

A novel target for treatment of chordoma: signal transducers and activators of transcription 3

Cao Yang,^{1,3} Joseph H. Schwab,^{1,3}
Andrew J. Schoenfeld,¹ Francis J. Hornicek,^{1,3}
Kirkham B. Wood,¹ G. Petur Nielsen,²
Edwin Choy,^{3,4} Henry Mankin,^{1,3}
and Zhenfeng Duan^{1,3}

Departments of ¹Orthopaedic Surgery and ²Pathology, ³Sarcoma Biology Laboratory, Center for Sarcoma and Connective Tissue Oncology, and ⁴Division of Hematology Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Abstract

A major obstacle in the effective treatment of chordoma is that there are no identifiable biomarkers capable of predicting prognosis. Recent research has indicated that signal transducers and activators of transcription (Stat3) may be an important prognostic marker in some cancers, but its role in chordoma tumors has not been elucidated. In this study, the expression of Stat3 was evaluated in chordoma tissue microarray that contains 70 chordoma samples. Cells in the tissue microarray showed nuclear staining for phosphorylated Stat3 in all instances. The level of phosphorylated Stat3 expression correlated with the survival and severity of the disease. Three chordoma cell lines were exposed to SD-1029, a novel inhibitor of Stat3 activation. MTT assay showed that the growth of all chordoma cell lines was inhibited by SD-1029. The expression of Stat3 signaling cascade was inhibited in all chordoma cell lines after treatment with SD-1029. The cytotoxicity of the combination of SD-1029 and chemotherapeutic drugs is significantly better than either agent alone. Phosphorylation of Stat3 in chordoma cells *in vitro* and cellular proliferation in three-dimensional culture were inhibited by SD-1029. In conclusion, the Stat3 pathway is constitutively activated in chordomas and the level of expression may serve as a predictor for prognosis. Blockade of the

Stat3 pathway represents a potential strategy for future treatment. [Mol Cancer Ther 2009;8(9):2597–605]

Introduction

Chordomas account for 2% to 4% of primary malignant bone tumors and represent the most common primary malignant bone tumor of the spine (1–3). Chordomas are considered to be radiation resistant and refractory to cytotoxic chemotherapy (4, 5). Therefore, the therapeutic approach to chordoma has traditionally relied heavily on surgical control (3, 6, 7). Many studies, however, have reported high rates of local recurrence despite surgery, and distant metastases have been reported in 5% to 44% of patients (7–13). The overall median survival time with chordoma has been estimated to be ~6 years (6).

Targeted chemotherapy is an area of great potential in cancer therapy and may be useful in the treatment of chordoma if the appropriate target can be found (4, 14–16). The identification of new targets for therapy and the design of agents created to affect targets in a clinically meaningful way may serve as a viable avenue for the treatment of chordoma.

Signal transducers and activators of transcription 3 (Stat3) belongs to a family of transcription factors that depend on cytokine receptor-generated signals being transduced into the nucleus (17). When activated, phosphorylated Stat3 (pStat3) homodimerizes, translocates to the nucleus, and then induces transcription of several Stat3-dependent genes. Aberrant Stat3 activation promotes uncontrolled tumor cell growth and survival through mechanisms including increased expression of the oncogenes c-myc and cyclin D as well as the antiapoptotic proteins Bcl-xL and MCL-1 (16, 18–20).

Constitutive activation of Stat3 has been documented in ovarian, breast, colon, prostate, and several other types of cancer (18–22). Reports indicate that activation of the Stat3 pathway correlates with clinical outcome in several of these cancers, and inhibition of Stat3 activity may exert an anti-cancer effect (16, 23, 24). Because normal cells tolerate the interruption of Stat3 signaling, Stat3 potentially represents an excellent molecular target for treatment (17, 25). In this study, expression of pStat3 in chordoma tumors was correlated with outcome and the effect of SD-1029, a Stat3 inhibitor (26), was measured using chordoma cell lines.

Materials and Methods

Patient Population

Chordoma patients treated at the Massachusetts General Hospital from 1985 to 2007 were identified using the Massachusetts General Hospital cancer registry and orthopedic oncology databases. Data obtained for each patient included

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Requests for reprints: Zhenfeng Duan, Massachusetts General Hospital and Harvard Medical School, 70 Blossom Street, GRJ 1115, Boston, MA 02114. Phone: 617-724-3144; Fax: 617-724-7396. E-mail: zduan@partners.org

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Table 1. Comparison of the clinical characteristics of 70 patients with pStat3 low-staining and pStat3 high-staining chordoma

Characteristic	pStat3		P
	Low staining*	High staining [†]	
Age at diagnosis (y)			
Median	61.9	57	
Range	29-88	31-83	0.12
Gender (%)			
Male	25 (71.4)	26 (74.3)	
Female	10 (28.6)	9 (25.6)	0.79
Location (%)			
Sacrum	23 (65.7)	21 (60)	
Mobile spine	12 (34.3)	14 (40)	0.69
Prognosis			
Survival	29 (82.9)	17 (48.6)	
Nonsurvival	6 (17.1)	18 (51.4)	0.0046

*n = 35.

[†]n = 35.

age, gender, date of birth, tumor location(s), dates of surgery, presence of local recurrence and/or metastases, date of death if applicable, and disease status at final follow-up. Those patients with archival tissue available through the Department of Pathology were reviewed. Chordomas with a conventional morphology were included in the

study, whereas dedifferentiated and chondroid chordoma subtypes were excluded. Furthermore, patients who were lost to follow-up, or had <1-year clinical follow-up, in the absence of demise, were also excluded. The protocols were reviewed and approved by our institutional investigational review board.

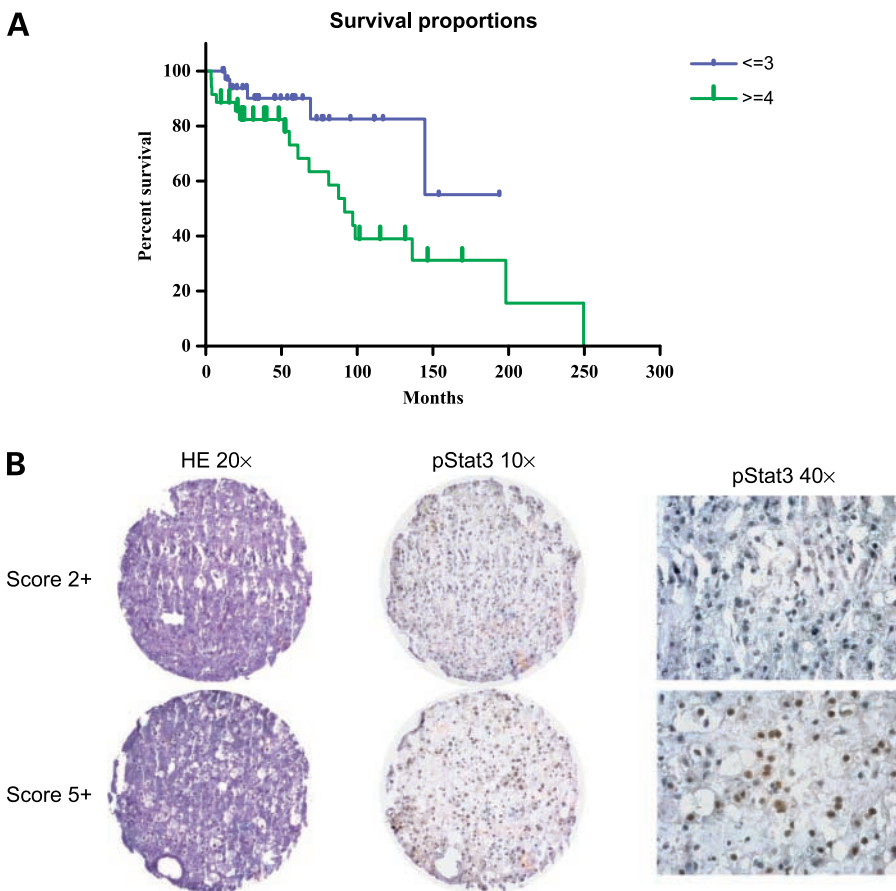
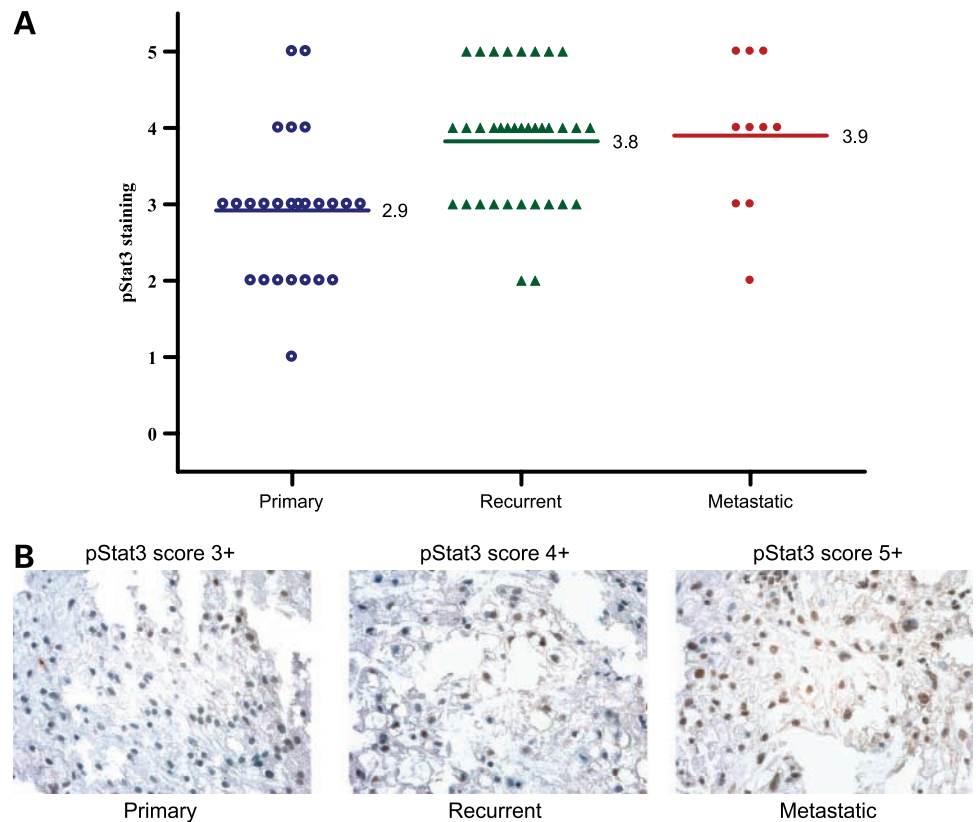


Figure 1. Relationship of overall prognosis to pStat3 expression. **A**, Kaplan-Meier survival analysis showing that the prognosis for patients with high expression of pStat3 was worse than those with low expression ($P = 0.039$). **B**, representative expression of pStat3 in low-staining (2+) and high-staining (5+) chordoma tissues.

Figure 2. Comparison of pStat3 expression in primary chordomas, with recurrent and metastatic lesions. **A**, distribution of pStat3 immunohistochemical staining in primary, recurrent, and metastatic lesions. **B**, representative expression of pStat3 in matched primary, recurrent, and metastatic chordomas.



Chordoma Tissue Microarray Slides and Immunohistochemistry

Tissue microarrays were prepared using a standard protocol. Archival blocks of chordoma tissues and the representative H&E slides from each case were reviewed microscopically by the coauthor (G.P.N.) and pathologist involved in this investigation. Areas of chordoma were identified on corresponding H&E-saffron-stained slides. 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 h, deparaffinized in xylene for 10 min, transferred through 100% ethanol for 5 min, and then rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by a 10-min incubation in 3% hydrogen peroxide in methanol. Antigen retrieval was processed with Target Retrieval Solution (Vector Laboratories) following the instruction of the manufacturer. After antigen retrieval, the slides were washed with PBS thrice at room temperature. Nonspecific protein blocking was done by incubating the slides in 5% normal goat serum and 1% bovine serum albumin in PBS for 1 h. Primary antibody was applied at 4°C overnight (1:100 dilution of pStat3 antibody; Cell Signaling Technology) in 1% bovine serum albumin with 5% normal goat serum. After 2-min rinses in PBS thrice, bound antibody was detected with the Vectastain ABC kit (Vector Laboratories) and visualized with 3,3'-diaminobenzidine high-sensitivity substrate (Vec-

tor Laboratories). Finally, the slides were counterstained with hematoxylin QS (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories) for long-term preservation.

Immunohistochemical Staining of Chordoma Tissue Microarray

Using light microscopy, the percentage of cells showing positive nuclear staining for pStat3 was independently evaluated by two investigators blinded to the origin of the sample. Staining patterns were categorized into six groups using the protocol described by Duan et al. (18): 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; and 5+, >75% positive cells. Chordoma patients were subgrouped into either a pStat3 low-staining group (scale 0-3: nuclear staining <50%) or a pStat3 high-staining group (scale 4 and 5: nuclear staining >51%). Light microscopic images were documented using a Nikon Eclipse Ti-U fluorescence microscope (Nikon) with an attached SPOT RT digital camera (Diagnostic Instruments).

Human Chordoma Cell Lines

The human chordoma cell line U-CH1 was obtained from the University Hospitals of Ulm (27). The human chordoma cell line GB 60 was provided by the Catholic University School of Medicine (28). The human chordoma cell line CH 8 was established in this laboratory. All three chordoma cell lines were cultured in DMEM supplemented with 10%

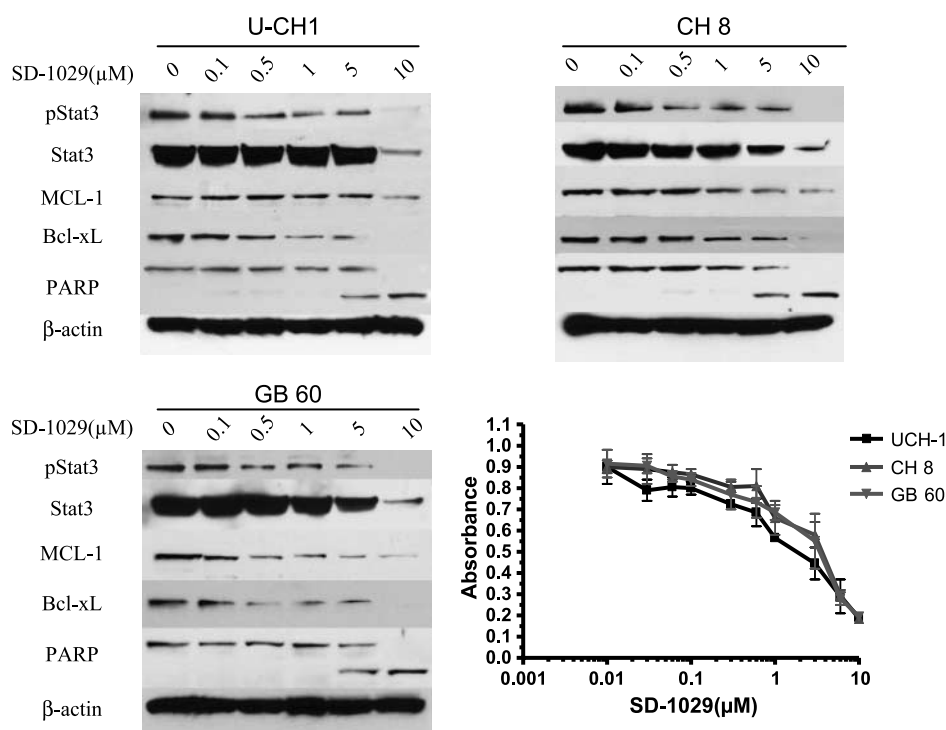


Figure 3. Western blot images for U-CH1, CH8, and GB 60 cells with increasing concentrations of SD-1029 from 0 to 10 μM . Note the inhibition of Stat3, pStat3, Bcl-xL, and MCL-1 at higher doses of SD-1029. Poly(ADP-ribose) polymerase (*PARP*) cleavage was also detected in all three chordoma cell lines following treatment with SD-1029. A plot of the MTT assay showing that the growth of all three chordoma cell lines was inhibited after treatment with SD-1029.

fetal bovine serum, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all obtained from Invitrogen).

Western Blot Analysis

Protein lysates from chordoma tissues and cells were generated through lysis with $1\times$ Radioimmunoprecipitation Assay Lysis Buffer (Upstate Biotechnology). The protein concentrations were determined using Protein Assay Reagents (Bio-Rad) and spectrophotometer (Beckman DU-640; Beckman Instruments). Forty micrograms of total protein were processed on Nu-Page 4% to 12% Bis-Tris Gel (Invitrogen) and transferred to a pure nitrocellulose membrane (Bio-Rad Laboratories). Antibodies directed against Stat3, pStat3, Bcl-xL, MCL-1, and poly(ADP-ribose) polymerase were obtained from Cell Signaling Technology. Antibodies directed against actin were obtained from Santa Cruz Biotechnology. Primary antibodies were incubated at 1:1,000 dilution in TBS (pH 7.4) with 0.1% Tween 20 and overnight at 4°C. Signal was generated through incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) incubated in TBS (pH 7.4) with 5% nonfat milk and 0.1% Tween 20 at 1:2,000 dilution for 1 h at room temperature. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Cytotoxicity Assay

Doxorubicin and cisplatin were obtained through unused residual clinical material provided by the pharmacy at the Massachusetts General Hospital. The stock solution of drugs was prepared according to the drug specifications and stored at -20°C . The Stat3 inhibitor SD-1029 was obtained through the National Cancer Institute (identifier:

NSC 371488; ref. 26). *In vitro* cytotoxicity assays were done by MTT assay (Sigma) as described previously by Yang et al. (29). Briefly, 3×10^3 cells per well were plated in 96-well plates. Cells were plated in DMEM containing SD-1029 and/or chemotherapeutic drugs doxorubicin or cisplatin. After culture in SD-1029 and/or chemotherapeutic drugs for 7 days, 10 μL MTT (5 mg/mL in PBS) was added to each well and the plates were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a SPECTRAMax Microplate Spectrophotometer. Experiments were done in duplicate. Dose-response curves were fitted with use of GraphPad PRISM 4 software (GraphPad Software).

Three-Dimensional Culture and Cytotoxicity Assay

Cytotoxicity assays were done on a reconstituted basement membrane using a previously described protocol (30). The Growth Factor Reduced Matrigel was added to each well of the eight-well glass chamber slides and spread evenly in the well to form the basement membrane measuring ~ 1 to 2 mm in thickness. A single-cell suspension was then seeded on the solidified layer. Cells were grown in an Assay Medium (DMEM/F-12 containing 2% horse serum, 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone, 100 ng/mL cholera toxin, 10 $\mu\text{g}/\text{mL}$ insulin, 100 units/mL penicillin G, and 100 mg/mL streptomycin) plus 5 ng/mL epidermal growth factor and 2% Matrigel. The cells were grown in a 5% CO_2 humidified incubator at 37°C. The cells were refed with Assay Medium containing 2% Matrigel and 5 ng/mL epidermal growth factor every 4 days. After culture for 4 days, the chordoma cells were treated with 6 $\mu\text{mol}/\text{L}$ SD-1029 for

8 days. The concentration of SD-1029 in three-dimensional culture was selected based on the results of MTT (Fig. 3). The chordoma cells were then isolated from the Matrigel culture using trypsin (0.25% trypsin, 0.1% EDTA). Cell proliferative activity was assessed by cell numeration using the protocol described by Debnath et al. (30) Experiments were done in duplicate.

Immunofluorescence

For the purposes of immunofluorescence, chordoma cells in three-dimensional culture were treated with 3 $\mu\text{mol/L}$ SD-1029 for 8 days. Nonlethal dose of SD-1029 was selected for immunofluorescence assay. After 8 days, chordoma cells were fixed in 2% paraformaldehyde for 20 min followed by permeabilization with 0.1% Triton X-100 (PBST). Permeabilized cells were blocked with 1% bovine serum albumin. Cells were then incubated with primary antibodies at 1:200 dilution in PBST at room temperature for 1 h. Antibodies directed against pStat3 were obtained from Cell Signaling Technology. The cells were washed with PBST and incubated with Alexa-conjugated secondary antibody (Invitrogen) at room temperature for 1 h. To counterstain nuclei, the chordoma cells were incubated with PBST containing 1 $\mu\text{g/mL}$ Hoechst 33342 (Invitrogen) for 1 min. Chordoma cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope (Nikon) equipped with a SPOT RT digital camera (Diagnostic Instruments).

Data Analysis

Values are representative of duplicate determinations in two or more experiments. The correlation between pStat3 expression level and prognosis was analyzed by Kaplan-

Meier survival analysis (GraphPad PRISM 4 software). A two-sided Student's *t* test (GraphPad PRISM 4 software) was used to compare the pStat3 intensity scores among primary tumors, recurrent tumors, and tumors with metastasis. Treatment effects of SD-1029 and chemotherapeutic drugs in chordoma cells were also evaluated using the two-sided Student's *t* test). Error bars are SD of averaged results and *P* values < 0.05 were accepted as a significant difference between means.

Results

Expression Level of pStat3 and Correlation with Clinical Prognosis

Eighty-nine patients treated for chordoma at Massachusetts General Hospital had archival tissue stored in the Massachusetts General Hospital Department of Pathology. Nineteen patients were excluded due to the diagnosis of nonconventional subtype of chordoma, or because of insufficient follow-up, leaving 70 samples eligible for study. The population included 51 males and 19 females. Average age at the time of presentation was 59.5 years (range, 29-88 years). The majority of tumors involved the sacrum ($n = 44$, 62.8%), whereas 26 involved the mobile spine. Immunohistochemical analyses determined that all tumors present on the tissue microarray had positive staining for pStat3 in the nucleus. The relative levels of pStat3 nuclear staining in the tumor sample sets from 70 individual patients were scored from <10% of tumor nuclei positive for pStat3 (1+) to >75% of tumor nuclei positive for pStat3 (5+). We subgrouped the low-staining and high-staining groups accordingly based on the criteria described above and compared

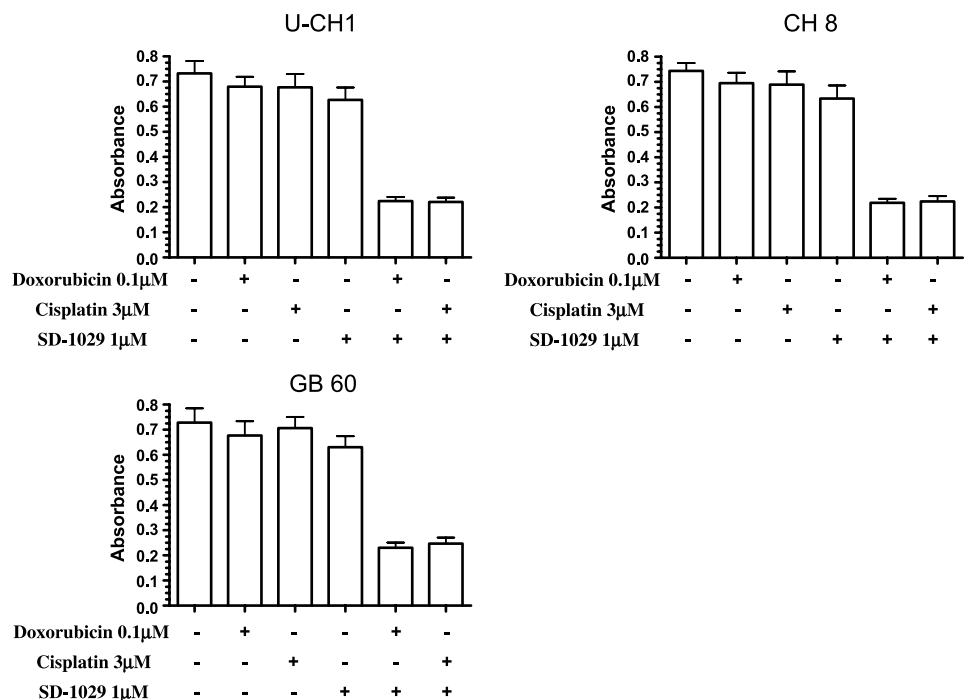


Figure 4. Cytotoxicity assays of chordoma cells treatment with SD-1029 and chemotherapeutic agents. Bar graphs of the cytotoxicity assays showing that growth of all three chordoma cell lines were inhibited by treatment with a combination of SD-1029 and chemotherapeutic agents.

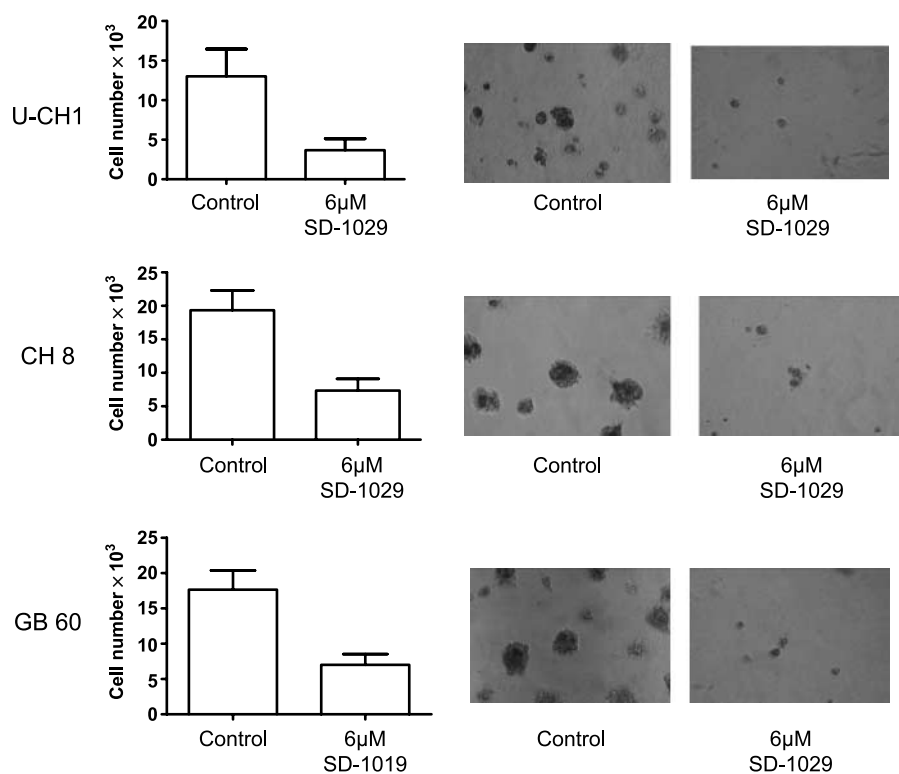


Figure 5. Chordoma cells treatment with SD-1029 in three-dimensional culture. Bar graphs and representative images of chordoma cells in three-dimensional culture treated with SD-1029.

patient prognosis with expression levels of pStat3. Of the 70 patients studied, there were 35 (50%) patients classified as low-staining and 35 (50%) as high-staining for pStat3 (Table 1). The average follow-up for patients in the low-staining and high-staining groups was 59.3 months ($n = 35$) and 68.1 months, respectively ($n = 35$; $P = 0.48$). When comparing the clinical characteristics of low-staining and high-staining chordoma, there was no significant relationship between pStat3 expression and age ($P = 0.12$), gender ($P = 0.79$), or tumor location ($P = 0.69$; Table 1). Kaplan-Meier survival analysis of chordoma patients between low-staining group and high-staining group showed that the prognosis for patient in the pStat3 high-staining group was significantly worse than those in the pStat3 low-staining group ($P = 0.039$; Fig. 1).

Increased Expression of pStat3 in Recurrent Chordoma and Chordoma Metastases

We next compared pStat3 expression in primary, recurrent, and metastatic chordomas. We observed greater levels of pStat3 ($P = 0.0002$) expression in the recurrent tumors compared with the primary tumors (Fig. 2). We also observed an increase in the intensity of pStat3 expression in tumors with metastasis compared with the primary tumors without metastasis ($P = 0.01$).

SD-1029 Blocks Stat3 Pathway and Inhibits Growth of the Human Chordoma Cell Lines

To evaluate if the Stat3 inhibitor, SD-1029, can block the Stat3 pathway, induce apoptosis, and inhibit growth of the chordoma cells, three chordoma cell lines, including U-CH1, CH 8 and GB 60, were treated with SD-1029. Western blot

analysis showed that the expression of Stat3 and pStat3 was inhibited in all three chordoma cell lines after treatment with SD-1029. The expression of the antiapoptotic proteins Bcl-xL and MCL-1 was also inhibited in three chordoma cell lines after treatment with SD-1029. We observed that the growth of all three chordoma cell lines, as measured by MTT, was inhibited after treatment with SD-1029. Poly (ADP-ribose) polymerase cleavage, an apoptosis-associated biochemical event, was detected in all three chordoma cell lines after treatment with SD-1029 (Fig. 3).

Cytotoxicity of the Combination of SD-1029 and Chemotherapeutic Drugs Is Significantly Better Than Either Agent Alone

To further confirm the role of Stat3 pathway in chordoma cells, we evaluated the effects of SD-1029 on cisplatin and doxorubicin induced cell death (chemotherapy drugs) by MTT. The chordoma cells were treated with SD-1029 and a nonlethal dose of chemotherapeutic drug. Cytotoxicity assay showed that the growth of all three chordoma cell lines was significantly inhibited after treatment with the combination of SD-1029 and cisplatin or the combination of SD-1029 and doxorubicin ($P < 0.01$). This indicates that the induction of chordoma cell death by the combination of SD-1029 and chemotherapeutic drugs is significantly better than either agent alone (Fig. 4).

SD-1029 Blocks Stat3 Pathway and Inhibits Growth of Chordoma Cells in Three-Dimensional Culture

As cellular architecture may have a significant effect on drug uptake, distribution, and efficacy, drug efficacy may be significantly lower in three-dimensional models in cancer

cell lines than in two-dimensional monolayer (31). To evaluate if the growth of chordoma cells could be inhibited by SD-1029 in three-dimensional culture, we measured the effect of SD-1029 on chordoma cell lines. Cell numeration showed that the growth of chordoma cells was significantly inhibited in three-dimensional culture after treatment with SD-1029 (Fig. 5). Immunofluorescence analysis showed that the expression of pStat3 was inhibited in all three chordoma cell lines after treatment with SD-1029 in three-dimensional culture (Fig. 6).

Discussion

At the present time, the effective treatment of chordoma remains a challenge. Even in the event of wide surgical resection, the tumor is known to frequently recur, and in such instances, lesions are more likely to behave aggressively and metastasize (5, 7, 11, 32, 33). A major obstacle in the effective treatment of chordoma is that, currently, there are no identifiable biomarkers capable of predicting prognosis. Recent research has indicated that Stat3 may be an important prognostic marker in some cancers (16, 23, 34–36), but its role in chordoma tumors has not been elucidated.

Stat3 is a major mediator of tumorigenesis (37), and activation of the Stat3 pathway in several cancers has been found to be associated with high histologic grade and advanced stage. Recent studies have also linked Stat3 to metastatic progression in several different cancers, including prostate, lung, ovary, and gastrointestinal tract (16, 18, 20–22). The influence of Stat3 on the metastatic potential of these cancers occurs through a variety of molecular mechanisms (35, 38–40). This study investigated the immunohistochemical staining of pStat3 in tissue samples of 70 patients with chordoma and sought to establish a relationship between expression and outcome.

Results indicate that Stat3 appeared to be activated in all chordoma tissue under investigation and 50% of samples showed a high level of staining for pStat3 (>50% of tumor nuclei positive). The overall survival for patients with tumors that stained highly for pStat3 was significantly reduced compared with those with tumors staining poorly. There was also a significant trend toward greater pStat3 expression in recurrent tumors, as well as tumors with metastasis, compared with primary lesions. This indicates that high expression of pStat3 in cancer lesions may be a useful biomarker for poor prognosis in patients with chordoma.

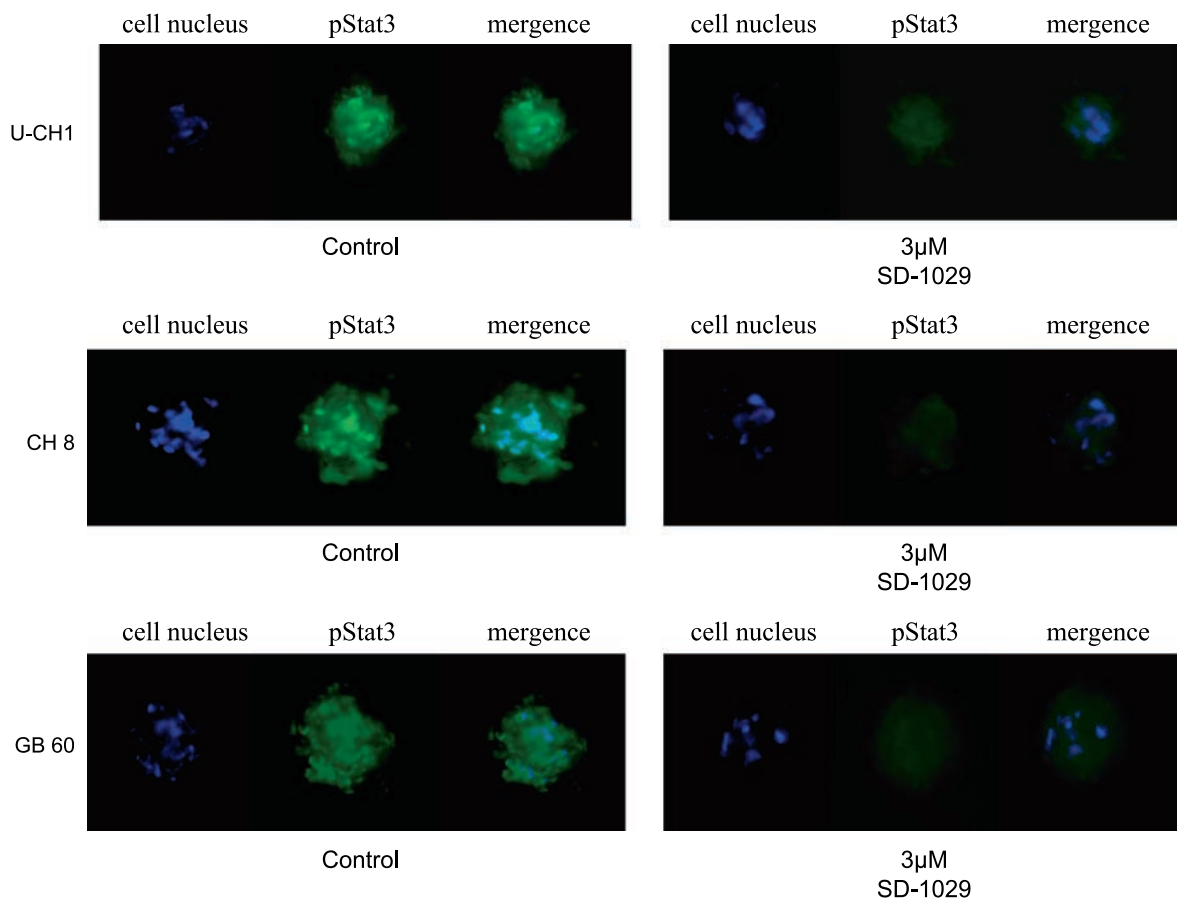


Figure 6. Immunofluorescent analysis of the expression of Stat3 in three-dimensional culture. Immunofluorescent images of chordoma showing the effect of SD-1029 on pStat3 expression in three-dimensional culture.

Stat3 has been shown to be vital for tumor cell growth, proliferation, and apoptosis (41–43). Inhibition of Stat3 in model systems has shown a reduction in survival and proliferation of tumor cells (24). The large body of data validating Stat3 as a potential target for cancer therapy, and the tolerance of normal cells to the loss of Stat3 function, has driven the effort to identify molecules capable of Stat3 inhibition (17, 25). SD-1029 has been identified as a novel inhibitor of Stat3 activation (26). This molecule blocks Jak activity with resultant inhibition of Stat3 phosphorylation, nuclear transport, and a decrease in Stat3-dependent transcription. Such actions have culminated in apoptosis in several human breast and ovarian cancer cell lines (26). The activation of Stat3 in chordomas indicates that this pathway may also serve as a potential target for the treatment of these tumors.

In this study, SD-1029 appeared to inhibit the expression and activation of Stat3 in three chordoma cell lines. This resulted in decreased expression of the antiapoptotic proteins Bcl-xL and MCL-1, poly(ADP-ribose) polymerase cleavage, and decreased growth of chordoma cell lines. These findings may indicate that SD-1029 is a viable agent for the treatment of chordoma.

Prior research has suggested that the efficacy of potential anticancer drugs *in vivo* differs when compared with *in vitro* studies using cancer cells grown in monolayer (31, 44). Because three-dimensional culture of cancer cell lines has long been advocated as a better model of the malignant phenotype *in vivo* (31, 45), we evaluated the effect of SD-1029 on chordoma cell lines in three-dimensional culture. Such testing confirmed that SD-1029 could also block the Stat3 pathway and inhibit the growth of chordoma even in cells in three-dimensional culture.

Although chordomas have long been known to be resistant to chemotherapeutic drugs, the mechanism behind this resistance remains unknown. Recently, several studies have shown that Stat3 is highly activated in drug-resistant ovarian cancer cell lines and interruption of Stat3 signaling could reverse resistance to chemotherapeutic agents (18, 46). Drug-resistant recurrent ovarian cancers have significantly greater pStat3 expression compared with matched primary tumors (18). Constitutive activation of the Stat3 pathway has also been shown to confer resistance to chemotherapy-induced apoptosis in numerous cancer cell lines (47–50). In this study, we found that both the constitutive activation of Stat3 in chordoma tissues and the expression level of pStat3 were correlated to disease severity and prognosis in chordoma patients. Additionally, the blockade of the Stat3 pathway by SD-1029 appeared to sensitize chordoma cells to chemotherapeutic agents.

These findings indicate that the Stat3 pathway may play a role in the chemotherapeutic resistance of chordoma as well. Although not specific for chordoma, SD-1029 may serve as a potential adjuvant for successful management of these tumors using conventional chemotherapeutic agents. More research in this regard must be done, however, before definitive conclusions can be drawn.

In summary, the present study shows that the Stat3 pathway is constitutively activated in chordoma and high ex-

pression of pStat3 in these lesions may serve as a biomarker for prognosis. Additionally, the effective blockade of the Stat3 pathway leads to apoptosis in chordoma cells and inhibits cellular proliferation *in vitro*. This highlights the potential for pStat3 to serve as a possible target for molecular therapy in the treatment of chordoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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