Establishment and Characterization of a Novel Dedifferentiated Chondrosarcoma Cell Line DDCS2

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Abstract

Background: The dedifferentiated variant of chondrosarcoma is highly aggressive and carries an especially grim prognosis. While chemotherapeutics has failed to benefit patients with dedifferentiated chondrosarcoma significantly, preclinical chemosensitivity studies have been limited by a scarcity of available cell lines. There is, therefore, an urgent need to expand the pool of available cell lines.

Methods: We report the establishment of a novel dedifferentiated chondrosarcoma cell line DDCS2, which we isolated from the primary tumor specimen of a 60-year-old male patient. We characterized its short tandem repeat (STR) DNA profile, growth potential, antigenic markers, chemosensitivity, and oncogenic spheroid and colony-forming capacity.

Results: DDCS2 showed a spindle to polygonal shape and an approximate 60-hour doubling time. STR DNA profiling revealed a unique genomic identity not matching any existing cancer cell lines within the ATCC, JCRB, or DSMZ databases. There was no detectable contamination with another cell type. Western blot and immunofluorescence assays were consistent with a mesenchymal origin, and our MTT assay revealed relative resistance to conventional chemotherapeutics, which is typical of a dedifferentiated chondrosarcoma. Under *ex vivo* three-dimensional (3D) culture conditions, the DDCS2 cells produced spheroid patterns similar to the well-established CS-1 and SW1353 chondrosarcoma cell lines.

Conclusion: Our findings confirm DDCS2 is a novel model for dedifferentiated chondrosarcoma and therefore adds to the limited pool of current cell lines urgently needed to investigate the chemoresistance within this deadly cancer.

Keywords
dedifferentiated chondrosarcoma, cell line, DNA profiling, chemotherapy, 3D culture

Highlights

(1) The dedifferentiated variant of chondrosarcoma is highly aggressive and resistant to currently used chemotherapeutics.

(2) There is a scarcity of dedifferentiated chondrosarcoma cell lines available for anticancer research.

(3) Our novel cell line DDCS2 accurately models the qualities of dedifferentiated chondrosarcoma and adds to the limited pool of quality cell lines.

Introduction

Chondrosarcoma is a heterogeneous cartilage-forming malignancy that is the second most common primary bone tumor

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following osteosarcoma.[1] As chondrosarcoma is resistant to both chemotherapy and radiotherapy, wide local excision remains the only treatment option for patients.[2] Current clinical outcomes largely correlate with the histological grading system described by the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone.[3] Chondrosarcomas are classified into several subtypes, including the central conventional subtype, which is the most common, and rarer subtypes such as secondary, periosteal, clear cell, and dedifferentiated.[4]

Dedifferentiated chondrosarcoma is a highly aggressive variant accounting for 11% of chondrosarcomas with a predilection for the humerus, femur, and pelvis.[5,6] Compared to the conventional subtype, dedifferentiated chondrosarcoma presents later in life as it mostly affects patients between 50 and 60 years of age.[7] Furthermore, dedifferentiated chondrosarcoma is highly metastatic and recurrent.[5] Its aggression coupled with the limitations of currently used therapies have led to a poor prognosis, with a median survival of approximately 7.5 months and a 5-year disease-free survival rate of only 7.1%.[8] These dismal patient outcomes highlight the urgent need for preclinical models of dedifferentiated chondrosarcoma so that novel chemotherapeutics can be more accurately investigated in this heterogenous cancer.[9] Currently, there is a scarcity of cell lines derived from dedifferentiated chondrosarcoma. Of those that do exist, their molecular and genetic features are limited and do not encompass the variability seen amongst patients. In this study, we report the establishment and characterization of a novel dedifferentiated chondrosarcoma cell line DDCS2, adding to the pool of cell lines available for future preclinical anticancer investigations.

Materials and Methods

Harvest and Culture of Cells

Tumor tissue was collected then cultured from a resected primary tumor of the right distal femur from a 60-year-old male patient with suspected L4 spinal metastasis. The diagnosis of dedifferentiated chondrosarcoma was confirmed by pathologists specializing in bone tumor pathology. Prior to surgery, written informed consent was obtained from the donor regarding removal and research of the tumor tissue. All procedures and experiments were approved and carried out in accordance with the UCLA Institutional Review Board (IRB#19-000096). No chemotherapy or radiotherapy was performed before resection of the primary tumor. The specimen was collected and anonymously encoded within the operating room. The tissue samples were placed in sealed microcentrifuge tubes with 0.9% sterile saline on ice for direct transport to the laboratory, where they were washed several times with sterilized PBS solution containing 500U/mL penicillin/streptomycin and minced with razor blades. The cells were harvested in flasks and cultured in complete RPMI

1640 media, supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, US) and 100U/mL penicillin/streptomycin (Life Technologies, US). The cells were trypsinized (0.25% trypsinization, Life Technologies, US) once the fragments adhered to the flasks and grew to confluence. The proliferating cancer cells were separated from normal fibroblasts *via* differential trypsinization and multiple passages.[10,11]

The human chondrosarcoma cell line SW1353, osteosarcoma cell lines MG63 and MNNG/HOS, Ewing's sarcoma cell line TC71, cervical cancer cell line Hela, and breast cancer cell line MCF7 were purchased from American Type Tissue Collection (ATCC, US). The chondrosarcoma cell line CS-1 was established in our laboratory as reported previously.[12,13] The SW1353, CS-1, MG63, MNNG/HOS, TC71, Hela, and MCF7 cells were cultured in complete RPMI 1640 media. All cell lines were grown in a humidified 37°C incubator with 5% CO_2.

Cell Growth Characterization

For cell growth and viability analysis, we seeded 2×10^3 tissue-derived DDCS2 cells into each well of a 96-well culture plate and incubated them to several time points over 7 days. At the end of each time point, 15 μL of MTT (5 mg/mL, Sigma-Aldrich, US) was added to each well, and the plates were incubated at 37°C for 4 h. 100 μL of acid isopropanol was used to dissolve the formazan products. We measured absorbance at a wavelength of 490 nm (A490) to determine the growth curve. All experiments were performed in triplicate.

Cell Line Identity

DNA isolation from the primary tissue specimens and cultured cell pellets was performed using the QIAamp DNA Micro kit (QIAGEN, US) according to the manufacturer's instructions. The DNA concentrations were assessed using ultraviolet light spectrophotometry at 260 nm, and the quality was checked through 1% agarose gel stained with ethidium bromide. Cell line authentication and identification were confirmed by Bio-Synthesis services (Lewisville, US) *via* analyzation of short tandem repeats (STR). STR DNA profiling of the samples was carried out with the AmpFℓSTR® Identifiler® for high resolution screening and intra-species cross-contamination detection. The genotype of the DDCS2 cells was matched to verify patient origin. We compared DDCS2 with a number of recognized repositories, including the ATCC, the Japanese Collection of Research Bioresources (JCRB), and the Deutsche Sammlung von Mikrorganismen und Zellkulturen (DSMZ). Data were analyzed using GeneMapper ID® v3.2 Software (Applied Biosystems, US).

Molecular Marker Assessment

We extracted protein lysates with 1×RIPA lysis buffer (Upstate Biotechnology, US) containing complete protease

inhibitor cocktail tablets (Roche Applied Science, US). Concentrations were determined using DC™ protein assay reagents (BIO-RAD, US) and a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., US). We separated equal amounts of protein by NuPAGE® 4–12% Bis-Tris Gel (Invitrogen, US) and transferred them to nitrocellulose membranes (BIO-RAD, US). The membranes were then blocked with 5% non-fat milk in 1×TBST for 1 h, then incubated at 4°C overnight with primary antibodies, including p53 (#2524, 1:1,000 dilution, Cell Signaling Technology, US), cytokeratin (#MA5-13203, 1: 1,000 dilution, Invitrogen, US), mouse double minute 2 homolog (MDM2, # sc-56154, 1:500 dilution, Santa Cruz, US), vimentin (#PA5-27231, 1: 5,000 dilution, Invitrogen, US), and β-actin (#A1978, 1:5000 dilution, Sigma-Aldrich, US). We then washed the membranes with $1 \times$ TBST three times for 5 minutes each before they underwent probing with secondary antibodies, IRDye® 800CW and IRDye® 680LT (LI-COR Biosciences, US) for 2 h at room temperature. The membranes were then washed with $1 \times$ TBST for 5 minutes three separate times before analysis of the bands with the Odyssey Infrared Fluorescent Western Blot Imaging System (Li-COR Bioscience, US) with Odyssey software 3.0 (Li-COR Bioscience, US). The optical density values were recorded with Image J software (NIH Image, Bethesda, MD) and the detection was collected in triplicate.

Immunofluorescence Assays

The expression of vimentin was evaluated by immunofluorescence analysis. Suspensions of 2×10^4 cells/mL of DDCS2, CS-1, and SW1353 were added into 24-well plates and incubated for 1 day. They were then fixed for 15 min with 3.7% paraformaldehyde, permeabilized for 20 min with ice-cold methanol, then blocked for 1 h with 1% bovine serum albumin (BSA). They were then incubated at 4°C overnight with the primary antibodies vimentin (#PA5-27231, 1:200 dilution), isocitrate dehydrogenase 2 (IDH2, #60322, 1:200 dilution, Cell Signaling Technology, US), cyclin-dependent kinase 4 (CDK4, #12790, 1:200 dilution, Cell Signaling Technology, US), and β-Actin (#A1978, 1:200 dilution). The following day, they were incubated for 1 h at room temperature with the secondary antibodies Alexa Fluor 488 (#A-11008, 1:1000 dilution, Invitrogen, US) and Alexa Fluor 594 (#A-11005, 1: 1000 dilution, Invitrogen, US). Hoechst 33342 (#H3570, 1: 10000, Life Technologies Corp., US) was applied for 5 min at room temperature for nuclear staining. The Nikon Eclipse Ti-U fluorescence microscope (Diagnostic Instruments Inc., US) attached with a SPOT RT™ digital camera was used for fluorescence images.

Chemotherapeutic Sensitivity Assays

MTT assays were performed to detect cytotoxicity *in vitro*. Our cell line DDCS2, the chondrosarcoma cell lines CS-1 and SW1353 were seeded at 2×10^3 cells/well alongside several

chemotherapeutics. Specifically, various concentrations of doxorubicin (up to 100 μM), cisplatin (up to 100 μM), or paclitaxel (up to 0.1 μM) were added during incubation for 3, 5, or 7 days. Every alternate day, absorbance values were evaluated at a wavelength of 490 on a SpectraMax Microplate® Spectrophotometer (Molecular Devices LLC, CA, US). The cell viability response to the various concentrations of chemotherapeutics was assessed using the same procedure in triplicate. The Zeiss microscope (Carl Zeiss, Inc., Germany) equipped with a Nikon D40 digital camera (NikonCorp., US) was used to take light microscope images after 5 days of chemotherapeutic treatment.

Ex Vivo Three-Dimensional Cell Culturing and Colony Formation

The *ex vivo* 3D culture model creates an artificial environment that simulates *in vivo* cell growth. The hydrogel *ex vivo* 3D culture system (Well Bioscience, US) was prepared according to the manufacturer's protocol. 250 μL of cell suspensions (2 × 10^4 cells/mL) of DDCS2, the chondrosarcoma cell lines CS-1 and SW1353, and the osteosarcoma cell lines MG63 and MNNG/HOS were mixed with the hydrogel 3D culture and seeded into 24-well culture plates. An additional 250 μL of complete RPMI 1640 media was used to cover the hydrogel for system preparation. We incubated the *ex vivo* 3D culture models at 37°C in a humidified atmosphere with 5% CO_2. The upper RPMI 1640 media component was replaced every other day to provide enough nutrients for cell growth and to prevent a shift in media osmolality. Over the 3-week period we captured light microscope images of DDCS2, CS-1, and SW1353 cell spheroids with a microscope equipped with a digital camera with image analyzing software (Carl Zeiss, Inc., Germany). At the end time point, the cells were incubated with 1 μM Calcein AM (Life Technologies, US) and images of the cell spheroids were taken with a Nikon Eclipse Ti-U inverted fluorescence microscope (Nikon Instruments Inc, US). The size of the cell spheroids was calculated using ImageJ software (NIH Image, Bethesda, MD). The *ex vivo* 3D culture models were conducted in triplicate.

For the clonogenic assays, 2×10^2 cells of DDCS2, CS-1, and SW1353 were added to each well of 6-well plates. The cells were cultured in RPMI 1640 media containing 10% FBS without antibiotics and incubated at 37°C for 2 weeks. After methanol-fixed and Giemsa-stained (Sigma-Aldrich, US), the cell colonies were counted under a microscope and images were captured. The experiments were conducted in triplicate.

Statistical Analysis

GraphPad PRISM 8 software (GraphPad Software, CA, US) was used for analyses, and data were expressed as mean ± SD. A two-tailed Student's t-test was performed to evaluate

significance between groups. Differences were considered statistically significant at P<0.05.

Results

Clinicopathological Data

The initial tissue specimen was retrieved from a resected primary tumor of the right distal femur from a 60-year-old male patient with dedifferentiated chondrosarcoma. Resection of the intercalary segment of the distal femur was performed and followed up with a thorough histological examination according to Evans histological grading criteria.[14] MRI of the primary tumor displayed an $8 \times 3 \times 3$ cm area of marrow replacement with bony destruction of the distal femoral metadiaphysis, breach of medial cortex, and periosteal reaction, findings consistent with a low-grade chondrosarcoma (Figures 1A,B). Chest CT showed metastatic subpleural nodularity. Histology of the primary tumor revealed the juxtaposition of two intramedullary cell populations, a grade I chondrosarcoma and a high-grade anaplastic sarcoma (Figures 1C,D). Specifically, there were typical components adjacent to hyaline cartilage with myxoid change, as well as proliferation of malignant spindle cells arranged in distinct fascicles. These densely arranged spindle cells extensively invaded the cortical bone and had high mitotic activity (Figures 1E,F).

Cell Line Establishment and Growth Pattern

The *in vitro* cultured DDCS2 cells were routinely passaged for over 50 generations in a 6-month period. Light microscopy showed the DDCS2 cells to have a spindle to polygonal shape, oval nuclei, and elongated cytoplasmic processes consistent with other established malignant spindle cell lines (Figure 2A). The cells reached confluence with intermittent piled-up foci. The *in vitro* growth pattern of DDCS2 was assessed with a cell growth curve (Figure 2B). The population doubling time of the DDCS2 cells in the logarithmic growth phase was 60–72 h.
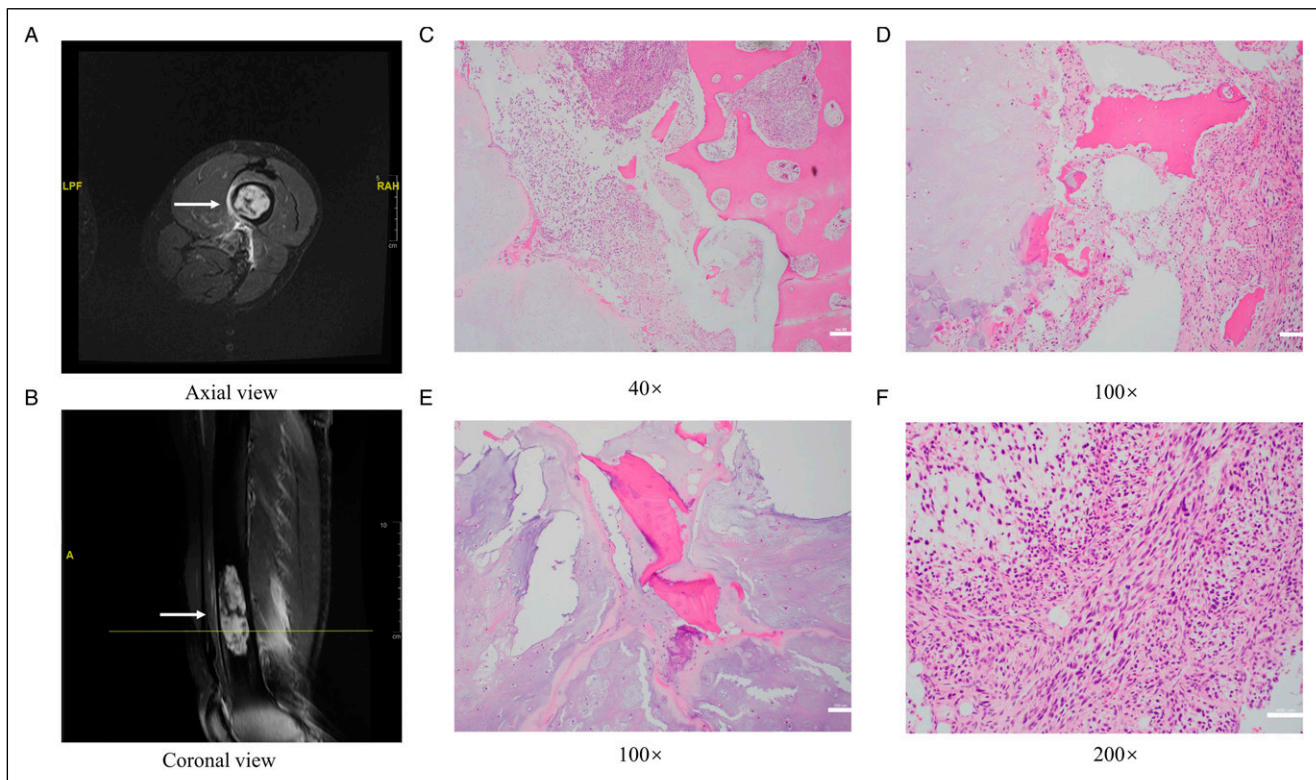
Figure 1. Clinical characteristics of the dedifferentiated chondrosarcoma donor. Radiographic features of the primary tumor. Axial **(A)** and coronal **(B)** views showed an $80 \times 26 \times 26$ mm focus of geographic marrow replacement/bony destruction involving the distal femoral metadiaphysis with marked attenuation/breach of the medial cortex, and early extraosseous spread of tumor/periosteal reaction of right femur. Arrow heads indicate the tumor areas. Pathologic features of the primary tumor. **(C)** Low-grade chondrosarcoma (bottom, left) adjacent to dedifferentiated chondrosarcoma was seen to invade cortical bone (right side) (H&E, 40×, scale bar: 50 μm). **(D)** Low-grade chondrosarcoma (left) and dedifferentiated chondrosarcoma (right side) in the same field (H&E, 100×, scale bar: 200 μm). **(E)** The permeate pattern of invasion of low-grade chondrosarcoma, surrounding fragments of cancellous bone (H&E, 100×, scale bar: 200 μm). **(F)** Dedifferentiated chondrosarcoma, characterized by a cellular proliferation of malignant spindle cells arranged in distinct fascicles (H&E, 200×, scale bar: 200 μm).
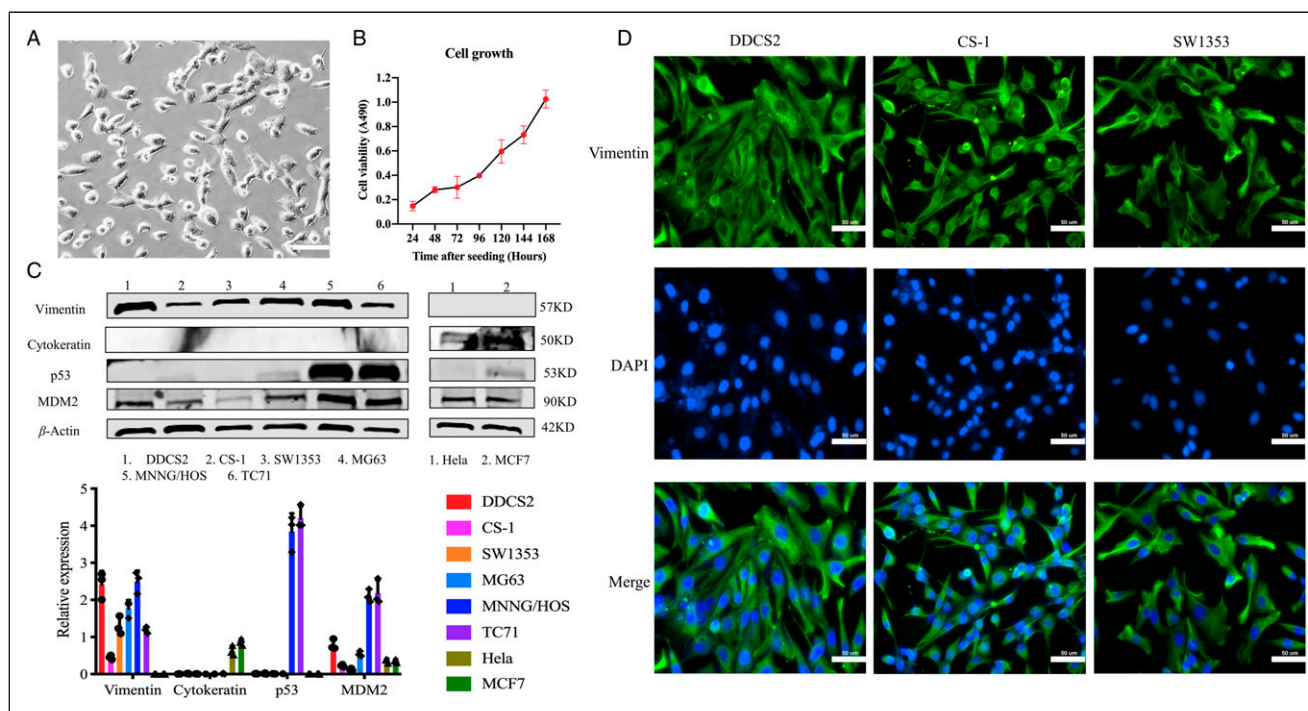
Figure 2. Morphologic characteristics and antigenic marker expression of the novel DDCS2 cell line. **(A)** DDCS2 cells display spindle to polygonal shape *in vitro* under light microscopy (200×, scale bar: 50 μm). **(B)** The growth curves of DDCS2 were plotted using cells plated in 96-well plates. **(C)** Western blots revealed the expression of vimentin, cytokeratin, p53, and MDM2 in various sarcoma cell lines compared with two epithelial carcinoma cell lines. Relative expressions of vimentin, cytokeratin, p53, and MDM2 were figured in different sarcoma and epithelial carcinoma cell lines compared with β-actin. **(D)** Immunofluorescence analysis of vimentin was conducted in DDCS2, CS-1, and SW1353 cell lines (200×, scale bar: 50 μm).

Tumor Marker Expression

The mesenchymal tissue marker vimentin, epithelial tissue marker cytokeratin, p53, and MDM2 were assessed by Western blot in several cell lines. The cell lines included dedifferentiated chondrosarcoma DDCS2, conventional chondrosarcomas CS-1 and SW1353, osteosarcomas MG63 and MNNG/HOS, Ewing's sarcoma TC71, and epithelial carcinomas including cervical cancer Hela and breast cancer MCF7. As expected, the sarcoma cell lines DDCS2, CS-1, SW1353, MG63, MNNG/HOS, and TC71 expressed vimentin but not cytokeratin, while the carcinoma cell lines Hela and MCF7 expressed cytokeratin but not vimentin. Loss or low expression of p53 was observed in DDCS2, CS-1, SW1353, MG63, Hela, and MCF7, while MDM2 was highly expressed in most cell lines except for the low-grade chondrosarcoma cell line SW1353. Relative expressions of vimentin, cytokeratin, p53, and MDM2 were compared with β-actin in sarcoma and epithelial carcinoma cell lines (Figure 2C). Our immunofluorescence analysis further confirmed expression of vimentin (Figure 2D), tumor-associated marker IDH2 and CDK4 (Supplementary Figure S2A) in DDCS2, a finding consistent with the conventional chondrosarcoma cell lines CS-1 and SW1353.

Cell Source Authentication

The identity of the DDCS2 cell line was confirmed by STR DNA profiling with bioinformatic analysis. To authenticate the cell line identity and exclude potential cross-contamination, we compared 15 core autosomal short tandem repeat (STR) loci and amelogenin in the original tumor tissue and passaged cells (Figure 3A). The data showed the DDCS2 cell DNA matched the original tumor tissue, was not contaminated with other cell types, and did not match existing cancer cell line profiles within the ATCC, JCRB, or DSMZ databases (Figure 3B). Overall, these STR assays further supported DDCS2 as a novel dedifferentiated chondrosarcoma cell line.

Chemotherapeutic Sensitivity

Viability assays were conducted on a monolayer of DDCS2 cells in standard culture conditions. The chemotherapeutic sensitivity of DDCS2 was compared with the chondrosarcoma cell lines CS-1 and SW1353. We evaluated their response to the three conventional chemotherapeutics doxorubicin, cisplatin, and paclitaxel. Similar to the CS-1 and SW1353 cell lines, the DDCS2 cells showed decreased cell viability in a dose and time-dependent manner to doxorubicin over 3, 5, and 7 days. Our MTT assay showed DDCS2 to have relative a
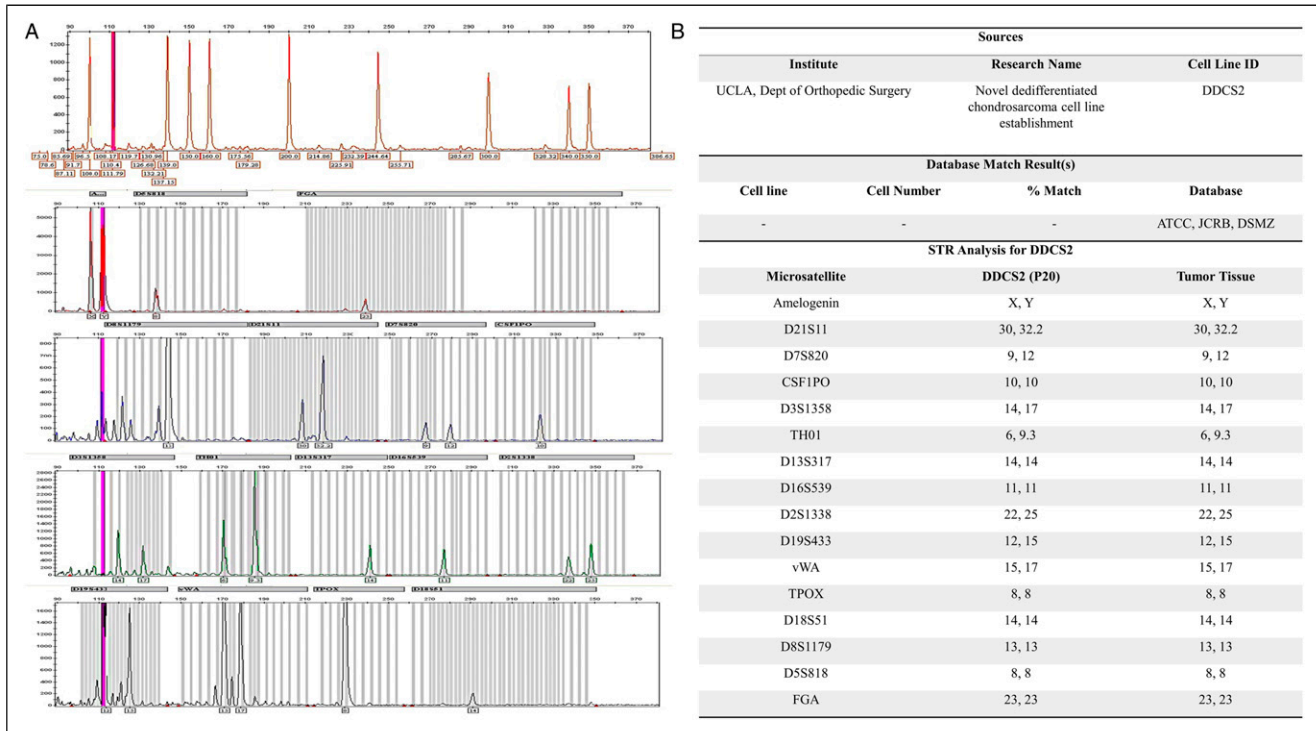
Figure 3. STR DNA profiling, comparison, and bioinformatic analysis of DDCS2. (A) Capillary electropherograms analyzed 15 core microsatellites and the gender identity locus amelogenin. The amplified STR peaks are in different colors and allele classifications. **(B)** On the basis of the obtained data, all detected STRs were identical between the original tumor tissue and the established DDCS2 cell line, without matching any existing human cancer cell line profiles in the ATCC, JCRB, or DSMZ databases.

resistance to doxorubicin, similar to CS-1 and SW1353 cells (*P*>0.05, Figure 4A). Similar chemoresponses were observed with cisplatin (*P*>0.05, Figure 4B) and paclitaxel (*P*>0.05, Figure 4C) in DDCS2, CS-1, and SW1353 cells. The IC$_{50}$ values were calculated in our current study (Table 1).

Morphologic changes were observed after 5 days of doxorubicin treatment. With increasing doses of doxorubicin (0, 0.003, 0.01, 0.3, 1, 3 μM) over 5 days, DDCS2, CS-1, and SW1353 cells showed pronounced morphologic signs of toxicity, including abnormal shape, appearance, lysis, and destruction. The DDCS2 and CS-1 cells began to show observable signs of toxicity in the form of cell reduction and abnormal cell morphology when treated with 1 μM doxorubicin, while the SW1353 cells showed these signs at only 0.3 μM of doxorubicin exposure. Cell death was increasingly more pronounced for DDCS2, CS-1, and SW1353 cells with up to 3 μM of chemotherapeutic (Supplementary Figure S1A). Similar morphologic changes were observed after 5 days of cisplatin (Supplementary Figure S1B) or paclitaxel (Supplementary Figure S1C) treatment.

Spheroid Formation in 3D Culture and Colony Formation in 2D Culture

DDCS2, chondrosarcoma cell lines CS-1 and SW1353, as well as the osteosarcoma cell lines MG63 and MNNG/HOS

were incubated in a 3D culture to mimic *in vivo* tumor growth. Much like the CS-1 and SW1353 cells, DDCS2 cells grew well and formed cell clusters by day 5, with increasing growth up to 21 days in the 3D culture (Figure 5A). The DDCS2 spheroids showed a similar growth rate (*P*>0.05, Figure 5B) and diameter (*P*>0.05, Figure 5C) to CS-1 and SW1353 cells. Spheroid formation curves of these three chondrosarcoma cell lines demonstrated no significant difference within the 3D culture (*P*>0.05, Figure 5D). Comparison of the ability of spheroid formation with osteosarcoma cells, the novel differentiated chondrosarcoma DDCS2 cell lines formed stable spheroids in 3D culture models by day 5 as well as osteosarcoma cell lines MG63 and MNNG/HOS, but with a relatively smaller volume. The osteosarcoma spheroids grew at a significant rate when observed on day 7 and tumor spheroids matured by day 15, while DDCS2 spheroids formation began to increase by day 10 and up to day 21 (Supplementary Figure S2B). In brief, DDCS2 cells formed the spheroids in a slight lower growth rate on the attachment 3D culture environment (*P*<0.05, Supplementary Figures S2C,D).

We also assessed the cell colony-forming ability of the novel dedifferentiated chondrosarcoma cell line DDCS2 and conventional chondrosarcoma cell lines CS-1 and SW1353 in 2D culture. The number of cell colonies formed by DDCS2, CS-1, and SW1353 cells showed no significant difference (*P*>0.05, Figures 5E,F). These findings support the spheroid
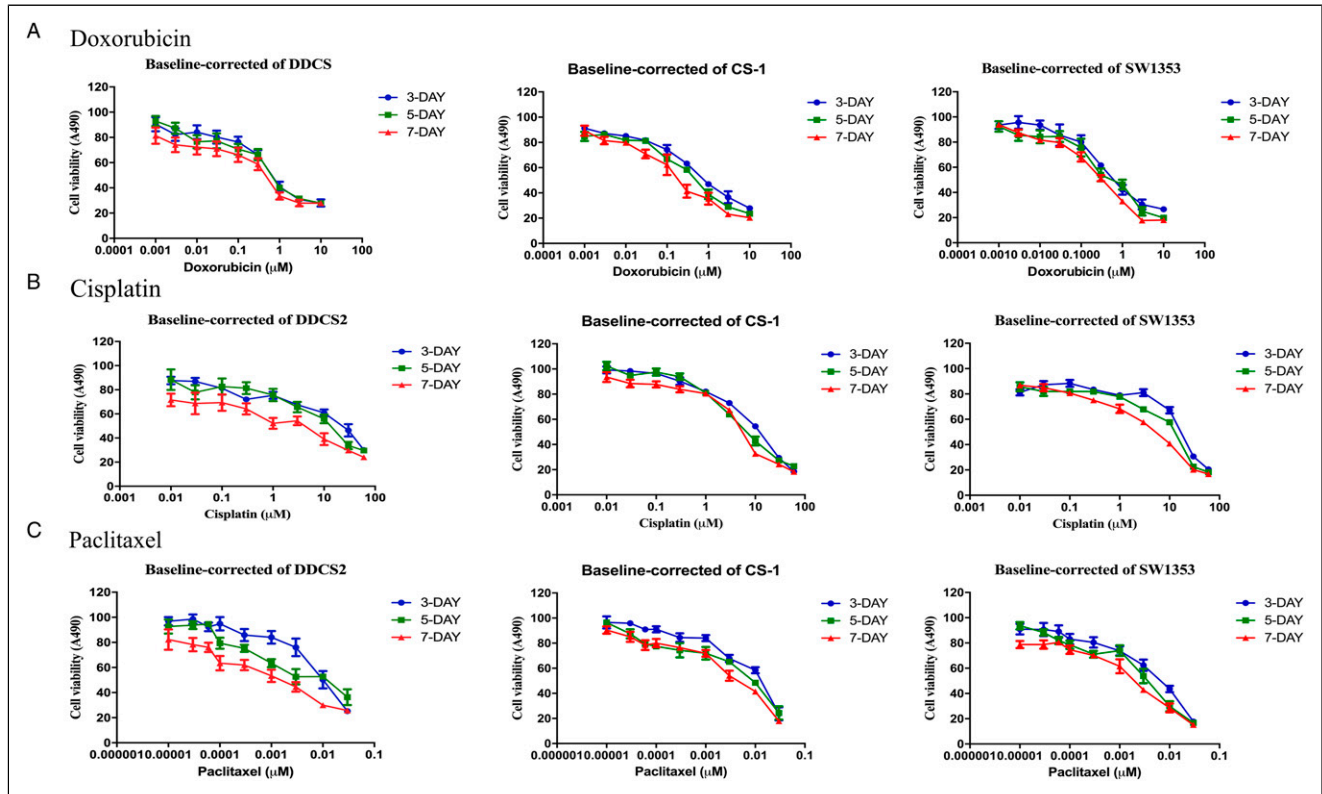
Figure 4. Cytotoxic assays of DDCS2, CS-1, and SW1353 cells. **(A)** MTT assays demonstrated a similar dose and time-dependent reduction of cell proliferation after doxorubicin treatment in DDCS2, CS-1, and SW1353 cell lines. **(B)** MTT assays demonstrated a similar dose and time-dependent reduction of cell proliferation after cisplatin treatment in DDCS2, CS-1, and SW1353 cell lines. **(C)** MTT assays demonstrated a similar dose and time-dependent reduction of cell proliferation after paclitaxel treatment in DDCS2, CS-1, and SW1353 cell lines.

Table 1. Sensitivity of primary chondrosarcoma cell lines DDCS2, CS-1, and SW1353 to doxorubicin, cisplatin, and paclitaxel.

Drugs Treatment Time (IC$_{50}$)	Chondrosarcoma Cell Line		
	DDCS2	CS-1	SW1353
Doxorubicin (nM)			
3-day	742.8±12.6	916.7±78.5	793.7±36.3
5-day	651.4±31.7	475.1±54.2	543.7±45.5
7-day	321.8±27.1	216.9±11.8	302.2±51.7
Cisplatin (μM)			
3-day	16.28±4.12	10.20±3.31	13.23±3.19
5-day	10.17±1.71	7.555±2.05	7.171±0.81
7-day	1.589±0.70	5.466±4.19	3.404±2.11
Paclitaxel (nM)			
3-day	9.445±2.15	9.628±3.77	4.811±0.51
5-day	6.807±1.97	5.936±2.53	2.651±1.83
7-day	1.182±0.21	3.434±2.12	1.427±0.55

and colony-forming ability of DDCS2 in both 3D and 2D culture conditions, which are consistent with a malignant cell line.

Discussion

Dedifferentiated chondrosarcoma is a rare but extremely aggressive primary bone tumor, characterized histologically by the presence of two distinct tumor portions: a well-differentiated cartilage tumor, which is generally low-grade chondrosarcoma, and a high-grade spindle-cell sarcoma component, in which osteosarcoma could be the most common malignant type.[8,15] Established and well-characterized cell lines are critical for describing the molecular mechanisms and therapeutic targets in this heterogeneous cancer. As summarized (Table 2), there are few conventional and dedifferentiated chondrosarcoma cell lines available, which has limited investigations to date.[12,16-24]

In the present study, we successfully established and characterized a novel dedifferentiated chondrosarcoma cell line DDCS2. The cells grew well in 2D and 3D cultures and exhibited a spindle to polygonal shape over 50 passages, findings which are consistent with a chondrosarcoma. Of note, the 60–72 hour population doubling time we observed in DDCS2 was much shorter than the conventional chondrosarcoma cell lines CS-1 and SW1353. STR DNA profiling
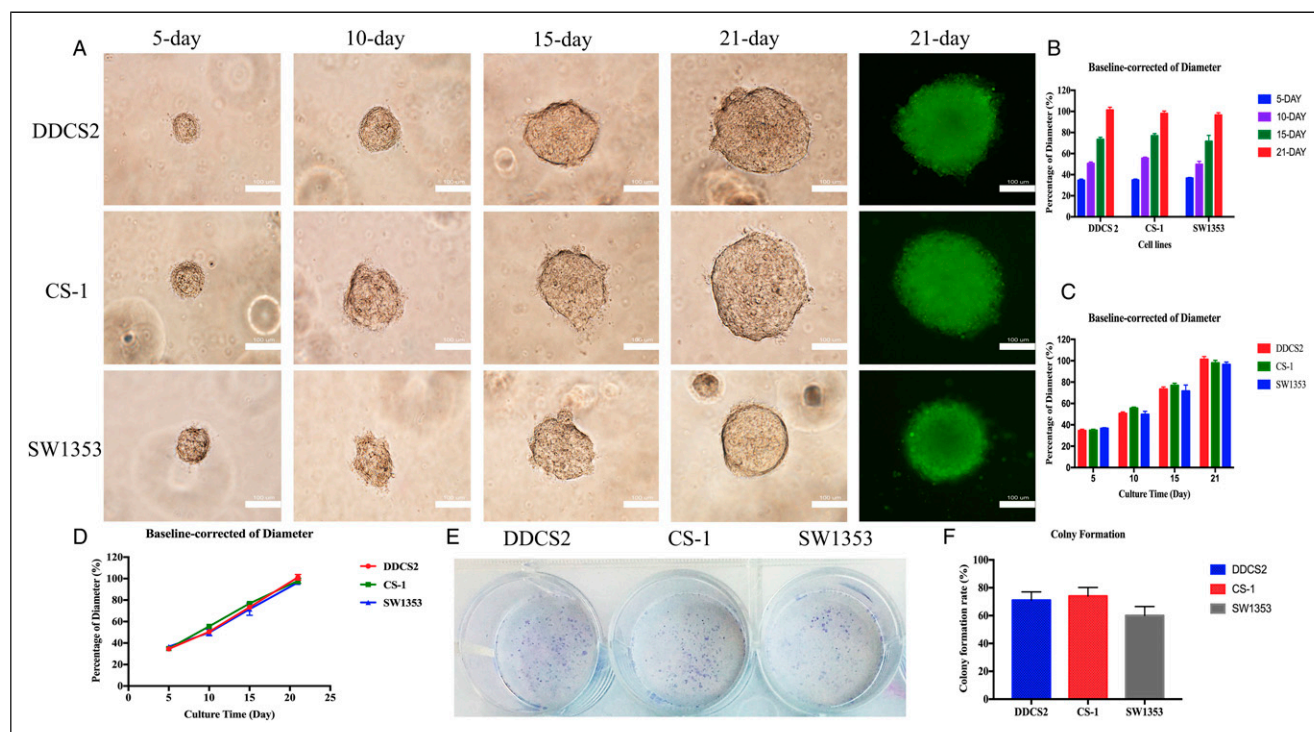
Figure 5. DDCS2 cell spheroid and colony growth in *ex vivo* 3D culture and 2D culture. **(A)** Representative images of a cell spheroid with rapid growth in DDCS2, CS-1, and SW1353 cells in 3D culture systems (200×, scale bar: 100 μm). The relative diameters of spheroids between DDCS2, CS-1, and SW1353 cell lines after 5, 10, 15, or 21 days **(B,C)** show no significant difference (*P*>0.05). **(D)** Spheroid formation curves of these three chondrosarcoma cell lines showed no significant difference in 3D culture conditions (*P*>0.05). **(E)** Representative cell colonies of DDCS2, CS-1, and SW1353 cells. **(F)** Quantification of clonogenic assays revealed similar cell colony formation between DDCS2, CS-1, and SW1353 cells. Experiments were performed in triplicate.

Table 2. Summary of current dedifferentiated and conventional chondrosarcoma cell lines.

Cell Line	Original Location	Tumor Subtype	Histological Grade	Passage	Reference
Dedifferentiated chondrosarcoma					
NDCS-1	Distal femur	Dedifferentiated	/	P20	[18]
L2975	Distal femur	Dedifferentiated	/	P58	[17]
L3252	Rib	Dedifferentiated	/	P21	[17]
BCSCH03	Femur	Dedifferentiated	/	P70	[19]
NCC-dCS1-C1	Rib	Dedifferentiated	/	P25	[16]
DDCS2	Distal femur	Dedifferentiated	/	P50	Present study
Conventional chondrosarcoma					
SW1353	Humerus	Solitary central	II	P12	ATCC
105 KC	Distal femur	Myxomatous	II	P26	[21]
OUMS27	Proximal humerus	Solitary central	III	P124	[20]
CH3573	Pelvis, ilium	Solitary central	II	P60	[22]
L835	Distal radius	Solitary central	III	P40	[17]
CS-1	Humerus	Solitary central	II	P30	[12]
CH2879	Rib	Solitary central	III	P80	[23]
HCS-TG	Pelvis, ilium	Solitary central	II	P45	[24]
BCSCH56	Humerus	Solitary central	III	P35	[19]

confirmed the unique genomic identity of DDCS2 which did not match any previous cancer cell lines within the ATCC, JCRB, or DSMZ repositories. As expected of a mesenchymal tumor, we observed increased vimentin expression without cytokeratin. Taken together, our results support DDCS2 as a novel dedifferentiated chondrosarcoma cell line suitable for future preclinical study.

Compared to the conventional subtype, dedifferentiated chondrosarcomas typically have higher grade, rates of recurrence and metastasis, and worse survival rates.[25-27] As abnormal regulation of p53 and MDM2 is especially prevalent in bone and soft tissue sarcomas,[28,29] we investigated these proteins within our novel cell line. Indeed, the DDCS2 cells showed a low expression of p53 and a high expression of MDM2. Loss of p53 is significant in sarcomas, as it correlates with tumor progression and malignancy.[30-33] MDM2 is an important inhibitor of p53-mediated transcription activation.[34,35] Specifically, overexpression of MDM2 is observed in more than one-third of human sarcomas retaining wild-type p53. [36] Overexpression of CDK4 and IDH2 indicate the malignant type and chondrosarcoma origin. CDK4 is reported as a trait of malignant tumors, and is expected to be served as an potential target for cancer therapy.[37,38] Cancer-associated IDH1/2 expressions have been considered as early stage events in chondrosarcoma development and could be served as a specific marker.[39,40]

With respect to treatment of dedifferentiated chondrosarcoma, complete resection remains the primary treatment modality, and prognosis remains dismal.[41] Chemotherapeutics trialed to date have failed to show significant results due to the well-described chemoresistance within this cancer. Reported researches confirmed that chemoresistance is one of the main biological features of chondrosarcoma. Cells reduced their sensitivity to doxorubicin and cisplatin through overexpression of efflux pumps or anti-apoptotic proteins.[42,43] The reported IC_{50} values of these drugs in monolayer chondrosarcoma cells are 50 nM–400 μM, with notable discrepancy between these IC_{50} values may be a result of using different cell lines, passages, culture conditions, or drug formulation.[19,43] Similar to the chondrosarcoma cell lines CS-1 and SW1353, DDCS2 exhibited similar resistance to the traditional chemotherapeutics doxorubicin, cisplatin, and paclitaxel and is therefore an accurate model of the barriers to currently used chemotherapeutics.

While the establishment of a cancer cell line is traditionally performed in monolayer, the lack of a 3D environment reduces its mimicry of *in vivo* growth. For chondrosarcoma, modeling growth is especially dependent on culturing conditions and their 3D environment. For example, primary chondrocytes grown in monolayer may lose their ability to produce their cartilaginous matrix.[44] We therefore confirmed the ability of DDCS2 cells to form spheroids in a 3D culture system before moving forward. Within the 3D culture, there was similarity in morphology and growth patterns to the conventional chondrosarcoma cell lines CS-1 and SW1353, but differences

appear with osteosarcoma cell lines MG63 and MNNG/HOS. As DDCS2 demonstrated reliable cellular architecture in 2D and 3D culture conditions, was consistent over multiple passages, and had a unique DNA profile, it is a promising option for future dedifferentiated chondrosarcoma *in vivo* studies.

We, therefore, report DDCS2 as a novel dedifferentiated chondrosarcoma cell line that adds to the pool of limited models currently available for investigating chondrosarcoma biology, chemoresistance, novel targets, and biomarkers.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

References

1. Tarpey PS, Behjati S, Cooke SL, et al. Frequent mutation of the major cartilage collagen gene COL2A1 in chondrosarcoma. *Nat Genet*. 2013;45:923-926. doi:10.1038/ng.2668

2. Ferguson JL, Turner SP. Bone Cancer: Diagnosis and Treatment Principles. *Am Fam Phys*. 2018;98:205-213.

3. Fletcher CDM, World Health Organization and International Agency for Research on Cancer. *WHO Classification of Tumours of Soft Tissue and Bone*. 4th ed. IARC Press; 2013: 468.

4. Leddy LR, Holmes RE. Chondrosarcoma of Bone. In: Peabody TD, Attar S, eds. *Orthopaedic Oncology: Primary and Metastatic Tumors of the Skeletal System*. Springer International Publishing; 2014: 117-130.

5. Staals EL, Bacchini P, Bertoni F. Dedifferentiated central chondrosarcoma. *Cancer*. 2006;106:2682-2691. doi:10.1002/cncr.21936

6. Frassica FJ, Unni KK, Beabout JW, et al. Dedifferentiated chondrosarcoma. A report of the clinicopathological features and treatment of seventy-eight cases. *J Bone Joint Surg Am*. 1986;68:1197-1205.

7. Capanna R, Bertoni F, Bettelli G, et al. Dedifferentiated chondrosarcoma. *J Bone Joint Surg Am*. 1988;70: 60-69.

8. Dickey ID, Rose PS, Fuchs B, et al. Dedifferentiated chondrosarcoma: the role of chemotherapy with updated outcomes. *J Bone Joint Surg Am*. 2004;86-A:2412-2418.

9. Bovee JV, Hogendoorn PC, Wunder JS, et al. Cartilage tumours and bone development: molecular pathology and possible therapeutic targets. *Nat Rev Canc*. 2010;10:481-488. doi:10.1038/nrc2869

10. Chifenti B, Morelli M, Zavaglia M, et al. Establishment and characterization of 4 new human pancreatic cancer cell lines: evidences of different tumor phenotypes. *Pancreas*. 2009;38:184-196. doi:10.1097/MPA.0b013e31818c746a

11. Ku JL, Shin YK, Kim DW, et al. Establishment and characterization of 13 human colorectal carcinoma cell lines: mutations of genes and expressions of drug-sensitivity genes and cancer stem cell markers. *Carcinogenesis*. 2010;31:1003-1009. doi:10.1093/carcin/bgq043

12. Shao L, Kasanov J, Hornicek FJ, et al. Ecteinascidin-743 drug resistance in sarcoma cells: transcriptional and cellular alterations. *Biochem Pharmacol*. 2003;66:2381-2395. doi:10.1016/j.bcp.2003.08.033

13. Liu X, Nielsen GP, Rosenberg AE, et al. Establishment and characterization of a novel chordoma cell line: CH22. *J Orthop Res*. 2012;30:1666-1673. doi:10.1002/jor.22113

14. Evans HL, Ayala AG, Romsdahl MM. Prognostic factors in chondrosarcoma of bone: a clinicopathologic analysis with emphasis on histologic grading. *Cancer*. 1977;40:818-831.

15. Gelderblom H, Hogendoorn PC, Dijkstra SD, et al. The clinical approach towards chondrosarcoma. *Oncologist*. 2008;13:320-329. doi:10.1634/theoncologist.2007-0237

16. Oyama R, Kito F, Takahashi M, et al. Establishment and characterization of a novel dedifferentiated chondrosarcoma cell line, NCC-dCS1-C1. *Human Cell*. 2019;32:202-213. doi:10.1007/s13577-018-00232-2

17. van Oosterwijk JG, de Jong D, van Ruler MA, et al. Three new chondrosarcoma cell lines: one grade III conventional central chondrosarcoma and two dedifferentiated chondrosarcomas of bone. *BMC Canc*. 2012;12:375. doi:10.1186/1471-2407-12-375

18. Kudo N, Ogose A, Hotta T, et al. Establishment of novel human dedifferentiated chondrosarcoma cell line with osteoblastic differentiation. *Virchows Arch*. 2007;451:691-699. doi:10.1007/s00428-007-0426-3

19. Monderer D, Luseau A, Bellec A, et al. New chondrosarcoma cell lines and mouse models to study the link between chondrogenesis and chemoresistance. *Lab Invest*. 2013;93:1100-1114. doi:10.1038/labinvest.2013.101

20. Kunisada T, Miyazaki M, Mihara K, et al. A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. *Int J Canc*. 1998;77:854-859. doi:10.1002/(sici)1097-0215(19980911)77:6<854::aid-ijc10>3.0.co

21. Block JA, Inerot SE, Gitelis S, et al. Synthesis of chondrocytic keratan sulphate-containing proteoglycans by human chondrosarcoma cells in long-term cell culture. *J Bone Joint Surg Am*. 1991;73:647-658.

22. Calabuig-Farinas S, Benso RG, Szuhai K, et al. Characterization of a new human cell line (CH-3573) derived from a grade II chondrosarcoma with matrix production. *Pathol Oncol Res*. 2012;18:793-802. doi:10.1007/s12253-012-9505-0

23. Gil-Benso R, Lopez-Gines C, Lopez-Guerrero JA, et al. Establishment and characterization of a continuous human chondrosarcoma cell line, ch-2879: comparative histologic and genetic studies with its tumor of origin. *Lab Invest*. 2003;83:877-887. doi:10.1097/01.lab.0000073131.34648.ea

24. Kudawara I, Araki N, Myoui A, et al. New cell lines with chondrocytic phenotypes from human chondrosarcoma. *Arch für Pathol Anat Physiol für Klin Med*. 2004;444:577-586.

25. Johnson S, Tetu B, Ayala AG, et al. Chondrosarcoma with additional mesenchymal component (dedifferentiated chondrosarcoma). I. A clinicopathologic study of 26 cases. *Cancer*. 1986;58:278-286.

26. Bruns J, Fiedler W, Werner M, et al. Dedifferentiated chondrosarcoma–a fatal disease. *J Canc Res Clin Oncol*. 2005;131:333-339. doi:10.1007/s00432-004-0648-6

27. Grimer RJ, Gosheger G, Taminiau A, et al. Dedifferentiated chondrosarcoma: prognostic factors and outcome from a European group. *Eur J Canc*. 2007;43:2060-2065. doi:10.1016/j.ejca.2007.06.016

28. Ozaki T, Nakagawara A. Role of p53 in Cell Death and Human Cancers. *Cancer*. 2011;3:994-1013. doi:10.3390/cancers3010994

29. Momand J, Jung D, Wilczynski S, et al. The MDM2 gene amplification database. *Nucleic Acids Res*. 1998;26:3453-3459. doi:10.1093/nar/26.15.3453

30. Liu Y, Chen C, Xu Z, et al. Deletions linked to TP53 loss drive cancer through p53-independent mechanisms. *Nature*. 2016;531:471-475. doi:10.1038/nature17157

31. Giacomelli AO, Yang X, Lintner RE, et al. Mutational processes shape the landscape of TP53 mutations in human cancer. *Nat Genet*. 2018;50:1381-1387. doi:10.1038/s41588-018-0204-y

32. Cancer Genome Atlas Research Network. Electronic address edsc and Cancer Genome Atlas Research N. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. *Cell*. 2017;171:950-965 e928. doi:10.1016/j.cell.2017.10.014

33. Roepke M, Diestel A, Bajbouj K, et al. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Canc Biol Ther*. 2007;6:160-169.

34. Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Canc*. 2013;13:83-96. doi:10.1038/nrc3430

35. Iwakuma T, Lozano G. MDM2, an introduction. *Mol Canc Res MCR*. 2003;1:993-1000.

36. Oliner JD, Kinzler KW, Meltzer PS, et al. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*. 1992;358:80-83. doi:10.1038/358080a0

37. Klein ME, Kovatcheva M, Davis LE, et al. CDK4/6 Inhibitors: The Mechanism of Action May Not Be as Simple as Once Thought. *Canc Cell*. 2018;34:9-20. doi:10.1016/j.ccell.2018.03.023

38. Whittaker SR, Mallinger A, Workman P, et al. Inhibitors of cyclin-dependent kinases as cancer therapeutics. *Pharmacol Ther*. 2017;173:83-105. doi:10.1016/j.pharmthera.2017.02.008

39. Waitkus MS, Diplas BH and Yan H. Biological Role and Therapeutic Potential of IDH Mutations in Cancer. *Canc Cell*. 2018;34:186-195. doi:10.1016/j.ccell.2018.04.011

40. Samuel AM, Costa J and Lindskog DM. Genetic alterations in chondrosarcomas - keys to targeted therapies? *Cell Oncol*. 2014;37:95-105. doi:10.1007/s13402-014-0166-8

41. Yokota K, Sakamoto A, Matsumoto Y, et al. Clinical outcome for patients with dedifferentiated chondrosarcoma: a report of 9 cases at a single institute. *J Orthop Surg Res*. 2012;7:38. doi:10.1186/1749-799X-7-38

42. Wyman JJ, Hornstein AM, Meitner PA, et al. Multidrug resistance-1 and p-glycoprotein in human chondrosarcoma cell lines: expression correlates with decreased intracellular doxorubicin and in vitro chemoresistance. *J Orthop Res*. 1999;17:935-940. doi:10.1002/jor.1100170619

43. van Oosterwijk JG, Herpers B, Meijer D, et al. Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma in vitro: BCL-2 family members cause chemoresistance. *Ann Oncol*. 2012;23:1617-1626. doi:10.1093/annonc/mdr512

44. Schorle CM, Finger F, Zien A, et al. Phenotypic characterization of chondrosarcoma-derived cell lines. *Canc Lett*. 2005;226:143-154. doi:10.1016/j.canlet.2004.11.022