

Analysis of IL6-protein complexes in chondrosarcoma

KARINA GALOIAN, SHIHUA LUO and PARTHIK PATEL

Miller School of Medicine, Department of Orthopedics, University of Miami, Miami, FL 33136, USA

Received September 15, 2017; Accepted October 26, 2017

DOI: 10.3892/br.2017.1016

Abstract. Cytokines produced in the tumour microenvironment serve important roles in cancer pathogenesis or in the suppression of disease progression. Metastatic chondrosarcoma is a cancer of the cartilage, and our group previously reported from a human ELISA assay that interleukin 6 (IL6) expression in JJ012 chondrosarcoma cells was 86-fold lower than that in C28 chondrocytes, indicating its role as an anti-inflammatory and anti-tumorigenic factor. Additionally, to the best of our knowledge, the study was the first to demonstrate downregulation of IL6 in a human chondrosarcoma cell line. To fully elucidate the effect of this IL6 downregulation, it is important to identify protein complexes and components that bind IL6 and potentially affect its gene expression directly or indirectly. To investigate IL6-protein interactions leading to these differences in IL6 expression, the current study performed a gel retardation electrophoretic mobility shift assay (EMSA), followed by 2D gel phoresis, in-gel trypsin digestion and proteomic mass spectral analysis. The results indicated a presence of ubiquitination enzymes in C28 chondrocytes, while none were identified in JJ012 chondrosarcoma cells. While it seems counterintuitive, it may be that the absence of ubiquitination of certain factors leads to the downregulation of IL6 expression in human chondrosarcoma. Therefore, dysregulated ubiquitination may be among the possible mechanisms for the markedly reduced IL6 expression in chondrosarcoma.

Introduction

Chronic inflammation and cytokines serve important roles in cancer. Cytokines are secreted proteins that are involved in immune response, cell communications and, depending on cellular context, may be anti or pro-tumorigenic (1). Chondrosarcoma is a cancer of the cartilage with systemic

involvement. It is a common primary bone malignancy, accounting for 25% of bone sarcomas (2). Tumors typically develop in the pelvis, long bones and spine, as well as the larynx, head and neck (3). More aggressive forms of chondrosarcoma are characterized by early metastasis, and the metastasis rate of primary chondrosarcoma may reach 42%, and up to 86% in patients with local recurrence (4).

Chondrosarcoma does not typically respond to conventional therapies such as chemotherapy and radiation, which necessitates the identification of novel alternative therapies (2-7). The etiological factors and molecular pathways leading to the transformation of mesenchymal cells into sarcoma cells are unknown; therefore, understanding of the involvement of certain cytokines in failed differentiation programs leading to cancer or lost tumor suppressive functions is critical and of current interest (1). A previous study by our group reported from a human ELISA assay that the expression of interleukin 6 (IL6) in JJ012 chondrosarcoma cells was 86-fold lower than that in C28 chondrocytes, indicating it to be an anti-inflammatory and antitumorigenic factor (7). Furthermore, downregulation of IL6 has been reported for a number of tumor types, including undifferentiated thyroid carcinoma and thyroid cancer (8-10). To investigate IL6-protein interactions leading to these differences in IL6 expression, the present study assessed IL6 complexes in JJ012 and C28 cells through nuclear extraction and an electrophoretic mobility shift assay (EMSA), followed by 2D gel phoresis, in-gel trypsin digestion (11) and proteomic mass spectrometry (MS) analysis.

Materials and methods

Cell culture. Complete growth medium for human JJ012 chondrosarcoma cells were obtained from the laboratory of Dr Joel Block (Rush University Medical Centre, Chicago, IL, USA) and for C28 chondrocytes from the laboratory of Dr Sean Scully (University of Miami, Miami, FL, USA) comprised of the following: Dulbecco's modified Eagle's medium supplemented with F12, 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 25 µg/ml ascorbic acid, 100 ng/ml insulin, 100 nM hydrocortisone and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cultures were incubated for 24 h in a humidified 5% CO₂ incubator at 37°C.

Correspondence to: Dr Karina Galoian, Miller School of Medicine, Department of Orthopedics, University of Miami, 1600 NW 10th Avenue, Suite 8012, Miami, FL 33136, USA
E-mail: kgaloian@med.miami.edu

Key words: cytokines, mass spectrometry, interleukin 6, chondrosarcoma, chondrocytes, ubiquitination

Nuclear extraction and EMSA, and SDS-PAGE and IL6 oligonucleotide forward and reverse sequence synthesis.

These procedures were performed as described in our recent study (7).

2D gel electrophoresis and in-gel trypsin digestion. The procedures were performed according to previously described methods (11) with slight modifications.

For the 2D gel electrophoresis (modified 2D EMSA), a gel shift EMSA kit (cat. no. 37341) from Active Motif, Inc. (Carlsbad, CA, USA) was used. The procedures from steps 1 to 5 as detailed in the EMSA kit manual were performed according to manufacturer's instructions. From step 6, gel transfer was performed to polyvinylidene difluoride membranes. For blot extraction, the membrane was divided into eight vertical slices and soaked twice in 1 ml extraction buffer (50 mM Tris-HCL, pH 9, 50 mM dithiothreitol and 0.5% Tween-20) for 2 h at room temperature with gentle shaking. The detergent was then removed using a Pierce Detergent spin column from Thermo Fisher Scientific, Inc. (cat. no. 87779), as instructed by the column manufacturer. The proteins in the extract were concentrated using Amicon Ultra 2 Centrifugal Filters with 10 kDa cut-off (cat. no. UGC501008; EMD Millipore, Billerica, MA, USA). The concentrated proteins were separated by SDS-PAGE as described previously (7).

For in-gel trypsin digestion, gel bands from the SDS PAGE were vertically cut into ten equal slices (1x1 mm). The gel pieces were dehydrated with acetonitrile (ACN) (Sigma-Aldrich; Merck KGaA), dried for 30 min and vacuumed for 10 min at 20°C, reduced in 150 μ l 10 mM dithiothreitol in 100 mM ACN at 56°C for 1 h, and then alkylated with 50 mM iodoacetamide (Sigma-Aldrich; Merck KGaA) in 100 mM ACN at room temperature for 1 h in the dark. Following these washing and dehydration steps, the gel cubes were digested in 30 μ l trypsin (20 ng/ μ l in 25 mM ACN) at 37°C overnight. The trypsin-digested peptides were subsequently extracted with 40 μ l 5% trifluoroacetic acid (Pierce; Thermo Fisher Scientific, Inc.) in 50% ACN for 1 h at room temperature. Finally, two rounds of drying by speed vacuum for 1 h at 45°C and resuspension in 20 μ l 0.1% trifluoroacetic acid were performed, followed by MS analysis.

Proteomic MS. Digestion mixtures were loaded onto a reversed-phase fused-silica capillary emitter column [75 μ m inner diameter x 15 cm, packed with Acclaim PepMap RSLC C18, 2 μ m, 100 Å (Thermo Fisher Scientific, Inc.)] connected to a precolumn [Acclaim PepMap 100, 75 μ m x 2 cm, packed with nanoviper C18, 3 μ m, 100 Å (Thermo Fisher Scientific, Inc.)]. For ultra high performance liquid chromatography (UHPLC), the column and precolumn were connected in-line to an Easy Nano LC 1000 UHPLC system (Thermo Fisher Scientific, Inc.) and were equilibrated and washed with water [Optima LC/MS Grade (W6-1) from Fisher Chemical; Thermo Fisher Scientific, Inc.] and were equilibrated and washed with water [Optima LC/MS Grade (W6-1) from Fisher Chemical; Thermo Fisher Scientific, Inc.]. Solvent A and solvent B were water and ACN [Fisher Chemical Optima LC/MS Grade (A955-1L); Thermo Fisher Scientific, Inc.] respectively. The peptides were gradient-eluted following application of solvent B from 2 to 98% at a flow of 350 nl/min for 1 h at 20°C, eluting 300 μ l at a maximum pressure of 980 bar. Following elution, the samples were resuspended in 2% ACN, and analyzed with

a Q Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Inc.). The column was fixed to a Nanospray Flex ion source from Thermo Fisher Scientific, Inc. Sheath, auxiliary and sweep gas were set to zero, and the run was in positive mode. The mass spectrometer was operated in data-dependent mode, with an automatic gain control target of $1e^6$ for full MS and $2e^5$ for data dependent-MS/MS. The isolation window was fixed to 1.3 m/z with a normalized collision energy of 28 eV. Bioinformatics analysis was performed using Thermo Proteome Discoverer v1.4 (Thermo Fisher Scientific, Inc.). Data were processed against *Homo sapiens* data in the Uniprot database (<http://uniprot.org>), allowing two missed cleavages, 10 ppm as a precursor mass tolerance and 0.02 Da for fragment mass tolerance.

Results

MS analysis of IL6-protein complexes. Tables I and II list the proteins complexed with IL6 identified by proteomic MS analysis in C28 chondrocytes and JJ012 chondrosarcoma cells, respectively. A population of ubiquitination enzymes (<2%) were detected in C28 chondrocytes, while none were expressed in JJ012 chondrosarcoma cells. Among these enzymes were enzymes of ubiquitination machinery, namely E3 ubiquitin ligases, including E3 ubiquitin-protein ligase SH3 domain-containing ring finger 1, E3 ubiquitin-protein ligase ring finger protein 169 and E3 SUMO-protein ligase Ran-binding protein 2, as well as ubiquitin carboxyl-terminal hydrolase 33 (USP33).

Discussion

Downregulation of IL6 expression has been observed in different tumors and is disease specific, though the cause remains unknown. Previous identification of significant downregulation of IL6 in human JJ012 chondrosarcoma cells compared with C28 chondrocytes prompted the current investigation into the mechanisms of this downregulation. In chondrosarcoma, IL6 may serve as an anti-inflammatory and anti-tumorigenic factor, based on our previous data that IL6 was downregulated by 86-fold in human chondrosarcoma compared with human C28 chondrocytes (7), indicating the possibility of a program in tumor cells with the ability to repress IL6 expression.

In the present study, a gel shift assay indicated the presence of IL6-protein complexes in C28 chondrocytes and JJ012 chondrosarcoma cells (data not shown), because we previously demonstrated (7). 2D gel electrophoresis and in-gel trypsin digestion of IL6 bands were subsequently performed to identify the IL6-protein complexes and the mechanisms involved in C28 and JJ012 cells by MS. The MS analysis detected presence of E3 ubiquitin protein ligases and USP33 hydrolase complexed with IL6 in C28 chondrocytes. Although this ligase machinery comprised a small percentage of the total proteins identified, which were overlapping between the C28 and JJ012 groups or unidentified proteins in general, this result is noteworthy, as no ubiquitination enzymes were identified in JJ012 human chondrosarcoma cells. While there is substantial data on the antitumorigenic effect of IL6 in other tumors, including undifferentiated thyroid carcinoma, thyroid cancer

Table I. Mass spectrometry analysis of proteins complexed with IL6 in human C28 chondrocytes.

Protein name	Accession no.
Ubiquitin carboxyl-terminal hydrolase 33 (fragment)	OS= <i>Homo sapiens</i> GN=USP33 PE=1 SV=7 - [E9PP47_HUMAN]
Ubiquitin-60S ribosomal protein L40	OS= <i>Homo sapiens</i> GN=UBA52 PE=1 SV=2 - [RL40_HUMAN]
Sterile alpha motif domain containing 11 splice variant ASV43	OS= <i>Homo sapiens</i> GN=SAMD11 PE=2 SV=1 - [I7G293_HUMAN]
Dapper homolog 1 (fragment)	OS= <i>Homo sapiens</i> GN=DACT1 PE=1 SV=1 - [C9JGV7_HUMAN]
Sarcoma antigen NY-SAR-29 (fragment)	OS= <i>Homo sapiens</i> PE=2 SV=1 - [Q86WF2_HUMAN]
TBC1 domain family member 15 (fragment)	OS= <i>Homo sapiens</i> GN=TBC1D15 PE=1 SV=1 - [F8VV61_HUMAN]
cDNA FLJ61508, moderately similar to ATP-binding cassette sub-family B member 6, mitochondrial	OS= <i>Homo sapiens</i> PE=2 [B4E055_HUMAN]
Beta chimaerin isoform B2-CHNdel ex2-8,11-12	OS= <i>Homo sapiens</i> GN=CHN2 PE=2 SV=1 - [B3VCF8_HUMAN]
40S ribosomal protein S14	OS= <i>Homo sapiens</i> GN=RPS14 PE=1 SV=3 - [RS14_HUMAN]
Tenascin	OS= <i>Homo sapiens</i> GN=TNC PE=1 SV=1 - [F5H7V9_HUMAN]
Polypeptide N-acetylgalactosaminyltransferase 7 (fragment)	OS= <i>Homo sapiens</i> GN=GALNT7 PE=2 SV=1 - [Q68VJ4_HUMAN]
DnaJ homolog subfamily B member 11 (fragment)	OS= <i>Homo sapiens</i> GN=DNAJB11 PE=1 SV=7 - [H7C2Y5_HUMAN]
Profilaggrin (fragment)	OS= <i>Homo sapiens</i> PE=4 SV=1 - [Q01212_HUMAN]
60S ribosomal protein L27a	OS= <i>Homo sapiens</i> GN=RPL27A PE=1 SV=2 - [RL27A_HUMAN]
Protein FAM117B	OS= <i>Homo sapiens</i> GN=FAM117B PE=1 SV=2 - [F117B_HUMAN]
Heterogeneous nuclear ribonucleoprotein K (fragment)	OS= <i>Homo sapiens</i> GN=HNRNPK PE=1 SV=1 - [Q5T6W2_HUMAN]
Neuropilin 2	OS= <i>Homo sapiens</i> GN=NRP2 PE=2 SV=1 - [B7ZL68_HUMAN]
Tubulin alpha-8 chain (fragment)	OS= <i>Homo sapiens</i> GN=TUBA8 PE=1 SV=1 - [C9J2C0_HUMAN]
KAT8 regulatory NSL complex subunit 1	OS= <i>Homo sapiens</i> GN=KANSL1 PE=1 SV=1 - [A0A0G2JQF5_HUMAN]
Putative EGF-like and EMI domain-containing protein 1	OS= <i>Homo sapiens</i> GN=EGFEM1P PE=5 SV=1 - [EGFEM_HUMAN]
CDNA FLJ25460 fis, clone TST09046	OS= <i>Homo sapiens</i> GN=hCG_2004368 PE=2 SV=1 - [Q96LI4_HUMAN]
Ribosomal protein S6 kinase alpha-5	OS= <i>Homo sapiens</i> GN=RPS6KA5 PE=1 SV=1 - [KS6A5_HUMAN]
60S ribosomal protein L7a (fragment)	OS= <i>Homo sapiens</i> GN=RPL7A PE=1 SV=1 - [Q5T8U3_HUMAN]
Serine/threonine-protein kinase 35	OS= <i>Homo sapiens</i> GN=STK35 PE=1 SV=2 - [STK35_HUMAN]
cDNA FLJ45139 fis, clone BRAWH3039623	OS= <i>Homo sapiens</i> PE=2 SV=1 - [Q6ZSX8_HUMAN]
Elongation factor 1-alpha 1	OS= <i>Homo sapiens</i> GN=EEF1A1 PE=1 SV=1 - [EF1A1_HUMAN]
E3 ubiquitin-protein ligase SH3RF1	OS= <i>Homo sapiens</i> GN=SH3RF1 PE=1 SV=2 - [SH3R1_HUMAN]

Table I. Continued.

Protein name	Accession no.
Keratin, type II cytoskeletal 1 PE=1 SV=6 - Solute carrier family 45 member 4	OS= <i>Homo sapiens</i> GN=KRT1 [K2C1_HUMAN] OS= <i>Homo sapiens</i> GN=SLC45A4 PE=1 SV=2 - [S45A4_HUMAN]
Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 WD repeat-containing protein 87	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B4DNJ5_HUMAN] OS= <i>Homo sapiens</i> GN=WDR87 PE=1 SV=2 - [E7ESW6_HUMAN]
Prolow-density lipoprotein receptor-related protein 1	OS= <i>Homo sapiens</i> GN=LRP1 PE=1 SV=2 - [LRP1_HUMAN]
78 kDa glucose-regulated protein	OS= <i>Homo sapiens</i> GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]
cDNA FLJ46818 fis, clone TRACH3038399, highly similar to eukaryotic translation initiation factor 2-alpha kinase 3 E3 SUMO-protein ligase RanBP2	(EC 2.7.11.1) [B3KY45_HUMAN] OS= <i>Homo sapiens</i> GN=RANBP2 PE=1 SV=2 - [RBP2_HUMAN]
Interferon receptor 1 isoform 4	OS= <i>Homo sapiens</i> PE=2 SV=1 - [A0A0U1SHW4_HUMAN]
cDNA FLJ16282 fis, clone NT2RI3005416, highly similar to F-box only protein 18 (EC 3.6.1.-) Vinculin	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B3KV95_HUMAN] OS= <i>Homo sapiens</i> GN=VCL PE=1 SV=4 - [VINC_HUMAN]
Chymotrypsinogen B	OS= <i>Homo sapiens</i> GN=CTRB1 PE=2 SV=1 - [CTRB1_HUMAN]
MAP7 domain-containing protein 3 (fragment)	OS= <i>Homo sapiens</i> GN=MAP7D3 PE=1 SV=1 - [A0A0A0MRP0_HUMAN]
Enolase 4	OS= <i>Homo sapiens</i> GN=ENO4 PE=4 SV=1 - [A6NI74_HUMAN]
Myozenin-2	OS= <i>Homo sapiens</i> GN=MYOZ2 PE=1 SV=1 - [MYOZ2_HUMAN]
Docking protein 6	OS= <i>Homo sapiens</i> GN=DOK6 PE=1 SV=1 - [DOK6_HUMAN]
Collagen alpha-1(XIV) chain	OS= <i>Homo sapiens</i> GN=COL14A1 PE=1 SV=3 - [COEA1_HUMAN]
Zinc finger protein 605	OS= <i>Homo sapiens</i> GN=ZNF605 PE=2 SV=1 - [ZN605_HUMAN]
E3 ubiquitin-protein ligase RNF169	OS= <i>Homo sapiens</i> GN=RNF169 PE=1 SV=2 - [RN169_HUMAN]
Keratin, type I cytoskeletal 9	OS= <i>Homo sapiens</i> GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]
ATP synthase subunit alpha, mitochondrial	OS= <i>Homo sapiens</i> GN=ATP5A1 PE=1 SV=1 - [ATPA_HUMAN]

and bladder carcinoma (7-10,12), to the best of our knowledge, this is the first report on the potential antitumorigenic and anti-inflammatory role of IL6 in a human chondrosarcoma cell line.

It appeared inconsistent that ubiquitination machinery was prevalent in C28 cells and lacking in JJ012 cells, considering that ubiquitination generally targets proteins to proteosomal degradation, and that our previous results indicated significant downregulation of IL6 in JJ012 cells compared with C28 chondrocytes (5). However, it is possible that the absence of

ubiquitination of certain unknown factors, to be determined in future studies, in JJ012 cells may lead to this downregulation of IL6.

Future *in vivo* experiments utilizing IL6 overexpression or knockdown in xenografts models may aid to elucidate the mechanisms of IL6 in human chondrosarcoma cells, and verify its antitumorigenic property and involvement in the process of differentiation. Following confirmation of the potential anti-proliferative function of IL6 *in vivo*, the next challenge will be 'targeting the absence' design experiments

Table II. Mass spectrometry analysis of proteins complexed with IL6 in human JJ012 chondrosarcoma cells.

Protein name	Accession no.
Nephronectin (fragment)	OS= <i>Homo sapiens</i> GN=NPNT PE=4 SV=1 - [H0YA60_HUMAN]
Keratin-associated protein 19-1	OS= <i>Homo sapiens</i> GN=KRTAP19-1 PE=2 SV=2 - [KR191_HUMAN]
Ubiquitin-60S ribosomal protein L40	OS= <i>Homo sapiens</i> GN=UBA52 PE=1 SV=2 - [RL40_HUMAN]
Epithelial cell-transforming sequence 2 oncogene-like (fragment)	OS= <i>Homo sapiens</i> GN=ECT2L PE=4 SV=1 - [B7ZBI6_HUMAN]
Sodium/potassium-transporting ATPase subunit beta-3	OS= <i>Homo sapiens</i> GN=ATP1B3 PE=1 SV=1 - [C9J6S2_HUMAN]
TBC1 domain family member 15 (fragment)	OS= <i>Homo sapiens</i> GN=TBC1D15 PE=1 SV=1 - [F8VV61_HUMAN]
cDNA FLJ61508, moderately similar to ATP-binding cassette sub-family B member 6, mitochondrial	OS= <i>Homo sapiens</i> PE=2 SV=1 [B4E055_HUMAN]
ATP-binding cassette sub-family B member 8, mitochondrial	OS= <i>Homo sapiens</i> GN=ABC8 PE=4 SV=1 - [F8WC43_HUMAN]
Heterogeneous nuclear ribonucleoprotein C-like 2	OS= <i>Homo sapiens</i> GN=HNRNPCL2 PE=4 SV=1 - [A0A0G2JNQ3_HUMAN]
Putative EGF-like and EMI domain-containing protein 1	OS= <i>Homo sapiens</i> GN=EGFEM1P PE=5 SV=1 - [EGFEM_HUMAN]
cDNA FLJ13888 fis, clone THYRO1001584	OS= <i>Homo sapiens</i> PE=2 SV=1 - [Q9H880_HUMAN]
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	OS= <i>Homo sapiens</i> GN=LHPP PE=1 SV=2 - [LHPP_HUMAN]
Uncharacterized protein (fragment)	OS= <i>Homo sapiens</i> PE=4 SV=2 - [H7C1N6_HUMAN]
40S ribosomal protein S14	OS= <i>Homo sapiens</i> GN=RPS14 PE=1 SV=3 - [RS14_HUMAN]
Chromosome 5 open reading frame 4, isoform CRA_a	OS= <i>Homo sapiens</i> GN=C5orf4 PE=2 SV=1 - [Q9UHK3_HUMAN]
60S ribosomal protein L21	OS= <i>Homo sapiens</i> GN=RPL21 PE=1 SV=1 - [G3V1B3_HUMAN]
S100P binding protein isoform 2	OS= <i>Homo sapiens</i> GN=S100PBP PE=2 SV=1 - [A0A0S2Z5S2_HUMAN]
cDNA FLJ26027 fis, clone PNC04328, highly similar to <i>Homo sapiens</i> translocase of outer mitochondrial membrane 34	(TOMM340) [Q6ZPD_HUMAN]
cDNA FLJ56407, highly similar to SLIT-ROBO Rho GTPase-activating protein 2	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B4DFE5_HUMAN]
60S ribosomal protein L27a	OS= <i>Homo sapiens</i> GN=RPL27A PE=1 SV=2 - [RL27A_HUMAN]
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B4DL99_HUMAN]
Neuropilin 2	OS= <i>Homo sapiens</i> GN=NRP2 PE=2 SV=1 - [B7ZL68_HUMAN]
60S ribosomal protein L31	OS= <i>Homo sapiens</i> GN=RPL31 PE=1 SV=1 - [RL31_HUMAN]
Protein spinster homolog 2	OS= <i>Homo sapiens</i> GN=SPNS2 PE=1 SV=2 - [SPNS2_HUMAN]
V-type proton ATPase proteolipid subunit	OS= <i>Homo sapiens</i> GN=ATP6V0C PE=1 SV=1 - [H3BNI4_HUMAN]
cDNA FLJ78084, highly similar to <i>Homo sapiens</i> cell division cycle associated 8, mRNA	OS= <i>Homo sapiens</i> PE=2 SV=1 - [A8K7A2_HUMAN]
Protein PMF1-BGLAP	OS= <i>Homo sapiens</i> GN=PMF1-BGLAP PE=4 SV=1 - [A0A087WT04_HUMAN]

Table II. Continued.

Protein name	Accession no.
Nuclear factor of-activated T-cells, cytoplasmic 4 (fragment)	OS= <i>Homo sapiens</i> GN=NFATC4 PE=1 SV=7 - [G3V5H0_HUMAN]
Scavenger receptor class F member 1	OS= <i>Homo sapiens</i> GN=SCARF1 PE=1 SV=1 - [A0A0A0MR54_HUMAN]
m7GpppN-mRNA hydrolase (fragment)	OS= <i>Homo sapiens</i> GN=DCP2 PE=1 SV=2 - [H0Y9T5_HUMAN]
Heterogeneous nuclear ribonucleoprotein K (fragment)	OS= <i>Homo sapiens</i> GN=HNRNPK PE=1 SV=1 - [Q5T6W2_HUMAN]
60S ribosomal protein L7a (fragment)	OS= <i>Homo sapiens</i> GN=RPL7A PE=1 SV=1 - [Q5T8U3_HUMAN]
Elongation factor 1-alpha 1	OS= <i>Homo sapiens</i> GN=EEF1A1 PE=1 SV=1 - [EF1A1_HUMAN]
78 kDa glucose-regulated protein	OS= <i>Homo sapiens</i> GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]
Zinc finger protein 90 homolog	OS= <i>Homo sapiens</i> GN=ZFP90 PE=1 SV=2 - [ZFP90_HUMAN]
Putative uncharacterized protein PCYOX1 (fragment)	OS= <i>Homo sapiens</i> GN=PCYOX1 PE=4 SV=1 - [Q584P1_HUMAN]
G-protein-signaling modulator 1	OS= <i>Homo sapiens</i> GN=GPSM1 PE=1 SV=2 - [GPSM1_HUMAN]
Vinculin	OS= <i>Homo sapiens</i> GN=VCL PE=1 SV=4 - [VINC_HUMAN]
DNA repair protein REV1	OS= <i>Homo sapiens</i> GN=REV1 PE=2 SV=1 - [A1L461_HUMAN]
CDNA FLJ25612 fis, clone STM01228	OS= <i>Homo sapiens</i> PE=2 SV=1 - [Q8N1H3_HUMAN]
Zinc finger protein 92 homolog	OS= <i>Homo sapiens</i> GN=ZFP92 PE=2 SV=3 - [ZFP92_HUMAN]
Leucine-rich repeat-containing protein 59	OS= <i>Homo sapiens</i> GN=LRR59 PE=1 SV=1 - [LRC59_HUMAN]
KN motif and ankyrin repeat domain-containing protein 2	OS= <i>Homo sapiens</i> GN=KANK2 PE=1 SV=1 - [KANK2_HUMAN]
Retrotransposon-derived protein PEG10	OS= <i>Homo sapiens</i> GN=PEG10 PE=1 SV=1 - [A0A087WZG9_HUMAN]
Fibrillin-1	OS= <i>Homo sapiens</i> GN=FBN1 PE=1 SV=3 - [FBN1_HUMAN]
Anthrax toxin receptor 2	OS= <i>Homo sapiens</i> GN=ANTXR2 PE=1 SV=1 - [J3KPY9_HUMAN]
ATP synthase subunit alpha, mitochondrial	OS= <i>Homo sapiens</i> GN=ATP5A1 PE=1 SV=1 - [ATPA_HUMAN]
ADAM9 protein	OS= <i>Homo sapiens</i> GN=ADAM9 PE=1 SV=1 - [A0AVL1_HUMAN]
cDNA FLJ50932, highly similar to Zinc finger protein basonuclin-1	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B7Z885_HUMAN]
Vigilin (fragment)	OS= <i>Homo sapiens</i> GN=HDLBP PE=1 SV=1 - [H7BZC3_HUMAN]
Alpha-protein kinase 1	OS= <i>Homo sapiens</i> GN=ALPK1 PE=2 SV=3 - [ALPK1_HUMAN]
Neuroblast differentiation-associated protein AHNAK	OS= <i>Homo sapiens</i> GN=AHNAK PE=1 SV=2 - [AHNK_HUMAN]
Zinc finger protein ZFP69	OS= <i>Homo sapiens</i> GN=ZFP69 PE=2 SV=2 - [ZFP69_HUMAN]
DNA polymerase epsilon catalytic subunit A	OS= <i>Homo sapiens</i> GN=POLE PE=1 SV=5 - [DPOE1_HUMAN]

Table II. Continued.

Protein name	Accession no.
cDNA FLJ58382, highly similar to Zinc finger protein 8	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B4DSF4_HUMAN]
Chymotrypsinogen B	OS= <i>Homo sapiens</i> GN=CTRB1 PE=2 SV=1 - [CTRB1_HUMAN]
Probable ATP-dependent RNA helicase DDX46	OS= <i>Homo sapiens</i> GN=DDX46 PE=1 SV=2 - [DDX46_HUMAN]
cDNA FLJ54652, highly similar to <i>Homo sapiens</i> podocan (PODN), mRNA	OS= <i>Homo sapiens</i> PE=2 SV=1 - [B4DUY6_HUMAN]
Myozenin-2	OS= <i>Homo sapiens</i> GN=MYOZ2 PE=1 SV=1 - [MYOZ2_HUMAN]
Thyroid adenoma-associated protein	OS= <i>Homo sapiens</i> GN=THADA PE=1 SV=1 - [THADA_HUMAN]
CLIP-associating protein 2	OS= <i>Homo sapiens</i> GN=CLASP2 PE=1 SV=1 - [E7ERI8_HUMAN]
Calcium/calmodulin-dependent protein kinase II alpha	OS= <i>Homo sapiens</i> GN=CAMK2A PE=2 SV=1 - [Q8IWE0_HUMAN]
Zinc finger protein 605	OS= <i>Homo sapiens</i> GN=ZNF605 PE=2 SV=1 - [ZN605_HUMAN]
Far upstream element-binding protein 2	OS= <i>Homo sapiens</i> GN=KHSRP PE=1 SV=4 - [FUBP2_HUMAN]
Putative uncharacterized protein XRCC5 (fragment)	OS= <i>Homo sapiens</i> GN=XRCC5 PE=4 SV=1 - [Q53T09_HUMAN]
Probable threonine-tRNA ligase 2, cytoplasmic	OS= <i>Homo sapiens</i> GN=TARSL2 PE=1 SV=1 - [SYTC2_HUMAN]
Translation initiation factor IF-2, mitochondrial	OS= <i>Homo sapiens</i> GN=MTIF2 PE=1 SV=2 - [IF2M_HUMAN]
Dystonin	OS= <i>Homo sapiens</i> GN=DST PE=1 SV=1 - [F8W9J4_HUMAN]
cDNA FLJ76304, highly similar to <i>Homo sapiens</i> ADAM metallopeptidase with thrombospondin type 1 motif, 4 (ADAMTS4), mRNA	OS= <i>Homo sapiens</i> [A8K6A8_HUMAN]
Polycystic kidney disease protein 1-like 1	OS= <i>Homo sapiens</i> GN=PKD1L1 PE=1 SV=1 - [PK1L1_HUMAN]
Centrosomal protein KIAA1731	OS= <i>Homo sapiens</i> GN=KIAA1731 PE=2 SV=4 - [K1731_HUMAN]
Ankyrin-3	OS= <i>Homo sapiens</i> GN=ANK3 PE=1 SV=3 - [ANK3_HUMAN]
ATP2B2 variant protein (fragment)	OS= <i>Homo sapiens</i> GN=ATP2B2 variant protein PE=2 SV=1 - [Q4LE63_HUMAN]

to investigate why there is downregulation of ubiquitination in chondrosarcoma, as well as the underlying mechanisms involved in this phenomena. A key approach will be to pursue studies on the posttranscriptional regulation of E3 ubiquitin ligase by miRNAs.

In conclusion, dysregulated ubiquitination may be a possible mechanism by which tumors exhibit the ability to repress IL6 expression. It is established that the microenvironments of cells, tissues and organs define gene expression. It has been demonstrated that IL6 is markedly downregulated in human chondrosarcoma cells compared with normal chondrocytes. This complies with the potential tumorigenicity and anti-inflammatory function of IL6 in chondrosarcoma. Therefore, identification of the mechanisms leading to IL6

downregulation may be important from a theoretical perspective and also for clinical practice, particularly regarding possible gene therapy applications.

Acknowledgements

The authors are thankful to Professor Sanjoy K. Bhattacharya and Ms. Maria del Carmen Piqueras from the Ophthalmology Mass Spectrometry shared instrument Core Facility of the University of Miami. The funding for mass spectrometry was provided by the National Eye Institute Ophthalmology Core Facility (grant no. P30EY14801; principal investigator Dr Vittorio Porciatti) and an unrestricted grant from Research to Prevent Blindness to the University

of Miami. The study was also supported in part by the Ratcliffe Foundation of the Miami Center of Orthopedic Research and Education.

References

1. Grivennikov SI and Karin M: Inflammation and oncogenesis: A vicious connection. *Curr Opin Genet Dev* 20: 65-71, 2010.
2. Andreou D, Ruppin S, Fehlberg S, Pink D, Werner M and Tunn PU: Survival and prognostic factors in chondrosarcoma: Results in 115 patients with long-term follow-up. *Acta Orthop* 82: 749-755, 2011.
3. Mirra J, Picci P and Gold RH: Bone Tumors: Clinical, Radiologic, and Pathologic Correlations. 2nd edition. Lea and Febiger, Philadelphia, 1989.
4. Ozaki T, Hillmann A, Lindner N, Blasius S and Winkelmann W: Metastasis of chondrosarcoma. *J Cancer Res Clin Oncol* 122: 625-628, 1996.
5. Galoian K, Scully S, McNamara G, Flynn P and Galoyan A: Antitumorigenic effect of brain proline rich polypeptide-1 in human chondrosarcoma. *Neurochem Res* 34: 2117-2121, 2009.
6. Galoian K, Temple HT and Galoyan A: mTORC1 inhibition and ECM-cell adhesion-independent drug resistance via PI3K-AKT and PI3K-RAS-MAPK feedback loops. *Tumour Biol* 33: 885-890, 2012.
7. Galoian K, Luo S, Qureshi A, Patel P, Price R, Morse AS, Chailyan G, Abrahamyan S and Temple HT: Effect of cytostatic proline rich polypeptide-1 on tumor suppressors of inflammation pathway signaling in chondrosarcoma. *Mol Clin Oncol* 5: 618-624, 2016.
8. Revel M: Growth regulatory functions of IL6 and antitumour effects. *Res Immunol* 143: 769-773, 1992.
9. Basolo F, Fiore L, Pollina L, Fontanini G, Conaldi PG and Toniolo A: Reduced expression of interleukin 6 in undifferentiated thyroid carcinoma: In vitro and in vivo studies. *Clin Cancer Res* 4: 381-387, 1998.
10. Mulé JJ, McIntosh JK, Jablons DM and Rosenberg SA: Antitumor activity of recombinant interleukin 6 in mice. *J Exp Med* 171: 629-636, 1990.
11. Jiang D, Jia Y and Jarrett HW: Transcription factor proteomics: Identification by a novel gel mobility shift-three-dimensional electrophoresis method coupled with southwestern blot and high-performance liquid chromatography-electrospray-mass spectrometry analysis. *J Chromatogr A* 1218: 7003-7015, 2011.
12. Tsui KH, Wang SW, Chung LC, Feng TH, Lee TY, Chang PL and Juang HH: Mechanisms by which interleukin-6 attenuates cell invasion and tumorigenesis in human bladder carcinoma cells. *Biomed Res Int* 2013: 791212, 2013.