

Signal Transducers and Activators of Transcription 3 Pathway Activation in Drug-Resistant Ovarian Cancer

Zhenfeng Duan,¹ Rosemary Foster,¹ Debra A. Bell,² Jennifer Mahoney,¹ Kathryn Wolak,¹ Ami Vaidya,³ Constanze Hampel,¹ Hang Lee,⁴ and Michael V. Seiden¹

Abstract Purpose: One of the major obstacles in the treatment of ovarian cancer is the development of multidrug resistance. Recent evidence shows that high-grade ovarian cancer often shows activation of the signal transducers and activators of transcription 3 (Stat3) pathway with subsequent transcription of genes that support tumor growth and survival. Less studied is the role of the Stat3 pathway in acquired drug resistance. There is no information on Stat3 expression in chemotherapy naïve ovarian cancer as compared with tumors collected later in the natural history of the disease. To further clarify the significance of Stat3 activation in ovarian cancer, here we investigated the Stat3 expression and activation in ovarian cancer and ovarian cancer multidrug resistance cell lines.

Experimental Design: Western blotting, electrophoretic mobility shift assay, luciferase assays, ELISA assay, and real-time reverse transcription-PCR determined interleukin-6 and Stat3 pathway expression and activation in cell lines. Stat3 expression in ovarian cancer tissue microarray was evaluated by immunohistochemistry.

Results: Activated (phosphorylated) Stat3 is overexpressed in most paclitaxel-resistant ovarian cancer cells. Inhibition of Stat3 activation results in significant decreases in paclitaxel resistance and enhanced apoptosis. Drug-resistant recurrent tumors have significantly greater phosphorylated Stat3 (pStat3) expression as compared with matched primary tumors. Tumors with associated inflammatory cell infiltrates also have a higher proportion of cells staining intensely for nuclear phosphorylated Stat3 as compared with tumors without inflammatory infiltrates, consistent with paracrine activation of the Stat3 pathway by immune-mediated cytokines.

Conclusions: These data support the hypothesis that interruption of Stat3 signaling could reverse resistance to paclitaxel and perhaps other chemotherapy agents in human cancer.

Ovarian cancer is the fourth most lethal cancer among women and the leading cause of gynecologic cancer deaths in the United States. The delivery of chemotherapeutic agents paclitaxel (Taxol) and carboplatin following aggressive surgical cytoreduction currently defines the standard treatment for this malignancy. Although this therapy generates tumor cytoreduction, high remission rates, and multi-year survival, cure of this malignancy remains uncommon. Most ovarian cancer survivors

eventually suffer from recurrent disease that will develop resistance to multiple types of chemotherapy. Progression of multidrug-resistant tumor will eventually lead to death. Specific mechanisms of acquired resistance *in vitro* have included overexpression of multidrug resistance gene 1 [*MDR1*; also known as *ABCB1*, which encodes the P-glycoprotein 1 (Pgp1) protein], differential expression of β -tubulin isotypes, and mutations in the β -tubulin gene (1, 2). However, the evidence linking these mechanisms to acquired drug resistance in tumors and clinically relevant drug resistance is lacking (3–5). Identifying the mechanisms that define acquired drug resistance in ovarian cancer is a high priority.

Constitutive activation of the signal transducers and activators of transcription 3 (Stat3) pathway has recently been shown to confer resistance to chemotherapy-induced apoptosis in epithelial malignancies. Stat3 can be activated by various protein tyrosine kinases including Janus tyrosine kinases (JAK) and the proto-oncogene tyrosine protein kinase Src, as well as membrane-bound growth factor receptor tyrosine kinases such as the epidermal growth factor receptor (6, 7). Several interleukin and IFN ligands and their receptors have been identified as upstream activators of receptor-associated tyrosine kinases that activate Stat3 (6, 8). Many studies have shown that epithelial ovarian cancer and several other malignancies are associated with elevated levels of epidermal

Authors' Affiliations: ¹Division of Hematology/Oncology, ²Department of Pathology, ³Division of Gynecologic Oncology, and ⁴Biostatistics Center, Massachusetts General Hospital, Boston, Massachusetts
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Requests for reprints: Zhenfeng Duan, Division of Hematology/Oncology, Massachusetts General Hospital, Boston, MA 02114. Phone: 617-724-3144; Fax: 617-724-3166; E-mail: zduan@partners.org.

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growth factor receptor, Src, and JAK2, as well as the cytokines interleukin-6 (IL-6) and oncostatin M (9–15). *In vitro* studies have implicated these various upstream pathways in drug resistance. For example, Src inhibition has been shown to sensitize ovarian cancer cells to chemotherapeutic agents such as paclitaxel and cisplatin (9, 16). Interestingly, Src inhibition can also resensitize paclitaxel-resistant cells to the cytotoxic effects of paclitaxel (9). Finally, *in vitro* studies with ovarian cancer cell lines show that generation of drug-resistant sublines is often associated with increased IL-6 mRNA expression and protein secretion (17).

Following phosphorylation, Stat3 homodimerizes, translocates to the nucleus, and induces transcription of several Stat3-dependent genes. Recent data show that aberrant Stat3 activation promotes uncontrolled tumor cell growth and survival through multiple mechanisms including increased expression of the oncogenes c-myc and cyclin D, as well as the antiapoptotic proteins Bcl-X_L, MCL-1, and survivin (6, 8). Several independent reports support the central role of the Stat3 pathway in controlling the apoptotic threshold in cancer. For example, persistent activation of Stat3 induces expression of the antiapoptotic gene *survivin* and confers resistance to apoptosis in human breast cancer cells (18). Inhibition of Stat3 signaling in myeloma cells or, alternatively, squamous cancer cells down-regulates Bcl-X_L expression and results in a dramatic induction of apoptosis (19). Studies also show that activation of Stat3 protects fibroblasts from apoptosis induced by UV light (20). Finally, recent studies have implicated Stat3 signaling in stimulating vascular endothelium associated with tumor and involves up-regulation of the vascular endothelial growth factor receptor in the tumor-associated endothelium (21, 22).

AG490, a JAK and Stat3 inhibitor, also enhances apoptosis induced by cisplatin in ovarian cancer cells (23). Likewise, dephosphorylation of Stat3 is followed by reduced expression of Bcl-2 with sensitization of tumor cells to paclitaxel both *in vitro* and *in vivo* through increased apoptosis (24). These results are consistent with recent findings showing that Stat3 mRNA expression was highest in cisplatin-resistant lung cancer cells and lowest in cisplatin-sensitive lung cancer cells (25). Similarly, phosphorylated Stat3 (pStat3) protein levels were specifically elevated in doxorubicin-resistant human neuroblastoma cells with concomitant increases in pStat3 nuclear translocation, DNA binding, and Bcl-X_L expression (26). These effects were not seen in the corresponding doxorubicin-sensitive cells. This suggests that Stat3 activation might be associated with evolving drug resistance *in vitro*.

Recently, Silver et al. (27) determined that constantly activated Stat3 levels correlate with high-grade ovarian carcinoma specimens. Gene transcription profile analysis also showed that high-grade ovarian cancers were characterized by the expression of genes associated with the Stat3-induced gene expression (28). Furthermore, small interfering RNA (siRNA)-mediated inhibition of Stat3 reduced the motility of ovarian cancer cells *in vitro*, which in turn was associated with less aggressive biological behavior (27). Less studied is the role of the Stat3 pathway in acquired drug resistance. There is no information on Stat3 expression in chemotherapy naïve ovarian cancer as compared with tumors collected later in the natural history of the disease. We have previously shown that progressive paclitaxel resistance *in vitro* is associated with increasing IL-6 expression in ovarian cancer cell lines and

showed that transfection of IL-6 (a Stat3 activating ligand) into some cell lines induced paclitaxel resistance (17, 29). In this study, we investigated Stat3 expression and activation in ovarian cancer cell lines. Studies presented here show that elevated constitutive expression of Stat3 is correlated with paclitaxel resistance in several resistant cell lines as well as in the recurrent ovarian cancer samples. More importantly, inhibition of Stat3 expression increases the sensitivity of these cell lines to paclitaxel treatment *in vitro*. In toto, these results provide support for targeting the Stat3 pathway as a strategy for reversing drug resistance.

Materials and Methods

Cell culture. The human ovarian cancer cell lines SKOV-3, SW626, NIH:OVCAR3, and Caov-3 were obtained from the American Type Tissue Collection (Rockville, MD). Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the human OV1063, IGROV1, OVCAR5, and OVCAR8 ovarian cancer cell lines. The human osteosarcoma cell line U-2OS was purchased from American Type Culture Collection. The paclitaxel-resistant SKOV-3_{TR}, OVCAR8_{TR}, SW626_{TR}, NIH:OVCAR3_{TR}, Caov-3_{TR}, OV1063_{TR}, IGROV1_{TR}, OVCAR5_{TR} and U-2OS_{TR} cell lines were established as previously reported (17, 30, 31). Briefly, the paclitaxel-resistant cell lines were selected over a period of 8 months by continuous culture in media containing stepwise increases in paclitaxel. The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Resistant cell lines were continuously cultured in paclitaxel. Paclitaxel was purchased from Bristol-Myers Squibb Co. (Princeton, NJ). All the resistant cell lines grow in the medium with varying concentrations of paclitaxel that would be lethal to the drug naïve parental lines.

Western blotting. The cells were lysed in 1× radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Charlottesville, VA) and protein concentration was determined by the DC Protein assay (Bio-Rad, Hercules, CA). Antibodies directed against Stat3, pStat3, Bcl-X_L, and MCL-1 were obtained from Cell Signaling Technologies (Cambridge, MA). Other antibodies used included the Pgp1 monoclonal antibody C219 (product ID SIG-8710, Signet, Dedham, MA) and a monoclonal antibody to human actin from Sigma-Aldrich (St. Louis, MO). Twenty-five micrograms of total protein were resolved on NuPage 4% to 12% Bis-Tris Gel (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween 20 and 5% nonfat milk (Bio-Rad) with gentle agitation overnight at 4°C as described in the instructions of the supplier. Horseradish peroxidase-conjugated secondary antibodies were incubated in TBS (pH 7.4) with 5% nonfat milk and 0.1% Tween 20 at 1:2,000 dilution for 1 hour at room temperature with gentle agitation. Positive immunoreactions were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared from 7 × 10⁶ cells with Nuclear Extract Kit from Panomics (Redwood, CA) following the instructions of the manufacturer. Stat3 binding reactions were done with the Stat3 EMSA "Gel-Shift" kit (Panomics). Briefly, 1 µg of poly(dI-dC) and 10 ng of biotin-labeled Stat3 probe were added to 5 µg of nuclear extract and incubated in binding buffer at 20°C for 30 minutes. The mixture was then separated on a nondenaturing polyacrylamide gel with subsequent transfer to a nitrocellulose membrane. After blocking, the membrane was incubated with streptavidin-horseradish peroxidase conjugate at room temperature for 15 minutes. Detection was accomplished with 1× detection buffer provided in the kit. The shifted bands corresponding to the Stat3/DNA complexes were visualized relative to the unbound double-stranded DNA after exposure of the membrane to X-ray film.

Luciferase assays. The pLucTKS3 vector (provided by Dr. Richard Jove, Lee Moffitt Cancer Center and Research Institute, Tampa, FL) contains seven copies of Stat3-binding sites in a thymidine kinase minimal promoter and its activation specifically depends on Stat3 status in the cell. The pLucTKS3 and pGL-3 (Promega) luciferase reporter plasmids were transfected into SKOV-3/SKOV-3_{TR}, OVCAR8/OVCAR8_{TR}, and U-2OS/U-2OS_{TR} cell lines, respectively, using Lipofectamine 2000 reagent. Luciferase activity was measured 48 hours posttransfection with the Promega Bright-Glo luciferase kit following the instructions of the manufacturer.

Real-time analysis of Stat3 nucleocytoplasmic translocation. On activation, Stat3 translocates from the cytoplasm to the nucleus. To study Stat3 nuclear translocation in live cells, a novel real-time cell-based assay was developed with the EGFP-Stat3 Assay System (Amersham Biosciences, Buckinghamshire, United Kingdom). This consists of transfected cells with a pCORON1000 EGFP-Stat3 vector (abbreviated as pEGFP-Stat3) that was generated by fusing Stat3 to the COOH terminus of enhanced green fluorescent protein (EGFP). The expressed EGFP fusion protein comprises a full-length functional Stat3. Detection of fluorescence can be used as a marker for Stat3 expression and localization and can thereby assess Stat3 activation in real time in the living cell. The pEGFP-Stat3 expression vector also has been transfected into the paclitaxel-sensitive ovarian cancer cell line SKOV-3 and its resistant daughter cell line SKOV-3_{TR}, as well as OVCAR8 and OVCAR8_{TR}. Subcellular location of EGFP-Stat3 was assessed by fluorescence microscopy.

Stat3 RNA interference assay. The Stat3 siRNA/siAB Assay kit (Dharmacon, Inc., Chicago, IL) with Dharmacon SMART technology was used according to the instructions of the manufacturer. For transfection, cells were either plated on 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays or plated on dishes for Western blot protein detection. Transfections were done with Lipofectamine reagent as directed by the manufacturer. For Stat3 inhibition, the final concentration of siRNA was 100 nmol/L. Medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum 24 hours after transfection. Total protein was isolated after 48 hours of Stat3 siRNA transfection.

Cytotoxicity assay. *In vitro* cytotoxicity was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (32). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma-Aldrich. Briefly, 2×10^3 cells per well were plated in 96-well plates. Cells were plated in RPMI 1640 containing increasing concentrations of paclitaxel. After 7 days of culture in paclitaxel, 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in PBS) were added to each well and the plates were incubated for 4 hours. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a BT 2000 Microkinetics Reader. The IC₅₀ was defined as the paclitaxel concentration required to decrease the A_{490} to 50% of the control (no paclitaxel) value. The absorbance values were normalized assigning the value of the parent line in medium without drug to 1.0 and the value of the no-cell control to 0. Experiments were done in duplicate.

Apoptosis assay. Apoptosis was evaluated using the M30-Apoptosense Elisa assay kit (Peviva AB, Bromma, Sweden; ref. 33). OVCAR8 or OVCAR8_{TR} cells were seeded at 8,000 per well in a 96-well plate for 24 hours before the addition of paclitaxel and AG490 (Calbiochem, La Jolla, CA). The cells were then treated with 0.1 μ mol/L paclitaxel, 30 μ mol/L AG490, or a combination of the two drugs. Twenty-four hours later, the cells were lysed by adding 10 μ L of 10% NP40 per well, then following the instructions of the manufacturer for apoptosis assay.

IL-6 ELISA assay and real-time reverse transcription-PCR for IL-6 receptor. IL-6 levels in culture supernatants were measured using quantitative ELISA kits (R&D Systems, Minneapolis, MN) as previously described (17). The absorbance of each well was read with a BT 2000 Microkinetics Reader (Bio-Tek Instrument, Inc., Winooski, VT) at 450 nm. A standard curve was constructed to quantitate the cytokine concentrations in the controls and samples. Total RNA was extracted

with Trizol Reagent (Invitrogen) and quantified with a spectrophotometer. Real-time quantitative PCR for the α -chain of the IL-6 receptor (IL-6R) was done with the Mx300P Real-time PCR System (Stratagene, La Jolla, CA) using BD QZyme real-time quantitative PCR assay reagents (BD Biosciences Clontech, Palo Alto, CA). Primers and probes were designed and validated by BD Biosciences Clontech. IL-6R mRNA expression was quantified in comparison with human β -actin mRNA. All assays were done in triplicate.

Evaluation of ovarian cancer cell line response to IL-6. To confirm IL-6 regulation of pStat3 protein expression, ovarian cancer cell lines were cultured for 24 hours in the absence of IL-6. Recombinant human IL-6 (R&D Systems) was used to treat different ovarian cancer cells. The cells were cultured in the presence of exogenous IL-6 (30 ng/mL) for 1 hour. After the treatment period, the cells were harvested, washed, lysed in 1 \times radioimmunoprecipitation assay lysis buffer, and pStat3 protein levels were determined by Western blotting as described above.

Ovarian cancer tissue microarray slides and immunohistochemistry. We used two different human ovarian cancer tissue microarrays for this study. The first microarray was composed of 94 epithelial ovarian cancers and included duplicate core biopsies (0.5 mm in diameter) from 30 serous, 18 endometrioid, 28 clear cell carcinoma, 9 transitional clear cell, and 9 mucinous formalin-fixed, paraffin-embedded tumors. The second human ovarian cancer tissue microarray was generated by the Tissue Microarray Core at the Dana-Farber/Harvard Cancer Center using matched sample sets obtained from 26 individual ovarian cancer patients. Each set was composed of the primary ovarian tumor, a metastatic tumor obtained at the time of the primary surgery, and a recurrent tumor. Three core biopsies (0.5 mm in diameter) were taken from histologically identified representative regions of each formalin-fixed, paraffin-embedded tumor. Slides of 5- μ m sections of the relevant arrays were baked at 60°C for 1 hour, deparaffinized in xylene for 10 minutes, transferred through 100% ethanol for 5 minutes, and then rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by a 10-minute incubation in 3% hydrogen peroxide in methanol. Antigen retrieval was processed with Target Retrieval Solution (Vector Laboratories, Burlingame, CA) following the instruction of the manufacturer. After antigen retrieval, the slides were washed with PBS (parathyroid hormone, 7.5) thrice at room temperature. Protein blocking was done by incubating the slides in 5% normal goat serum and 1% bovine serum albumin in PBS for 1 hour. Primary antibody was applied at 4°C overnight (1:100 dilution of total Stat3 and pStat3 antibody; Cell Signaling Technologies) in 1% bovine serum albumin with 5% normal goat serum. After 2-minute rinses in PBS thrice, bound antibody was detected with the Vectastain ABC kit (Vector Laboratories) and visualized with 3,3'-diaminobenzidine high-sensitivity substrate from Vector Laboratories. Finally, the slides were counterstained with hematoxylin QS (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories) for long-term preservation.

Evaluation of immunohistochemical staining and statistical analysis. Immunostained slides were scored under a microscope. For total Stat3 and Pgp, the staining intensity pattern was scored as follows: 1+, weak staining; 2+, moderate staining; and 3+, intense staining. Most of the ovarian cancer tissues showed staining in >60% of area. For specimens that were not interpretable or were missing most of the cancer cells, a score of not applicable (N/A) was given. For pStat3, the percentage of cells showing positive nuclear staining for pStat3 was calculated by reviewing the entire spot. On the basis of the percentage of cells with positive nuclear staining, the staining patterns were categorized into six groups: 0, no nuclear staining; 1+, <10% of cells stained positive; 2+, 10% to 25% positive cells; 3+, 26% to 50% positive cells; 4+, 51% to 75% positive cells; 5+, >75% positive cells. In addition, the inflammatory cell infiltrates in the tumor tissues were classified as negative (–) or positive (+). Descriptive analysis of the observed distribution of Stat3 intensity pattern and pStat3 showed that all the Stat3 intensity score outcomes were moderate (2+) to intense (3+), and pStat3 scores ranged from 0 to 5+. The statistical analysis of

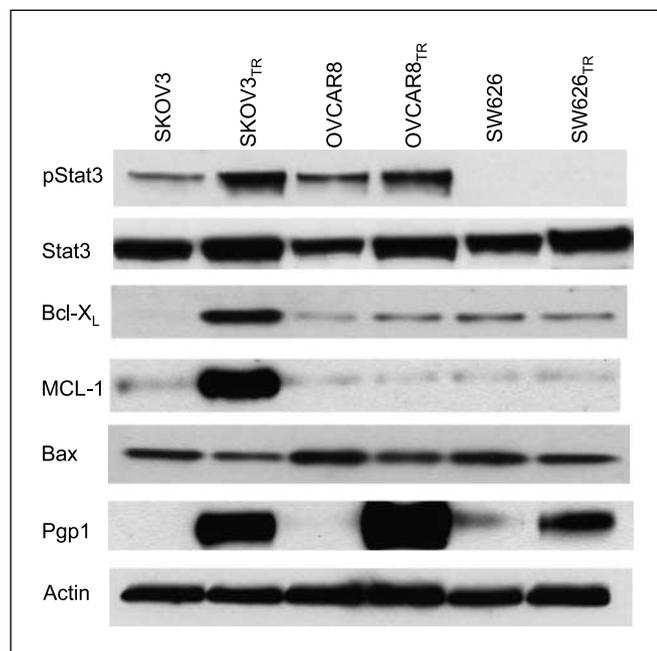


Fig. 1. Western blot analysis of Stat3, Stat3 target proteins, and Pgp1 in paclitaxel-sensitive and paclitaxel-resistant cell lines. Expression of Stat3, pStat3, and Pgp1 was assessed with total cellular protein isolated from the indicated cell lines and immunoblotted with specific antibodies as described in Materials and Methods. The blots were also probed with an anti-actin monoclonal antibody to assess relative protein levels in the sample lanes.

Stat3 was done to compare the proportions of 2+ and 3+. For pStat3 analysis, a comparison of mean scores was done. Both analyses were carried out with the Generalized Estimating Equations (34, 35) implemented in SAS proc GENMOD (36) to handle the correlated outcomes arising from the matched triplicates. The link function of the Generalized Estimating Equations to compare Stat3 intensity patterns among primary, metastasis, and recurrent tumors was logit (i.e., logistic regression of longitudinally correlated dichotomous outcomes), and that of pStat3 intensity scores among the three tumor type triplicates and within the primary tumor between with and without inflammatory cell infiltrates was identity link function. $P < 0.05$ was considered significant.

Results

Analysis of pStat3 expression and activation in paclitaxel-resistant cell lines. To determine the relationship between pStat3 expression and activation and multidrug resistance in ovarian cancer, we first analyzed pStat3 expression in several paired cell lines. Western blot analysis determined that pStat3 was overexpressed in the paclitaxel-resistant cell lines SKOV-3_{TR} and OVCAR8_{TR}, but not in SW626_{TR} (Fig. 1). In total, we found pStat3 was overexpressed in 6 of 9 (67%) resistant cell lines (Table 1). To confirm that increased pStat3 levels correlated with increased Stat3 activation and trafficking into the nucleus, we did electrophoretic mobility shift assay analysis with three pairs of ovarian cancer cell lines. As shown in Fig. 2A, increased Stat3 DNA-binding activity was present in nuclear extracts prepared from SKOV-3_{TR} and OVCAR8_{TR}, but not in similar extracts from SW626_{TR}. The electrophoretic mobility shift assay data are consistent with the Western blot results and correlate with the high level of activated pStat3 detected in SKOV-3_{TR} and OVCAR8_{TR}, but not in SW626_{TR}. To specifically analyze

pStat3 activity, we transfected three independent pairs of paclitaxel-resistant and paclitaxel-sensitive cancer cell lines with the Stat3-dependent luciferase reporter construct pLucTKS3. This vector contains multidimerized Stat3-specific binding sites upstream of a luciferase reporter gene. Analysis of relative luciferase expression in each cell line confirmed higher Stat3-dependent transcriptional activity in the paclitaxel-resistant cell lines (Fig. 2B). Additionally, Stat3 was preferentially localized to the nucleus in resistant cell lines with increased pStat3 levels (Fig. 2C). These studies support the hypothesis that the majority of paclitaxel-resistant ovarian cancer cell lines show increased activation of the JAK-Stat3 pathway even without the addition of exogenous IL-6.

Reversal of paclitaxel resistance using Stat3 siRNA in vitro. Based on the observation that the majority of drug-resistant cell lines have elevated levels of pStat3, we examined whether disruption of the Stat3 pathway would also increase paclitaxel sensitivity in ovarian cancer. The paclitaxel-resistant cell line OVCAR8_{TR} or SKOV-3_{TR} was transfected with Stat3 targeted siRNAs that resulted in a significant knockdown of Stat3 levels (Fig. 3A). This approach showed a 5- to 10-fold reduction in paclitaxel resistance as determined by fold-change in the LD₅₀. This effect was not seen when cells were transfected with a nonspecific siRNA control (Fig. 3B and C).

Effects of inhibition of Stat3 activity on paclitaxel-induced apoptosis. The induction of apoptosis is believed to be the principal final step of chemotherapy associated cytotoxicity and failure to activate the intrinsic apoptotic program is a newly recognized mechanism of drug resistance (37, 38). As stated above, the constitutive activation of Stat3 may contribute to the survival advantage of ovarian cancer cells and provide at least a partial explanation for acquired drug resistance to conventional chemotherapy in ovarian cancer. To examine whether interruption of the Stat3 pathway could induce apoptosis when combined with chemotherapy, we analyzed the apoptotic effect

Table 1. Comparison of IL-6, pStat3, and *IL-6R* expression in parent and multidrug-resistant cancer cell lines

Cell line	IL-6 (pg/mL)	pStat3	<i>IL-6R</i>
SKOV-3/SKOV-3 _{TR}	35/2,100*	↑ [†]	+ [‡]
Caov-3/Caov-3 _{TR}	400/1,400	↑	N/M
OV1063/OV1063 _{TR}	820/980	↑	N/M
OVCAR5/OVCAR5 _{TR}	400/360	↑	+
OVCAR8/OVCAR8 _{TR}	360/380	↑	+
SW626/SW626 _{TR}	6/150	-	+
IGROV-1/IGROV-1 _{TR}	6/70	-	N/M
U-2OS/U-2OS _{TR}	12/50	↑	+
A2780/A2780CP70	3/3	-	N/M

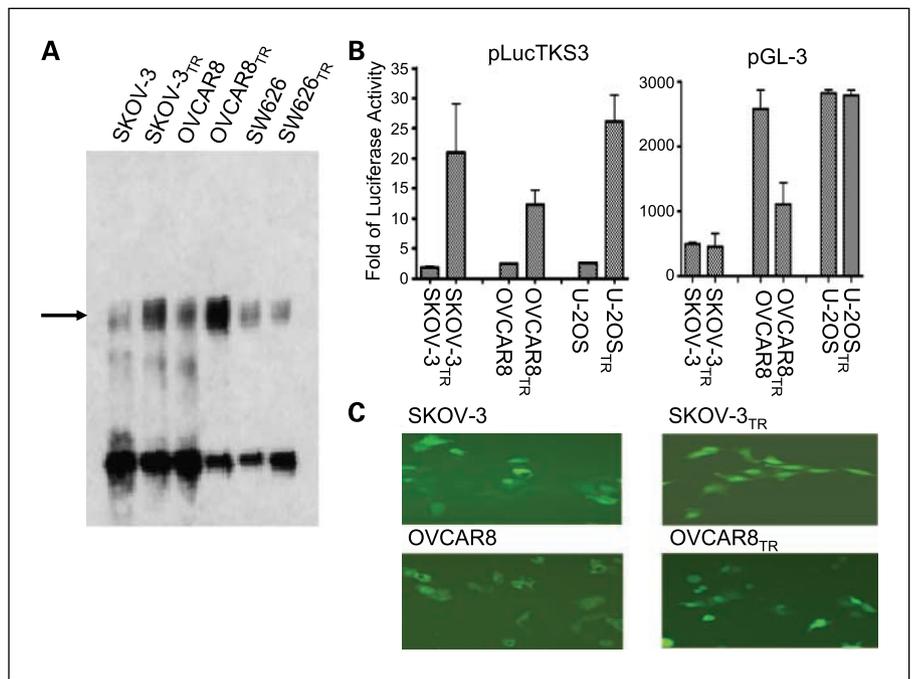
*Culture supernatants were collected and IL-6 levels were analyzed by ELISA. Data represent the average value of triplicate experiments.

[†]An upward arrow indicates the expression was higher in drug-resistant cell lines as compared with sensitive cell lines. "-" indicates expression were nearly equal in drug-resistant cell lines as compared with sensitive cell lines. pStat3 expression was determined by Western blot analysis.

[‡]"++" indicates positive expression of *IL-6R*. "N/M" indicates not measured. *IL-6R* expression was determined by quantitative reverse transcription-PCR.

Fig. 2. Comparison of Stat3 activity in paclitaxel-sensitive and paclitaxel-resistant cell lines.

A, electrophoretic mobility shift assay of Stat3 DNA binding activity in multidrug-sensitive and multidrug-resistant cell lines. The formation of Stat3-DNA complexes was determined by incubating the biotin-labeled Stat3 probe with different nuclear extract proteins. The mixture was then separated on a nondenaturing polyacrylamide gel and the shifted bands (*arrow*) corresponding to the pStat3/DNA complexes were visualized relative to the unbound double-stranded DNA. **B**, Stat3-dependent transcriptional activation in paclitaxel-sensitive and paclitaxel-resistant cell lines. The Stat3-dependent luciferase reporter construct pLucTKS3 or the luciferase reporter control construct pGL-3 was transfected into the indicated cell lines and the cells were harvested for luciferase activity analysis 48 hours posttransfection. **C**, preferential nuclear localization of Stat3 in multidrug-resistant lines. The multidrug-resistant (*TR*) and multidrug-sensitive cell lines were transfected with the pEGFP-Stat3 expression vector. Subcellular localization of the EGFP-Stat3 fusion protein was examined by fluorescence microscopy 24 hours posttransfection.



of the JAK and Stat3 inhibitor AG490 in the presence or absence of paclitaxel. Treatment of OVCAR8 and OVCAR8^{TR} cells *in vitro* with paclitaxel, AG490, or a combination of both drugs resulted in apoptosis by 24 hours with the combination of the two agents resulting in significantly greater cell death than either drug alone (Fig. 3D). Similar results have been obtained using AG490 or other novel Stat3 inhibitor knockdown of Stat3 in the breast cancer MDA-MB-468 cell line with increased pStat3.⁵ Furthermore, Stat3 inhibition increased paclitaxel-induced apoptosis even in the paclitaxel-resistant cell line (Fig. 3D).

Analysis of IL-6 and Pgp1 expression in drug-resistant cell lines. Our previous studies showed that IL-6 is preferentially overexpressed in a subset of paclitaxel-resistant human cancer cell lines as compared with the drug naïve parental cell lines (17). In this study, we extended those studies to several more pairs of cell lines. We examined 18 cell lines (9 pairs of drug-sensitive and drug-resistant lines) including 7 paclitaxel-sensitive/paclitaxel-resistant ovarian cancer cell line pairs and one cisplatin-sensitive/cisplatin-resistant ovarian cancer cell line pair. This evaluation shows that IL-6 secretion increased in 6 of 9 (67%) resistant cell lines as compared with their drug-sensitive parental lines. Significantly, high levels of IL-6 (>200 pg/mL) correlate with the increased expression of pStat3 in the multidrug-resistant cell lines (Table 1). Evaluation of IL-6R α -chain levels in four of these lines by quantitative reverse transcription-PCR analysis showed that all lines were positive for the receptor with a constant IL-6R/actin ratio, suggesting that this receptor is expressed in ovarian cancer cell lines and its relative expression is not changed in the drug-resistant cell lines (Table 1).

IL-6 mediated up-regulation of pStat3 expression in ovarian cancer cell lines. Stat3 is a major downstream component of the IL-6-JAK signaling pathway. To examine IL-6 regulation of

pStat3 protein expression, we evaluated the effect of IL-6 on the levels of pStat3 in several ovarian cancer cell lines. We found that IL-6 induced phosphorylation of Stat3 in several, but not all, of the examined cell lines (Fig. 4). The lack of enhanced Stat3 phosphorylation in a subset of the cell lines may be due to suboptimization of IL-6 dose, absence of an intact IL-6R-JAK-Stat3 axis, or saturation of the IL-6R from autocrine secretion of IL-6, thereby limiting augmentation of pathway activation through the addition of exogenous IL-6. Furthermore, it is possible that Stat3 could be activated through IL-6-independent mechanisms such as Src, epidermal growth factor receptor, or other cytokines like oncostatin in different cancer cells (6–9, 12, 18).

Stat3 and pStat3 expressions in different histologic subtypes of ovarian cancer. High levels of pStat3 have been found to correlate with high-grade tumors and thus aggressive clinical behavior (27, 28). However, none of these studies have evaluated the association of pStat3 and Stat3 expression and different histologic subtypes of epithelial ovarian cancer or ovarian tumors over their natural history. Using pStat3- and Stat3-specific antibodies, tissue microarrays containing clear cell carcinoma, transitional clear cell, mucinous, serous, and endometrioid tumors were analyzed (Fig. 5A and B). All tumor specimens from a variety of histologic subtypes showed at least modest Stat3 expression. Similar ranges of pStat3 expression were observed in clear cell, transitional cell, serous, and endometrioid tumors. Because this was an exploratory analysis without a prior hypothesis, it was not subjected to formal statistical analysis although it seems that mucinous tumors have less pStat3 expression than the other histologic subtypes of epithelial ovarian cancer (Fig. 5B).

Increased expression of pStat3 and inflammatory infiltration in recurrent and metastatic ovarian cancer tissues. We next examined total Stat3, pStat3, and Pgp1 expression in primary, metastatic, and recurrent ovarian cancer tissues. This analysis was done in a tumor tissue microarray that included the

⁵ Unpublished data.

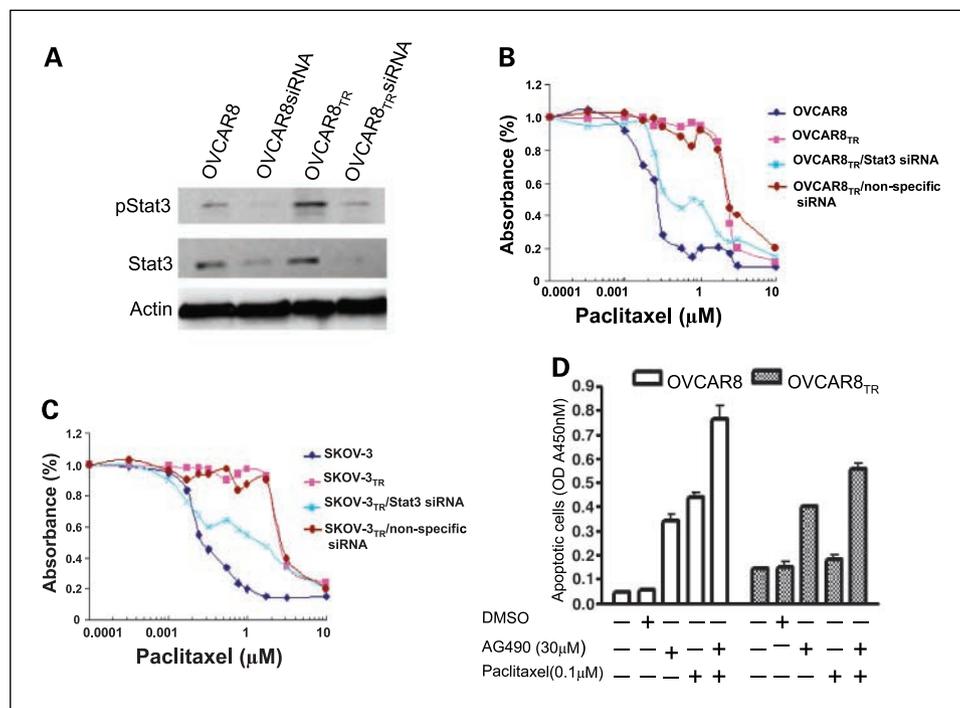


Fig. 3. Effect of Stat3 inhibition on paclitaxel sensitivity and apoptosis in ovarian cancer cells. **A**, siRNA-mediated inhibition of Stat3 expression in human cancer cell lines. Cells were transfected with Stat3 SMARTpool siRNA. Total protein was isolated 48 hours posttransfection and Stat3 expression was analyzed by Western blotting with anti-pStat3 (*top*) or anti-Stat3 (*middle*) antibodies. **B** and **C**, reversal of paclitaxel resistance by Stat3 siRNA. The paclitaxel-resistant ovarian cancer cell line OVCAR8_{TR} (**B**) or SKOV-3_{TR} (**C**) was transfected with either SMARTpooling Stat3 siRNA or a nonspecific siRNA. The relative sensitivity of each line to paclitaxel was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis 96 hours posttransfection. **D**, inhibition of Stat3 activity enhances paclitaxel-induced apoptosis. OVCAR8 or OVCAR8_{TR} cells were seeded and treated with the indicated concentrations of paclitaxel and AG490 for 24 hours followed by the apoptosis assay as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD.

primary tumor, a synchronous metastasis, and a metachronous metastasis from the same patient with the metachronous metastasis being collected at the time of tumor recurrence after treatment with platinum- and taxane-based chemotherapy. Immunohistochemical analyses determined that all tumors present on the microarray had moderate to strong staining for total Stat3 and >60% of ovarian tumors had strong pStat3 staining in the nucleus (Fig. 6A and B). The relative levels of pStat3 nuclear staining in the tumor sample sets from 26 individual patients were scored from no detectable nuclear pStat3 staining (0+) to >75% of tumor nuclei positive for pStat3 (5+). There were trends towards greater Stat3 ($P < 0.038$) and pStat3 ($P < 0.015$) expression in the recurrent tumors as compared with the matched primary tumors (Fig. 6B and C). There was also an increase in the intensity of pStat3 expression in the recurrent metastatic lesion as compared with the primary metastasis ($P < 0.012$). Furthermore, there were no differences in the intensity of Pgp1 staining between primary, metastatic, and recurrent tumors as previously reported (Fig. 6C).

Intensity of pStat3 nuclear staining was then compared with the relative levels of inflammatory infiltrates in each sample. Fifteen of 128 primary tumors show brisk inflammatory infiltrate. All of these tumors show at least 3+ and, most commonly, 5+ pStat3 staining with a mean score of 4.1, which is greater than the mean pStat3 scores in tumors without inflammatory infiltrates ($P < 0.0001$; Fig. 7A and B).

Discussion

Several recent studies have addressed the role of Stat3 in tumor cell growth and apoptosis, including in ovarian cancer specimen. However, the role of Stat3 expression and activation in the acquisition of the multidrug resistance phenotype in ovarian cancer has not been investigated. In this study, we provide evidence that supports the hypothesis that Stat3

expression and activation may promote multidrug resistance and apoptosis resistance in a large subset of ovarian cancers.

Our analyses of the Stat3 pathway show that Stat3 is often overexpressed and activated in many paclitaxel-resistant ovarian cancer cells as compared with paired parental cell lines that are paclitaxel naïve. These findings were further supported by results showing that, in several multidrug-resistant cell lines, there is evidence of greater nuclear levels of activated Stat3 as determined by both electrophoretic mobility shift assay and Stat3-dependent luciferase reporter assay. These results are consistent with recent findings showing that mRNA expression of Stat3 was highest in cisplatin-resistant lung cancer cells and lowest in cisplatin-sensitive lung cancer cells (25). Similar findings are also seen in doxorubicin-resistant human neuroblastoma cells (26).

Our results support the hypothesis that development of paclitaxel resistance *in vitro* is accompanied in many, but not all, cases with increased expression of pStat3 and downstream

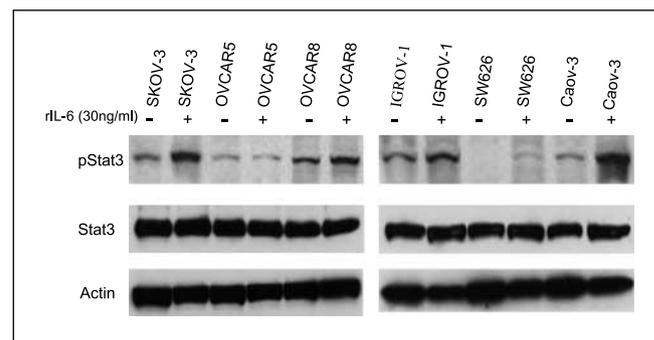


Fig. 4. IL-6 dependent up-regulation of pStat3 expression in ovarian cancer cell lines. Drug-sensitive ovarian cancer cells were treated with 30 ng/mL IL-6 for 1 hour as indicated. Cell lysates were subjected to Western blot analysis with anti-pStat3, anti-Stat3, or anti- β -actin antibodies.

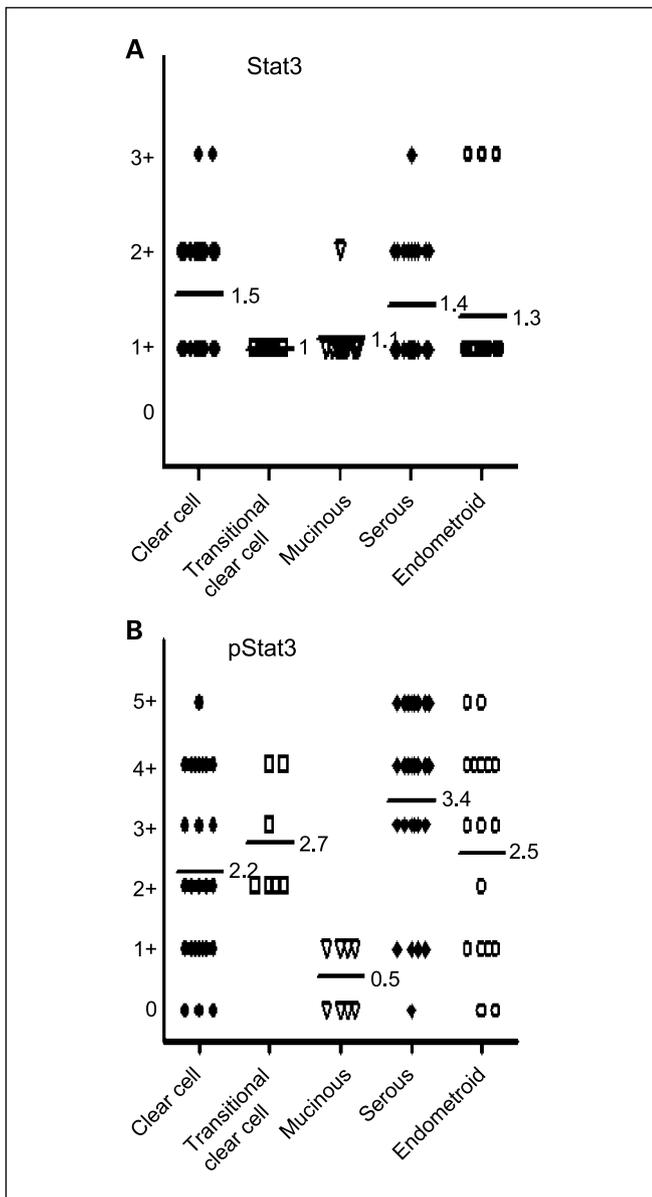


Fig. 5. Distribution of pStat3 and Stat3 immunohistochemical staining scores in the different histologic types of ovarian cancer. The graph depicts the distribution of samples according to the staining intensities of Stat3 (A) and pStat3 (B) expression as scored by percent of cells with strong nuclear staining.

activation of Stat3-dependent genes. Pathway activation is likely multifactorial. In many tumors and cell lines, this may be due to increased expression of IL-6 through either autocrine or paracrine signaling via the IL-6R, which seems to be widely expressed in ovarian cancer cell lines. There is currently no evidence that increased receptor activation is due to changes in receptor copy number or receptor mutation although the latter possibility has not been formally excluded. Interestingly, we have also shown that many of these drug-resistant lines overexpress *MDR1* (Pgp1) as compared with the drug-sensitive parental lines. Curiously, we and others have shown that whereas baseline *MDR1* expression may define, at least in part, intrinsic drug resistance in tumors, the acquisition of additional drug resistance in tumors (as compared with cell lines) is not

associated with increased expression of the *MDR1* protein Pgp1 (3, 4, 30, 31).

There is a strong correlation between activation of Stat3 in tumors and the presence of a brisk intratumoral inflammatory cell infiltrate (Fig. 7A and B) providing support for the hypothesis that, in a subset of ovarian cancers, activation of the Stat3 pathway may be via paracrine mechanisms (i.e., from the intratumoral production of IL-6 and perhaps other cytokines by inflammatory cells in the tumor microenvironment). In other cases, the activation of the Stat3 pathway is seen in the absence of inflammation, suggesting either autocrine activation, perhaps by the tumor cell production of IL-6, or,

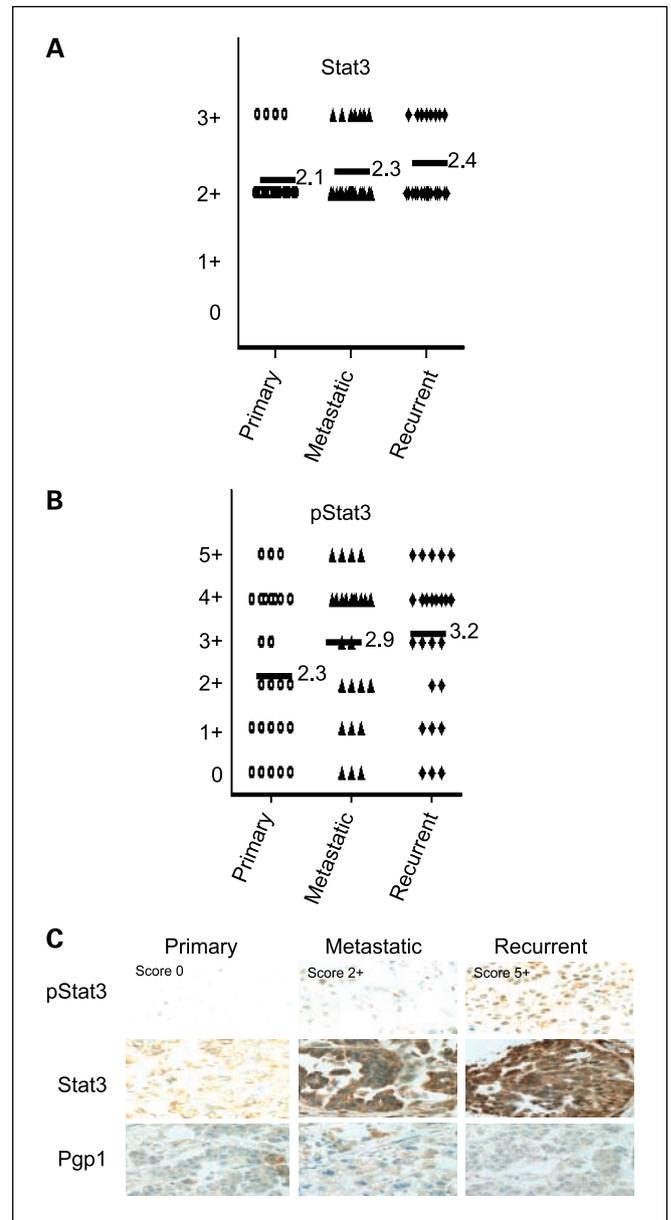


Fig. 6. Comparison of pStat3 and Stat3 expression in primary, metastatic, and recurrent ovarian cancer. A, distribution of Stat3 immunohistochemical staining scores in primary, metastatic, and recurrent ovarian cancer. B, distribution of pStat3 immunohistochemical staining scores in primary, metastatic, and recurrent ovarian cancer. C, representative expression of pStat3, Stat3, and Pgp1 in matched primary, metastatic, and recurrent ovarian cancers.

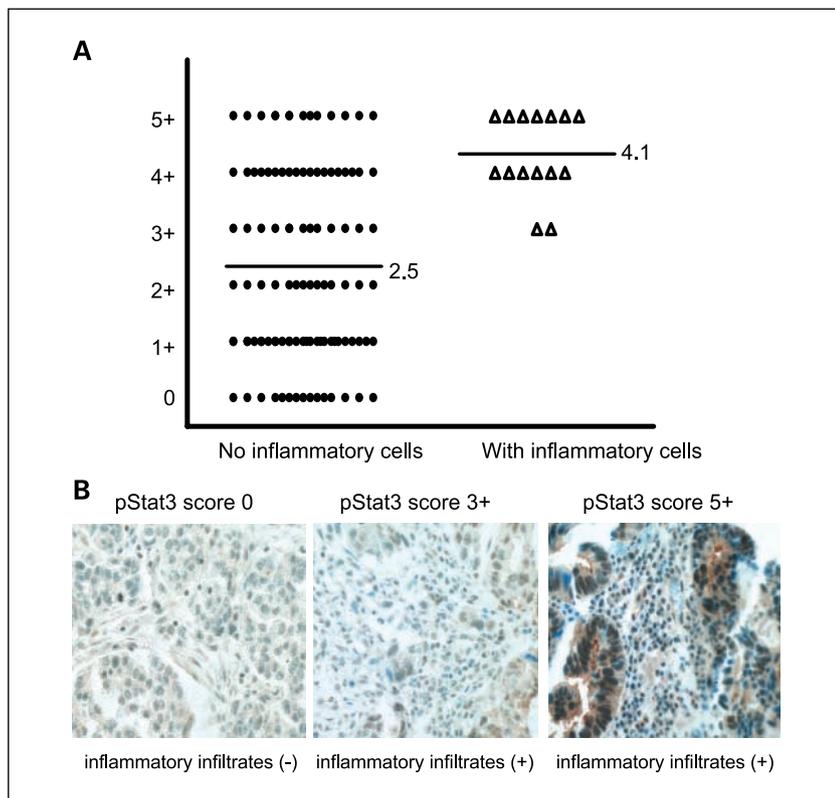


Fig. 7. Correlation of pStat3 expression with presence of intratumoral inflammatory cell infiltration. **A**, distribution of pStat3 immunohistochemical staining scores in primary ovarian tumors with inflammatory cell infiltration and without inflammatory cell infiltration. **B**, representative inflammatory cell infiltration correlation with higher pStat3 immunohistochemical staining scores.

alternatively, constant activation of the Stat3 pathway through alternative mechanisms such as Src or epidermal growth factor receptor. Mechanisms of cytokine independent Stat3 pathway activation have recently been described in individuals with myeloproliferative disorders through mutations in JAK2 that result in increased constitutive activity of the mutant kinase as compared with the wild-type protein (39). High levels of activated JAK2 have been reported in several ovarian cancer cell lines and, thus, this mechanism may explain Stat3 activation in a subset of tumors (10).

These results suggest that the drug-resistant phenotype in a subset of ovarian cancers may be explained, at least in part, by an activated Stat3 pathway. Data supporting this hypothesis come from clinical specimens treated with both paclitaxel and other chemotherapeutic agents; thus, it is possible, perhaps likely, that agents such as carboplatin may have induced or selected for Stat3 activation. Whether Stat3 is activated by taxanes, nontaxanes, or both, there is evidence that inhibition of Stat3 activated antiapoptotic Bcl-X_L by small-molecule inhibitor increases the activity of chemotherapeutic drug and radiation (40). Src inhibition has also been shown to sensitize resistant cells to paclitaxel (9). We showed that inhibition of constitutively active Stat3 significantly reverses the paclitaxel-resistant phenotype as well as enhances paclitaxel-induced apoptosis (Fig. 3). These results, along with the observation that Stat3 is frequently expressed in high-grade and recurrent ovarian cancer,

suggest that manipulation of Stat3 pathway activity is a viable strategy for the reversibility of drug resistance. There are several approaches that could target the Stat3 pathway, including antibodies that bind IL-6 or its receptor; inhibition of gp130, Src, or JAK kinase activity; or small molecules that inhibit Stat3 dimerization, nuclear translocation, or DNA binding activity. In addition, Dalwadi et al. (41) have recently shown that IL-6 secretion and Stat3 activation are dependent on cyclooxygenase-2 expression and that inhibition of cyclooxygenase-2 may also prove useful for reversing Stat3 activation.

In summary, this report provides evidence that the Stat3 is often activated in drug-resistant ovarian cancer cell lines and notably activated in ovarian tumors. Most importantly, recurrent ovarian tumors or tumors with brisk setting of inflammatory infiltrates have the highest level of pStat3. Activation of this pathway *in situ* may be explained by intratumoral IL-6 or other cytokines produced either by tumor cells or surrounding inflammatory cells. The potential usefulness of interrupting Stat3 pathway activation deserves further investigation.

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