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Leukemia Inhibitory Factor Promotes Aggressiveness of Chordoma

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Chordomas are rare tumors of the spine and skull base that are locally destructive and resistant to chemotherapy and radiation therapy, with a poor prognosis and limited therapeutic options. Chordoma patients have a long life expectancy with high mortality from the disease. Cancer stem cells, which are known to exist in chordomas, have extensive proliferative and self-renewal potential and are responsible for maintaining tumor heterogeneity along with chemotherapy and radiotherapy resistance. Leukemia inhibitory factor (LIF) has multiple functions in stem cell biology, the immune response, and cancer, and is potentially a key molecule that allows cancer stem cells to self-renew. The purpose of this study was to determine whether LIF increases the aggressive traits of chordoma cells and leads to a poor prognosis in patients. Chordoma cell lines were treated with LIF, and functional tests were done. Twenty skull base chordoma samples were checked for levels of LIF and a correlation with clinicopathological features. The whole transcriptome microarray was used to observe changes in gene expression. We observed increased migration, invasion, tumorosphere formation, colony formation, epithelial–mesenchymal transition, and chemoresistance accompanied by a dramatic elevation in inflammatory gene networks and pathways in chordomas. The expression of LIF was associated with tumor size and a poorer overall survival. Microarray and quantitative real-time polymerase chain reaction assessments suggest that LIF can facilitate tumor-promoting inflammation. Results indicate that LIF plays a role in maintaining cancer stem cells in chordomas.

Key words: Chordoma; Leukemia inhibitory factor (LIF); Tumor inflammation; Prognosis

INTRODUCTION

Chordoma is a primary bone tumor that occurs along the vertebral column and is believed to originate from remnants of embryonic notochord. It is a rare tumor, with an incidence of 0.08 per 100,000 population in the US. The tumor is resistant to current radiotherapy and chemotherapy treatments and has a high relapse rate[1,2]. Chordoma is observed predominantly at the sacrum (29.1%) and skull base (32%), with a male/female ratio of about 2:1[1,3]. Overall, 5-, 10-, and 20-year survival rates are 68%, 40%, and 13%, respectively[4]. Chordomas are known to possess cancer stem cells, which can explain the high relapse rate, chemoresistance, and radioresistance of the tumor[5]. In order to establish effective treatment options, a greater focus on molecules that might influence the disease outcome is needed.

During the evolution of a solid tumor, complex cell-to-cell interactions occur in the tumor microenvironment, orchestrated by physical and chemical mediators[6]. As a member of the interleukin-6 (IL-6) cytokine family, leukemia inhibitory factor (LIF) is a pleiotropic molecule acting on different types of cells under a variety of conditions. LIF binds to the LIF receptor to activate a number of pathways, such as JAK/STAT3, MAPK, Ras/Raf/MEK/ERK, and PI3K[7–11]. LIF also acts as a proinflammatory cytokine in conditions such as arthritis[12,13] and spinal cord injury[14]. Currently, the relationship between LIF and cancer is ambiguous, but LIF overexpression has been observed in breast and pancreatic cancers[15,16]. A relatively small number of studies reveal that LIF has a diverse effect on various types of cancer. LIF can suppress or promote the differentiation of cancer cells and may contribute to disease progression or reduce growth, depending on the tumor type[17–19].

The aims of this study were to evaluate the functional role of LIF in chordoma cell lines and to evaluate the

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relationships between LIF expression level and the clinicopathological features of chordoma.

MATERIALS AND METHODS

Human Chordoma and Nucleus Pulposus Tissue Samples

Over a period of 12 years (2005 through 2016), chordoma tissue samples were obtained from 20 patients at the time of surgery at the Department of Neurosurgery, Yeditepe University Medical School in Istanbul, Turkey. None of the patients had undergone chemotherapy. All chordoma tissues were immediately frozen and stored at −80°C until RNA extraction. Nucleus pulposus primary cells, which were obtained as described previously[5] from patients with acute disc herniation, were used as a healthy control. To exclude fibrous cells, patients with chronic disc herniation were not included in the study.

Clinicopathological data including age, gender, tumor location, diagnosis, tumor origin, surgery margin, tumor location, recurrence, metastasis, and adjuvant therapy were collected retrospectively (Table 1). The follow-up time was calculated from the date of surgery until the patient's death or last follow-up. Samples pathologically defined as chordoma were included, while samples with insufficient patient information or a follow-up time of less than 3 months were excluded from the study.

The study was approved by the institutional review board of Yeditepe University Hospital (IRB 98-3943B and 101-4621B), and written informed consent was obtained from all participants.

Cell Culture and LIF Treatment

Chordoma cell lines U-CH1[20] and MUG-Chor1[21], available from the ATCC Bioresource Center, were provided by the Chordoma Foundation (Durham, NC, USA). Cells were cultured in culture flasks or well plates coated with 0.1% gelatin (Cat. No. G1890-100G; Sigma-Aldrich) and IMDM/RPMI (4:1) containing 10% fetal bovine serum and 1% antibiotics. Cell lines were checked routinely for molecular markers. Polymerase chain reaction (PCR)-based mycoplasma screening and Sanger sequencing were used for short tandem repeat to verify retaining of chordoma character. Recombinant human LIF was obtained from Millipore (Cat. No. LIF 1050) and administered to cells in medium in a 100 ng/ml concentration.

ICC and Flow Cytometry

LIF-treated cells were detached, washed, and resuspended in PBS without Ca^{2+} and Mg^{2+}. This procedure was followed with the addition of FcR-blocking human reagent (Cat. No. 120-000-442; Miltenyi Biotech, Germany), and the cells were incubated for 5 min on ice. For each experimental group, cells were separated into one unstained and one stained group. Antibodies recognizing CD15 conjugated with FITC (Cat. No. 130-081-101; Miltenyi Biotech), CD133/1 conjugated with APC (Cat. No. 130-090-826; Miltenyi Biotech), and CD338 conjugated with PerCP-Cy5.5 (Cat. No. 561460; BD Biosciences, San Jose, CA, USA) were incubated for 30 min and then washed with PBS without Ca^{2+} and Mg^{2+} to eliminate unbound antibodies. Both groups were stained with DAPI (Cat. No. 422801; BioLegend, San Diego, CA, USA). Histograms were generated for the stained population, and signal intensity was recorded for each surface marker after the samples were run in FACSAria III (BD Biosciences). The unstained control was used to determine threshold values. DAPI was used to eliminate nonviable cells.

To visualize LIF receptors on chordoma cell surfaces, cells grown on gelatin-coated coverslips were fixed using the paraformaldehyde–methanol method after 1 and 8 weeks of LIF treatment. Fixed cells were incubated with LIFR conjugated with FITC (Cat. No. sc-9995; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and washed twice with PBS. DAPI stain (Cat. No. 422801; BioLegend) was added to the specimen and visualization was done under the fluorescence microscope.

Cell Migration and Invasion Assays

Cell culture inserts with 8.0-μm pore size (Cat. No. PIEP12R48; Millipore) were used to determine the invasiveness and migration capacity of LIF-treated cells. For the invasion, assay chambers were coated with 100 μl of 250 μg/ml basement Matrigel (Cat. No. 354234; BD Biosciences) and incubated overnight at 37°C. Cells treated with LIF for 1, 3, 5, and 8 weeks were seeded into the upper chambers at a density of 4×10^4 in 100 μl serum-free IMDM/RPMI (4:1) medium. The lower chambers were filled with 1 ml of IMDM/RPMI (4:1) containing 10% fetal bovine serum. After 24 h of incubation, the membranes were fixed with 3.7% formaldehyde for 4 min and made permeable with methanol for 20 min, and then stained with crystal violet for 2 min. Migrating and invading cells were visualized and counted in four different fields of 400-fold magnification under the inverted microscope.

Colony Formation Assay

The self-renewal capacity of LIF-treated cells under nonadhesive conditions was determined with the colony formation assay. Six-well plates were coated with 1 ml of IMDM/RPMI (4:1) containing 10% fetal bovine serum and 1% antibiotics with 1% low-melting agarose (Cat. No. A9045; Sigma-Aldrich, Germany). After 20 min of incubation at 37°C, 1×10^4 cells were mixed with 1 ml of IMDM/RPMI (4:1) containing 10% fetal bovine serum and 1% antibiotics with 0.7% low-melting agarose. The medium was changed every 3 days. After 3 weeks

Table 1. Clinical Characteristics of the Chordoma Patients

Sample No.	Age/ Gender	Origin	Location	Surgery	Recurrence	Metastasis (Location)	Adjuvant Therapy	Follow-up After Surgery (Months)	Last Control Status
1	35/F	Primary	Clivus	Transoral tumor resection	No	No	Radiotherapy	46	NED
2	66/F	Primary	Petroclival	Left lateral suboccipital craniotomy	Yes	No	Radiotherapy	38	DOD
3	32/M	Primary	Superior Clivus	Transphenoidal endonasal approach	No	No	Radiotherapy	94	NED
4	10/M	Primary	Clivus	Left anterior paramedian approach	Yes	No	Radiotherapy	85	DOD
5	48/F	Primary	Clivus	Sublabial transphenoidal approach	Yes	No	Cyberknife	56	AWD
6	26/M	Primary	Clivus	Sublabial transphenoidal approach	No	No	Radiotherapy	40	NED
7	75/F	Primary	Clivus	Transphenoidal endonasal approach	No	No	Radiotherapy	49	NED
8	56/F	Primary	Left cavernous sinus	Left pterional approach	No	No	Radiotherapy	40	NED
9	47/F	Primary	Clivus	Craniorbitozygomatic approach	Yes	No	Radiotherapy/ cyberknife	60	DOD
10	48/M	Primary	Sphenoid Sinus	Transphenoidal endonasal approach	No	No	None	29	NED
11	64/M	Recurrent	C1 anterior	Transoral tumor resection	Yes	No	None	71	NED
12	71/M	Recurrent	Clivus	Right pterional craniotomy	Yes	No	Radiotherapy	37	DOD
13	47/F	Primary	Clivus+cranivertebral junction	Transoral tumor resection	No	No	Radiotherapy	125	NED
14	27/F	Primary	Petroclival	Right lateral suboccipital+ sublabial transfenoidal	No	No	Radiotherapy	36	NED
15	42/F	Primary	Clivus	Transphenoidal endonasal approach	No	No	Radiotherapy	24	NED
16	28/F	Primary	Clivus	Transphenoidal endonasal approach	No	Yes (lumbar)	Radiotherapy	96	NED
17	64/M	Primary	Clivus	Left pterional transcavernous approach	No	No	Radiotherapy	100	NED
18	52/F	Recurrent	C1 and T3	Prone position and multifocal tumor excision	Yes	Yes (thoracal)	None	120	DOD
19	33/F	Primary	Left cavernous sinus	Left craniorbitozygomatic approach	No	No	None	10	NED
20	53/F	Primary	Clivus and dorsum sella	Sublabial transphenoidal approach	No	No	Radiotherapy	30	NED

AWD, alive with disease; NED, no evidence of disease; DOD, died of disease; F, female; M, male. Origin was defined as primary for patients undergoing initial treatment at our facility and recurrent if the patient was previously treated at another institution.

in culture, the plates were stained with 0.05% crystal violet for quantifying colonies. Colonies containing more than 20 cells were counted under an inverted light microscope.

Tumorosphere Formation Assay

LIF-treated cells (4×10^5 at 1, 3, 5, and 8 weeks) were seeded in 25-cm^2 culture flasks coated with 2% agarose and 0.9% NaCl. The medium used for sphere formation was IMDM/RPMI (4:1) with 1% antibiotics, 20 ng/ml human FGF-2 (BD Biosciences), 20 ng/ml EGF (BD Biosciences), 1× N2 supplement (Gibco, USA), and 1× B27 supplement (Gibco) during the culturing of cells at 37°C in a 5% CO_2 incubator. Clusters formed by MUG-Chor1 cells were checked routinely and were dispersed by gentle pipetting. After 3 weeks, spheroid bodies were transferred to 96-well plates and quantified under an inverted microscope (Leica, Germany) at 400× magnification.

Chemoresistance Assay

To evaluate the effect of LIF on chemoresistance, 2.5×10^4 cells were seeded into 24-well plates in duplicate after 1, 2, 5, and 8 weeks of LIF administration. Freshly prepared 0.012 μM paclitaxel and 0.012 μM bortezomib in chordoma medium were added to the cells and changed every 2 days. An MTS assay was conducted after 7 days to assess the relative viability of LIF-treated cells and controls.

Microarray

For microarray analysis, total RNA isolated with TRIzol (Cat. No. 15596026; Thermo Fisher Scientific, Waltham, MA, USA) from U-CH1 and MUG-Chor1 cells treated with LIF for 3 weeks was used for whole transcriptome analysis with the Human Gene 2.1 ST Array (GeneAtlas System; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. The resulting data were analyzed with Transcriptome Analysis Console 3 software (Affymetrix).

Quantitative Gene Expression Analyses

Twenty fresh-frozen primary clival chordoma tissues were homogenized by using liquid nitrogen with a mortar and pestle, followed by the immediate addition of TRIzol according to the manufacturer's protocol. Cell lysates of six cultured nucleus pulposus cells from patients with acute disc herniation were used to acquire total RNA with the TRIzol method, as described previously[5]. RNA was quantified using a NanoPhotometer (Implen, Munich, Germany). Total RNA was isolated from LIF-treated cells and their controls with the TRIzol method according to the manufacturer's protocol. All mRNA levels were measured through a two-step real-time PCR with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Relative mRNA levels were detected with the $2^{-\Delta\Delta}$Ct method with GAPDH as the housekeeping gene. Analyses were conducted by normalizing with the control groups.

Statistical Analysis

The microarray data were analyzed with one-way repeated-measures ANOVA (paired) using two controls and two LIF-treated samples. Clinicopathological features age, gender, tumor location, recurrence, metastasis, and survival were analyzed for their correlation with LIF expression level. Correlations were evaluated with Spearman's rank correlation test ($n=12$). The overall survival graph was sketched using Kaplan–Meier survival analysis with the Mantel–Cox test ($n=20$). All other p values were obtained with two-tailed Student's t-test using at least triplicates ($n>3$) for each experiment. A value of $p<0.05$ was considered statistically significant. The high- and low-LIF groups of patients were separated into groups of equal number according to the relative median LIF levels. Significant outliers were evaluated for each experiment and removed from analysis.

RESULTS

Surface LIFR Increases in a Time-Dependent Manner Upon LIF Treatment

Fluorescence microscopy images revealed that chordoma cell lines U-CH1 and MUG-Chor1 have cell surface LIF receptors (Fig. 1a), and these receptors increased as cells were exposed to LIF in a paracrine manner. The presence of LIF receptors was greater in week 8 compared to week 1, which indicates a time-dependent increase in cellular response to LIF exposure.

LIF Treatment Increases Invasion and Migration Abilities and Induces EMT-Related Genes

We used Transwell chamber inserts to investigate the effect of LIF on migration and invasion. As representative images from week 1 indicate (Fig. 1b), LIF treatment drastically improved the migration (Fig. 1c) and invasion abilities (Fig. 1d) of both cell lines. This effect decreased over time and was neutralized at week 8. The effect of LIF on EMT gene levels was also monitored. E-Cad and CK19 were suppressed by LIF treatment, whereas the EMT marker ZEB2 had a significant peak at either week 3 or week 5. EMT marker levels at the corresponding weeks were consistent with the migration and invasion abilities. MET, which plays an important role in local invasion by chordoma cells, also escalated in weeks 1 and 3 (Fig. 1e). No significant transformation of cells to a mesenchymal phenotype in morphology was observed. LIF treatment did not significantly affect T expression in chordoma cell lines.

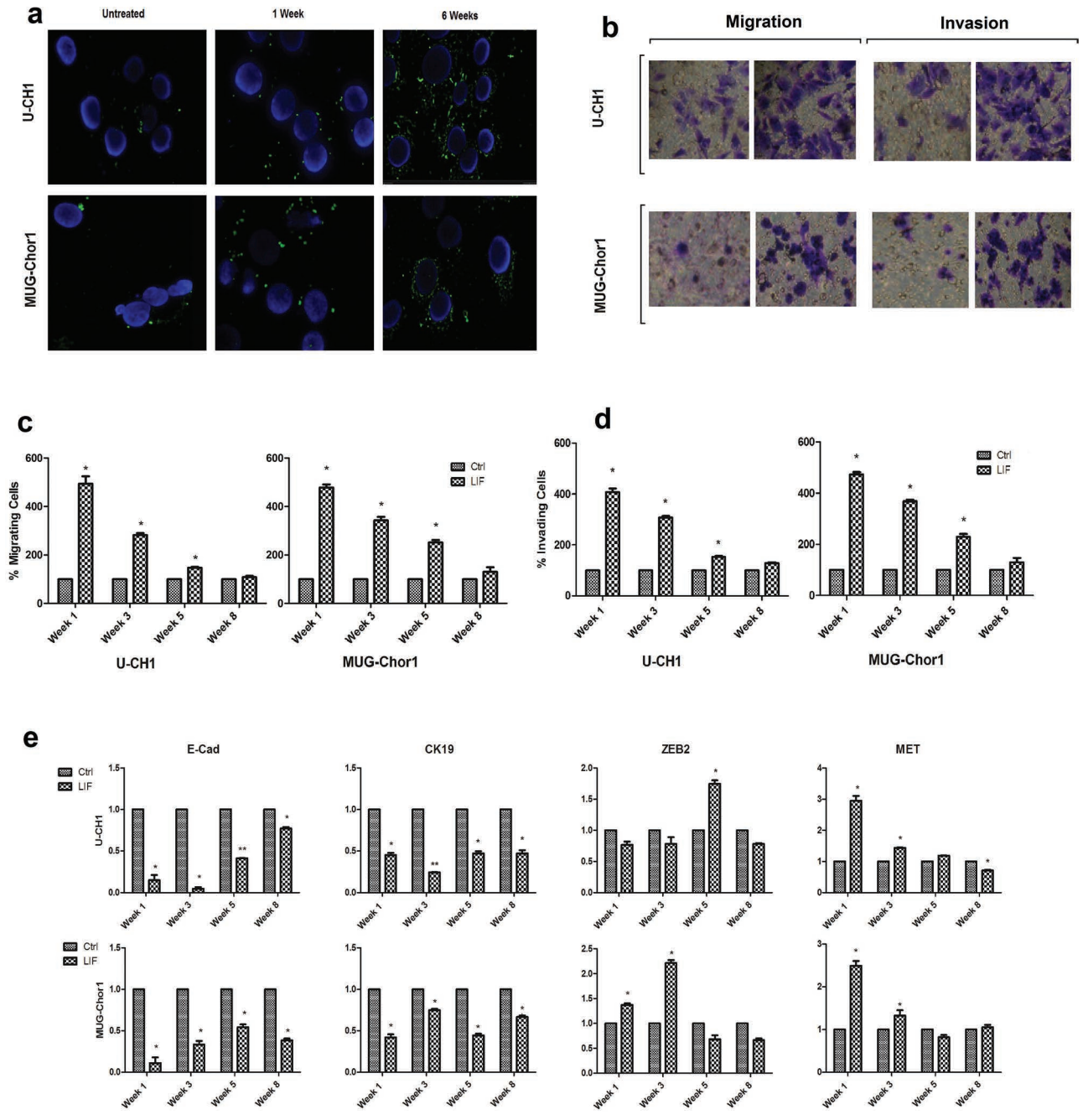
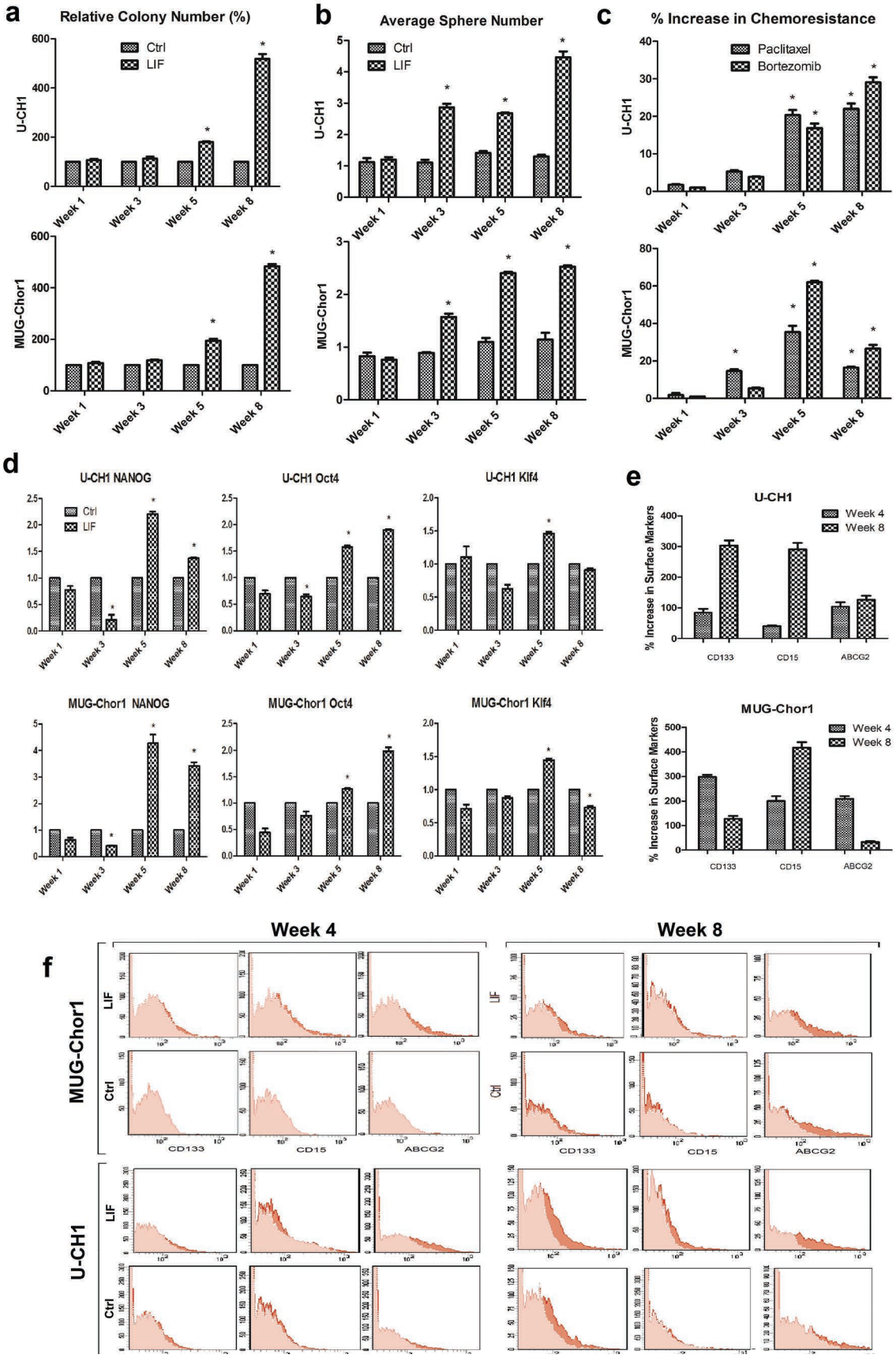


Figure 1. LIF treatment increases invasion and migration abilities and induces EMT-related genes in chordomas. (a) Anti-LIFR antibody (green) was used to visualize the LIFR level of U-CH1 and MUG-Chor1 after LIF treatment of 1 and 8 weeks. The control group was treated with complete chordoma medium. (b) Representative images of migration and invasion assays at week 1. (c, d) LIF promoted the migration of U-CH1 and MUG-Chor1. Data were normalized with the control group. The highest rate occurred at week 1 and steadily decreased thereafter. (e) LIF treatment decreased the relative level of epithelial markers E-Cad and CK19 while increasing the late EMT marker ZEB2 level at weeks 1 and 3 for MUG-Chor1 and at week 5 for U-CH1 normalized with the control. Proinvasive MET was significantly increased at weeks 1 and 3, a pattern consistent with the invasive character at the time interval. $*p<0.05$; $**p<0.01$.


a
Relative Colony Number (%)
Ctrl
LIF
U-CH1
MUG-Chor1
Week 1
Week 3
Week 5
Week 8
b
Average Sphere Number
c
% Increase in Chemoresistance
Paclitaxel
Bortezomib
d
U-CH1 NANOG
U-CH1 Oct4
U-CH1 Klf4
MUG-Chor1 NANOG
MUG-Chor1 Oct4
MUG-Chor1 Klf4
e
% Increase in Surface Markers
Week 4
Week 8
CD133
CD15
ABCG2
f
Week 4
Week 8
LIF
Ctrl

LIF Promotes Anchorage-Independent Growth, Tumorosphere-Forming Ability, and Chemoresistance of Chordoma Cells, Indicating an Increase in Cancer Stem Cell (CSC) Character

LIF promoted the anchorage-independent growth of chordoma cells in soft agar, as LIF treatment increased the number of colonies formed by U-CH1 and MUG-Chor1 cells after 5 and 8 weeks in a time-dependent manner (Fig. 2a). Furthermore, LIF-administered cells formed a significantly higher number of tumorospheres when compared to cells grown in medium without LIF (Fig. 2b). The highest number of tumorospheres was formed after 8 weeks of LIF treatment, and no significant increase was observed at week 1 in either cell line.

LIF treatment increased the chemoresistance of U-CH1 and MUG-Chor1 against bortezomib and paclitaxel from the third week (Fig. 2c). As these functional tests implicate an increase in stem-like behavior, CSC and drug transport markers were increased in our experimental group, as indicated by real-time PCR. LIF treatment increased the levels of CSC markers NANOG, Oct4, and Klf4 predominantly at week 5 (Fig. 2d). Potential chordoma CSC cell surface markers CD133 and CD15 and the drug transporter CD338 (ABCG2) levels were consistent with our findings that LIF treatment transforms chordoma cells into a more stem-like state. CD133, CD15, and ABCG2 levels increased in both cell lines at weeks 4 and 8 (Fig. 2e and f). There was no significant increase in any of the three markers at week 1 (data not shown). No significant change was observed in the surface markers.

LIF Escalates Tumor Inflammatory and Antiapoptotic Pathways

As a result of microarray analysis, a total of 31 genes were upregulated, and 16 genes were downregulated in LIF-treated U-CH1 and MUG-Chor1 cell lines after 3 weeks. Of the 31 upregulated genes, 21 relate to inflammatory mechanisms (Fig. 3a). In addition, six antiapoptosis genes were upregulated in the apoptosis pathway. Gene networks related to inflammation, such as the Toll-like receptor signaling pathway, tumor necrosis factor-α (TNF-α), TWEAK signaling, IL-1 signaling, IL-6 signaling, and RANKL/RANK pathways, were effected with numerous genes that were dysregulated as a result of LIF treatment (Table 2). The proinflammatory gene TNFAIP2 level was checked in LIF-treated cells and found to be consistent with the invasion and migration abilities, as well as EMT markers, declining after week 3 (Fig. 3b).

LIF Correlates With TNFAIP2, KLF4, and MET Gene Expression and Overall Survival and Tumor Size

Patient-derived chordoma and nucleus pulposus samples were checked for LIF mRNA levels, and the chordoma samples had a higher LIF level average than nucleus pulposus samples, but the result was insignificant (Fig. 4a). Tumor size was positively correlated with LIF level ($p<0.05$) (Fig. 4b). We checked our sample cohort for a Spearman's rank correlation between LIF and genes that were affected in the LIF treatment experiments on cell lines. E-Cad ($R=-0.308$), CK19 ($R=-0.235$), ZEB2 ($R=0.341$), TNFAIP2 ($R=0.653$), KLF4 ($R=0.574$), and MET ($R=0.793$) showed a consistent correlation with our results with U-CH1 and MUG-Chor1. Among these, TNFAIP2 ($p=0.0114$), KLF4 ($p=0.0320$), and MET ($p=0.0007$) were statistically significant (Fig. 4c). NANOG and Oct4 genes exhibited inconsistent results as they were inversely related to LIF levels, unlike in vitro-treated cell lines, but the results were statistically insignificant ($p=0.1692$ and $p=0.5426$, respectively). Kaplan–Meier survival analysis revealed that patients with a higher LIF level in their tumors had a lower chance of overall survival than the low-LIF group ($p<0.05$) (Fig. 4d). In our cohort, the LIF level did not correlate with age, metastasis, or recurrence.

DISCUSSION

In this study, we found that chordoma cells respond to LIF with respect to motility, invasive ability, chemoresistance, tumorosphere-forming capacity, and anchorage-independent growth in vivo, which indicates a potential for tumor-promoting inflammation. EMT, CSC characteristics,

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Figure 2. LIF promotes the anchorage-independent growth, tumorosphere-forming ability, and chemoresistance of chordoma cells, indicating an increase in cancer stem cell (CSC) character. (a) LIF treatment promoted anchorage-independent growth of U-CH1 and MUG-Chor1 cells in soft agar at weeks 5 and 8. The figure shows the relative number of colonies formed as a percentage of the control group. (b) The tumorosphere formation abilities of U-CH1 and MUG-Chor1 cells on attachment-free conditions increased after week 3 and peaked at week 8. Graph columns represent the average tumorosphere number for each well in a 96-well plate. (c) LIF treatment increased the chemoresistance of chordoma cells against paclitaxel and bortezomib at weeks 5 and 8. Chemoresistance was calculated as the percentage viability of LIF-treated cells over untreated cells for each week and each cell line. (d) Relative CSC marker levels against the control group were determined with real-time polymerase chain reaction (PCR). The timing of increased CSC marker expression was consistent with the results of the functional tests. (e, f) Flow cytometry results of CSC markers CD133, CD15, and the drug efflux protein ABCG2. The columns indicate the percentage increase of these surface markers after LIF treatment compared with the control group. In the histograms, pale red represents unstained cells, whereas dark red represents stained cells. *$p<0.05$.

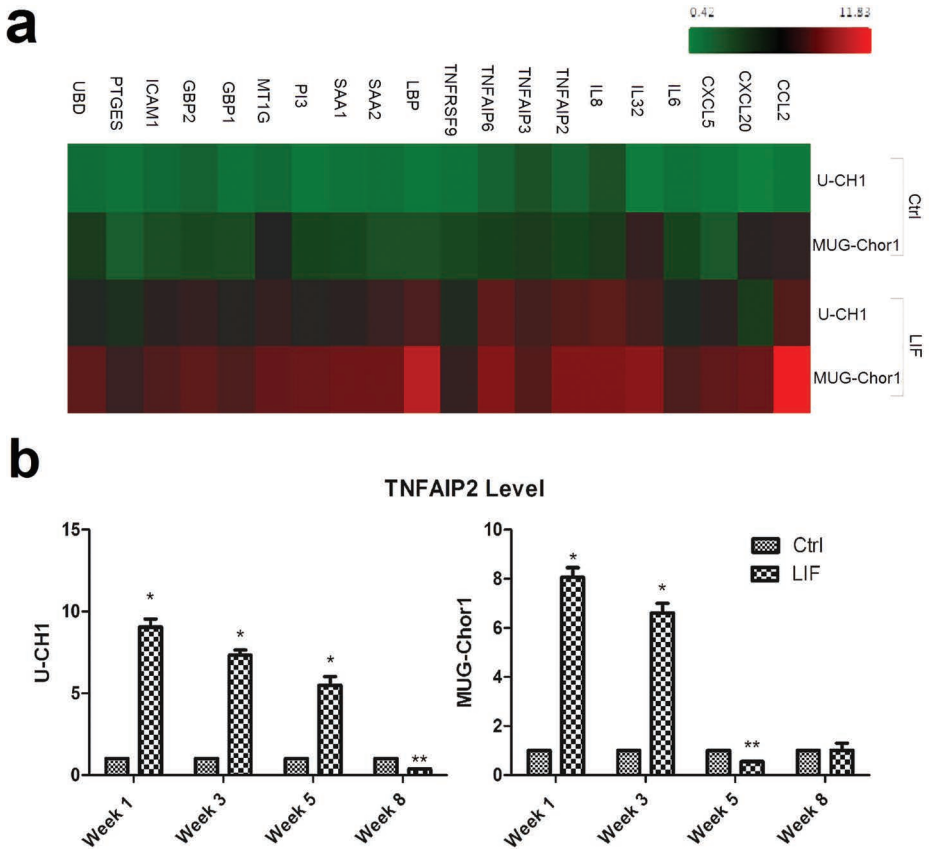

Figure 3. LIF escalates tumor inflammatory and antiapoptotic pathways in chordomas. (a) A heat map representing the differential expression of 21 inflammation-related genes after 3 weeks of LIF treatment. (b) TNFAIP2 levels were checked as a marker of activated inflammation after LIF treatment in chordoma cells by using real-time PCR. *$p<0.05$, **$p<0.01$.

Table 2. Pathways Regulated by LIF in Chordomas

Pathway	No. of Positive Regulators
Inhibition of apoptosis	6
Toll-like receptor signaling pathway	8
Photodynamic therapy-induced NF-κB survival signaling	7
TNF-α signaling pathway	7
TWEAK signaling	5
Interleukin-1 (IL-1) signaling pathway	5
IL-6 signaling pathway	3
RANKL/RANK	3
JAK/STAT	3

The number of dysregulated genes in the respective pathways that positively regulate the pathway. All but two remarkable pathways were related to inflammation. Data were obtained by using the Transcriptome Analysis suite (Affymetrix).

and tumor-promoting inflammation signature genes have been identified upon exogenous LIF administration to two chordoma cell lines, U-CH1 and MUG-Chor1. We found that the LIF level correlated with TNFAIP2, MET, and KLF4 expression, as well as tumor size and overall survival in chordoma patient samples.

Chordomas are rare tumors of the spine exhibiting excessive chemoresistance and radioresistance and lacking any confirmed drug therapy. Studies of the molecular biology of this orphan disease are insufficient to provide prognostic biomarkers and drug targets, which are necessary for better treatment strategies. Results from our study suggest that LIF increases the aggressive features of chordoma cells. LIF promotes the anchorage-independent growth of chordoma cells in soft agar, and LIF treatment increased in vitro Transwell migration and invasion at the first and third weeks of treatment.

LIF is a versatile cytokine with a variety of functions and a complex role in cancer. LIF is an essential actor in embryonic implantation and inhibits the differentiation of mouse embryonic stem cells[22]. In recent studies, LIF has

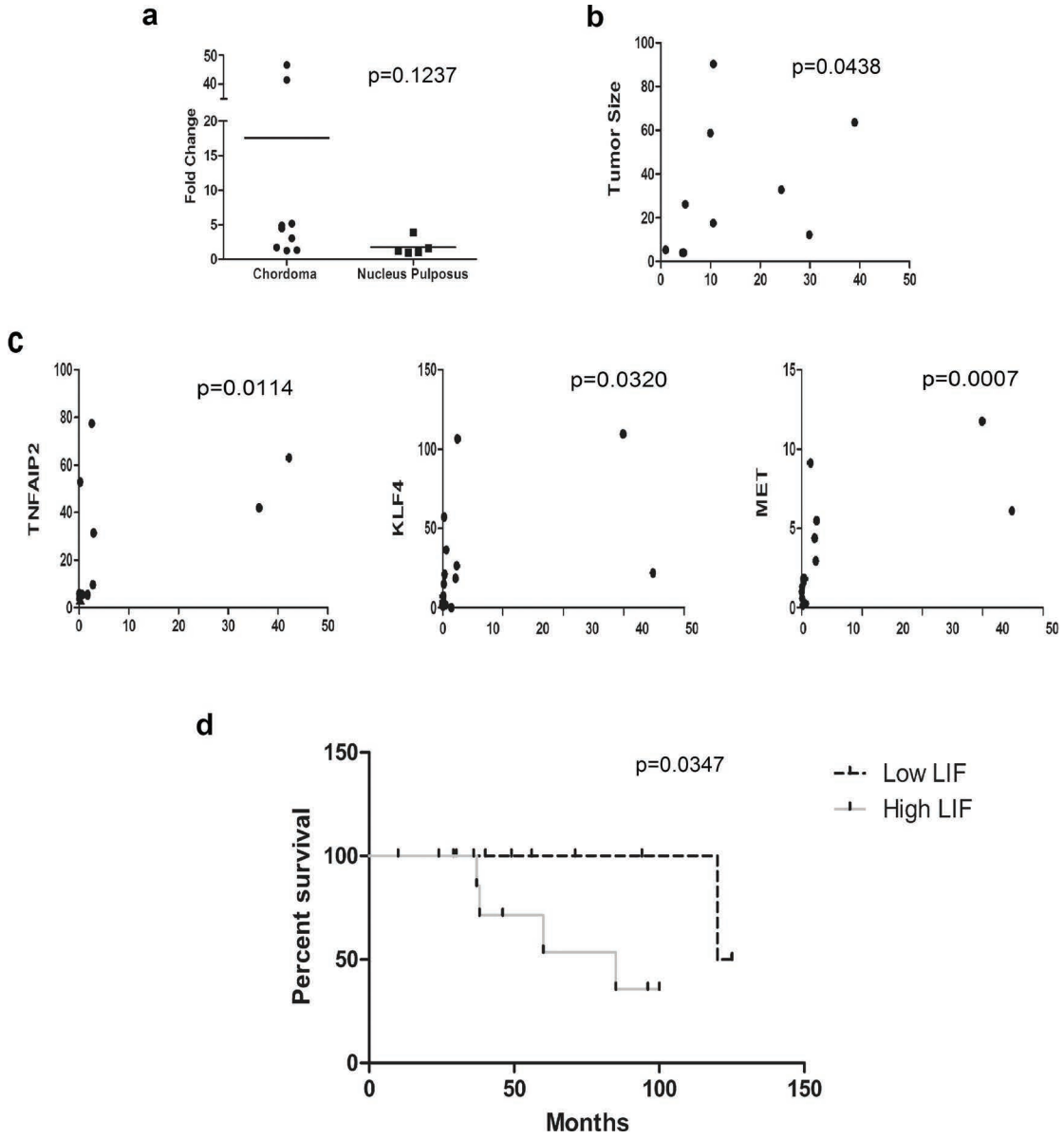

Figure 4. LIF is correlated with TNFAIP2, KLF4, and MET genes; tumor size; and overall survival in chordoma patient samples. (a) Dot plot comparing the level of LIF expression in chordoma tumor samples against that in nucleus pulposus samples. The horizontal bar represents the average value. (b) Correlation of LIF level (*x*-axis) versus tumor size, and (c) LIF level (*x*-axis) versus TNFAIP2, KLF4, and MET. (d) Kaplan–Meier survival plot of our patient cohort. High- and low-LIF groups were defined according to the relative LIF expression levels. The high-LIF group had a poorer overall survival.

been shown to promote the development and progression of cancer[23,24]. Both intrinsic and extrinsic LIF can increase proliferation in vitro and promote tumor growth in vivo in melanoma, breast, and colorectal cancers[23,25,26]. LIF overexpression was reported to promote resistance to chemotherapy and radiotherapy in studies on a variety of tumors, including breast, colorectal, lung, head and neck, and nasopharyngeal carcinoma[23]. LIF can activate a range of signaling pathways including JAK/STAT, MTOR and MAPK, and p53[26]. These pathways are known to be dysregulated in chordoma tumors[27]. Still, little is known about the underlying mechanisms by which LIF can lead to tumors, as the studies in this field are limited.

In this study, the MET gene expression level, along with a number of EMT markers, dramatically increased upon LIF treatment. As previously reported[28–31], MET is a key gene for local invasion and patient outcome in chordomas. The downregulation of E-cadherin expression is a

prognostic marker for chordoma, correlating with poorer survival and a high rate of recurrence[32]. In our study, LIF decreased the E-cadherin and CK19 levels while it increased ZEB2 abundance at week 5, which suggests that it may play a role in EMT transition in chordoma cells. As a late EMT marker, ZEB2 elevation happened at week 5 in both cell lines, whereas the epithelial markers were suppressed throughout the entire 8 weeks, which was a result of EMT progression.

Surface cancer stem cell marker levels on chordoma cells are consistent with these data, and mRNA levels of NANOG, Oct4, and KLF4 were elevated. Chemoresistance is also increased by LIF treatment, which was supported by cell viability and the assessment of ABCG2 gene expression. On both the functional and molecular levels, we observed that LIF-treated cells have a more motile and invasive character at the first and third weeks of treatment. As these traits steadily decreased, we observed an increase in tumorosphere formation and anchorage-independent growth alongside an increase in CSC and drug resistance markers at the fifth and eighth weeks. This process mimics events during metastasis, in which tumor cells invade through the tissue envelope and migrate, eventually settling in other tissues.

The evaluation of gene networks and pathways that were influenced by LIF provides intriguing results. Although the conventional LIF pathways such as mTOR, AKT, and JAK/STAT3 were not affected, the mRNA profile of LIF-treated chordoma cells showed an outstanding increase in inflammatory pathways. Previously, it has been observed that LIF-induced cell survival does not act through the JAK/STAT pathway in mammary carcinomas in mice[33]. Tumor-promoting inflammation is considered to be one of the hallmarks of cancer[6]. LIF can act as a proinflammatory molecule in specific tissues, including in the spinal cord, as shown by overexpression and knockout studies[14,34]. LIF can pass through the blood–brain barrier during inflammation with the help of TNF[35]. LIF has recently been shown to activate fibroblasts for a proinvasive role, promoting cancer cell invasion[36]. In a previous study, the expression of programmed cell death ligand 1 (PD-L1) was correlated with tumor-infiltrating lymphocytes in chordomas. Furthermore, the data suggest that these lymphocytes are associated with metastasis in a chordoma patient cohort[37].

We checked the levels of the TNFAIP2 gene, which is downstream of TNF-α in the proinflammatory NF-κB pathway, to see if extrinsic LIF treatment of chordoma cell lines or intrinsic levels of LIF in our tissue samples can affect this important molecule. The TNFAIP2 level positively correlated with LIF in chordoma patient samples and cell lines. Our data suggest that inflammatory pathways, activated by LIF, might be the driving force of EMT, migration, invasion, and cancer stem-like cell characteristics in chordomas. Our current study and previous knowledge about tumor-infiltrating lymphocytes in chordoma suggest that chordoma tumors are vulnerable to tumor-promoting inflammation, which can explain their high locally invasive character. We showed that chordoma cells respond to extrinsic LIF, to which they are likely to be exposed from the inflammatory tumor microenvironment. Tumor samples in our cohort also expressed high levels of intrinsic LIF, which may activate proinflammatory pathways intracellularly, meanwhile creating an inflammatory tumor microenvironment through secretion of the molecule.

The effects of the intrinsic LIF level in chordoma tumor samples were in harmony with the extrinsic effects on chordoma cell lines. In our patient cohort, MET and KLF4 levels were significantly correlated with the intrinsic LIF level. Our study presents the first data demonstrating that the LIF level is positively correlated with tumor size in patients. Our data further show that LIF expression is associated with shorter overall survival in chordoma patients.

There are few studies of LIF and its relationship to cancer, and, to date, the connection between LIF and inflammation in cancer has not been reported. As one of the hallmarks of cancer, tumor-promoting inflammation is a promising field of study for chordoma tumor progression, but these studies are very limited. Because of the rarity of chordomas, a relatively small number of patient samples were used in this study, which limits the survival analysis. Furthermore, tumor grade was not included in our statistical analysis because of limitations in acquiring data. Further studies with larger cohorts should be carried out to determine whether LIF is a reliable biomarker for survival or other prognostic features in patients with chordomas. Additionally, the role of LIF in the chordoma microenvironment, the ability of chordoma cells to secrete LIF, and the effects of in vivo LIF knockdown and overexpression should be investigated.

In summary, this study presents data on the effects of LIF on chordoma migration, invasion, chemoresistance, EMT, clinicopathological factors, and cancer stem cell character. LIF plays a vital role in promoting tumor inflammation. Our results also emphasize tumor-promoting inflammation as a pathway that drives chordoma progression.

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