. Two other tumors not included in the figure demonstrated the presence of JAK3 mRNA, however, one of these tumors did not show JAK3 mRNA in the benign colonic tissue and the other lacked corresponding archival benign tissue. Human 18s rRNA was used as endogenous control. Original magnifications, $\times 200$ (A).

pJAK3 may exert its oncogenic effects via interaction with other signal transduction pathways, for example ERK-MAP kinase pathway, or the activation of other members of STAT family of transcription proteins,^{30,31} and not only through the activation of STAT3. Importantly, pJAK3 and pSTAT3 were expressed in a subgroup of colonic adenomas and the frequency of their expression increased with the progression of the disease because 77.7% of the metastatic tumors expressed pSTAT3 and 100% expressed pJAK3. These findings imply that the expression of pSTAT3 and pJAK3 may be directly related to the progression of the disease. However, the number of patients included in our study is relatively small and further studies are needed to validate this hypothesis.

We sought to evaluate further the biological significance of JAK3/STAT3 activation in the pathogenesis of colon cancer cells. Our approach was to use a pharmacological JAK3 inhibitor, AG490 (tyrphostin).^{19,32–35} In addition, we selectively blocked STAT3 using AdSTAT3DN.¹⁵ We found that AG490 induces concentration-dependent down-regulation of pJAK3 and pSTAT3. No changes were noted in JAK3 and STAT3 proteins. Similarly, infection of colon cancer cells with AdSTAT3DN induced a concentration-dependent decrease in pSTAT3, whereas STAT3 level did not show similar changes. Down-regulation of pJAK3 and pSTAT3 was associated with a gradual decrease in the number of viable cells. The decrease in cell viability can be attributable to the significant increase in apoptotic cell death and the occurrence of cell cycle arrest after inhibition of JAK3/STAT3 signaling.

We have also investigated the molecular basis for the occurrence of apoptotic cell death and cell cycle arrest in colon cancer cells. JAK3/STAT3 signaling is known to control the transcription of key regulators of apoptosis and cell cycle progression.^{36–40} Treatment of colon cancer cells with AG490 or AdSTAT3DN resulted in a concentration-dependent decrease in cyclin D2, Mcl-1, Bcl-X_L, and Bcl-2. Concurrently, there was an increase in p21^{waf1/cip1} and p27^{kip1}.

Our findings are the first to illustrate the mechanistic role of JAK3/STAT3 signaling in colon cancer cells. They also demonstrate novel evidence that JAK3 is expressed and appears to play an important role in STAT3 phosphorylation and activation in colon carcinoma cells. Our study highlights the importance of the downstream targets of JAK3/STAT3 signaling that possess significant anti-apoptotic activity, namely Bcl-2, Bcl-X_L, and Mcl-1, in colon cancer pathogenesis. Also, our findings confirmed that several downstream targets of JAK3/STAT3 signaling that are involved in cell-cycle regulation, including cyclin D2, p21^{waf1/cip1}, and p27^{kip1}, play a major role in cell cycle regulation in colon cancer cells. Previous studies have demonstrated the significant role that these proteins play in colonic tumorigenesis.^{41–47} Survivin is another downstream target of the JAK-STAT signaling pathway that possesses significant anti-apoptotic effect.⁴⁸ Recently, Mahboubi and colleagues,⁴⁹ have shown that IL-11-induced up-regulation of survivin expression in endothelial cells is mediated via STAT3. Previous studies have demonstrated that survivin is important in the pathogen-

esis of colon cancer cells.^{50,51} The absence of significant changes in survivin levels after inhibition of JAK3/STAT3 signaling in our study may be explained by the fact that other pathways, such as Akt, are also involved in the regulation of survivin expression.^{52,53}

In conclusion, the present study demonstrates that JAK3/STAT3 signaling pathway plays an important role in the pathogenesis of human colon cancer by promoting cell survival and counteracting apoptotic cell death. Our study also illustrates that targeting JAK3/STAT3 signaling could represent a novel approach for therapy in this aggressive neoplasm.

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