Establishment and characterization of a chordoma cell line from the tissue of a patient with dedifferentiated-type chordoma

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OBJECTIVE Chordoma is a rare bone tumor of the axial skeleton believed to originate from the remnants of the embryonic notochord. The available tumor cells are characteristically physaliferous and express brachyury, a transcription factor critical for mesoderm specification. Although chordomas are histologically not malignant, treatments remain challenging because they are resistant to radiation therapy and because wide resection is impossible in most cases. Therefore, a better understanding of the biology of chordomas using established cell lines may lead to the advancement of effective treatment strategies. The authors undertook a study to obtain this insight.

METHODS Chordoma cells were isolated from the tissue of a patient with dedifferentiated-type chordoma (DTC) that had recurred. Cells were cultured with DMEM/F12 containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Cell proliferation rate was measured by MTS assay. Cell-cycle distribution and cell surface expression of proteins were analyzed by fluorescence-activated cell sorting (FACS) analysis. Expression of proteins was analyzed by Western blot and immunocytochemistry. Radiation resistance was measured by clonogenic survival assay. Tumor formation was examined by injection of chordoma cells at hindlimb of nude mice.

RESULTS The putative (DTC) cells were polygonal and did not have the conventional physaliferous characteristic seen in the U-CH1 cell line. The DTC cells exhibited similar growth rate and cell-cycle distribution, but they exhibited higher clonogenic activity in soft agar than U-CH1 cells. The DTC cells expressed high levels of platelet-derived growth factor receptor–β and a low level of brachyury and cytokeratins; they showed higher expression of stemness-related and epithelial to mesenchymal transition–related proteins than the U-CH1 cells. Intriguingly, FACS analysis revealed that DTC cells expressed marginal surface expression of CD24 and CD44 and high surface expression of CXCR4 in comparison to U-CH1 cells. In addition, blockade of CXCR4 with its antagonist AMD3100 effectively suppressed the growth of both cell lines. The DTC cells were more resistant to paclitaxel, cisplatin, etoposide, and ionizing radiation than the U-CH1 cells. Injection of DTC cells into the hindlimb region of nude mice resulted in the efficient formation of tumors, and the histology of xenograft tumors was very similar to that of the original patient tumor.

CONCLUSIONS The use of the established DTC cells along with preestablished cell lines of chordoma may help bring about greater understanding of the mechanisms underlying the chordoma that will lead to therapeutic strategies targeting chordomas.

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KEY WORDS chordoma; dedifferentiated-type chordoma; brachyury; CXCR4; oncology

ABBREVIATIONS DTC = dedifferentiated-type chordoma; EGFR = epidermal growth factor receptor; EMT = epithelial to mesenchymal transition; FACS = fluorescence-activated cell sorting; FBS = fetal bovine serum; IR = ionizing radiation; p- = phosphorylated; PBS = phosphate-buffered saline; PDGFR = platelet-derived growth factor receptor; PTEN = phosphatase and tensin homolog; RT = room temperature; TBST = Tris-buffered saline with Tween 20.


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Chordomas are rare, slow-growing bone tumors that mostly arise from remnants of the notochord. Histologically, chordomas are classified as conventional (classic), chondroid, and dedifferentiated type. Although rare, these tumors can be life-threatening due to their local aggressiveness, invasiveness, and progressive nature. The standard treatment for chordoma is adjuvant radiotherapy after excision. However, this current treatment is greatly ineffective possibly because of the local invasiveness followed by recurrence.

Because research in this field is limited, the establishment of various types of chordoma cell lines would be a beneficial tool to move the research forward. Although several studies have reported the establishment of chordoma cell lines, only a few have been validated. In addition, among the validated chordoma cell lines, only a few were reported to be useful as xenograft models. For example, in the NOD/SCID/interleukin 2 receptor [IL2r]null mouse model, the U-CH1 cell line was able to produce a xenograft tumor whose morphological features and immunohistochemical staining features resembled typical conventional-type chordoma. However, a dedifferentiated-type chordoma (DTC) cell line has yet to be established. Therefore, more defined chordoma cell lines capable of forming tumors in mice would be a valuable resource for understanding the biology of the tumor and to identify therapeutic targets.

In this study, we attempted to establish a chordoma cell line from human tissue obtained in a patient with a DTC, characterizing the cell line in vitro and in vivo by comparing it to the preexisting conventional-type chordoma cell line, U-CH1.

Methods
Clinical Presentation
A 48-year-old female patient developed pain in her coccyx and radiating pain in her lower extremities. Initial MRI showed a well-enhancing mass of the S2–4 along with sacral nerve root compression. The tumor was resected from the sacral region, and sacral nerve root decompression was performed. Postoperative histological diagnosis confirmed that the tumor was a DTC that was composed of mainly typical conventional chordoma areas, with predominantly epithelioid features and exhibiting occasional physaliferous cells and a focal high-grade sarcomatous area (Fig. 1A). After surgery and recovery, the patient was discharged from the hospital with relief of her preoperative symptoms. However, 4 months later, she returned to our clinic, suffering from right leg pain and weakness. MRI revealed a recurrent tumor mass at the S-1 vertebral body (Fig. 1B). A signal indicative of a recent bleed was observed. A second operation was performed, and the pathological diagnosis was DTC exhibiting ovoid to spindle highly malignant sarcomatous tumor cells (Fig. 1C). Conventional chordoma-like areas were not identified in this time. This indicates an aggressive transformation has occurred. Tumor tissue from the second operation was used to develop a primary cell line.

Reagents
Antibodies against platelet-derived growth factor receptor (PDGFR)–α, PDGFR-β, cytokeratin 8/18, brachyury, c-Met, CD24, CD44, Oct4, ZEB1, CD133, c-Myc, and β-actin were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated (p)–Akt (T308 and S473), p-STAT3 (Y705 and S727), p-ERK1/2, p-JNK, p-mTOR, Snail, Slug, phosphatase and tensin homolog (PTEN), and Musashi were obtained from Cell Signaling Biotechnology. Sox2 was purchased from R&D Systems. Antibodies against epidermal growth factor receptor (EGFR), E-cadherin, and N-cadherin were obtained from BD Biosciences (Becton Dickinson). Antibodies against Nestin, Notch1, and Nanog were acquired from Millipore. Antibodies against Twist, FoxM1, and CXCR4 were obtained from Abcam. Vimentin was purchased from thermo Scientific and growth factor–reduced Matrigel was purchased from BD Biosciences.

Cell Culture
Human primary chordoma cells were obtained from a fresh specimen with the approval of institutional review board as a part of the study protocol. The chordoma specimen was cut into fine pieces with a scalpel and subsequently cultured in DMEM/F12 (Cellgro) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/
ml penicillin, 100 µg/ml streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (FBS; Gibco) in a humidified incubator containing 5% CO₂ at 37°C. The established chordoma cell line U-CH1 was provided by the Chordoma Foundation (Durham, NC) and cultured in Iscove/RPMI (4:1) medium containing 10% FBS, 2 mM glutamate, and antibiotics as described above.

MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assays

Cells were seeded in 96-well (5 × 10³ cells/well) plates and incubated at 37°C under 5% CO₂ incubator for 24 hours. The medium was replaced with 100 µl of complete medium with or without various drugs (cisplatin, paclitaxel, etoposide, AMD3100, AMD3465 [Sigma-Aldrich]) and ionizing radiation (IR) at the indicated concentrations and doses, respectively. After 48 hours of incubation for drugs and 96 hours of incubation for IR exposure, cells were replaced with fresh medium (100 µl/well) with the addition of 20 µl of MTS solution (CellTiter 96 Aqueous MTS Reagent Powder) and 1 µl of phenazine methosulfate in a each well, followed by incubation for 2 hours at 37°C. After incubation, optical density was measured at 490 nm using plate reader (BioRad).

Immunocytochemistry

Chordoma cells were fixed with 4% paraformaldehyde with 0.1% Triton X-100 for 10 mins at room temperature (RT). After washing with phosphate-buffered saline (PBS) 3 times for 5 mins each, cells were blocked with blocking solution (5% bovine serum albumin and 0.5% Triton X-100 in PBS) for 1 hour at RT, stained with primary antibodies in blocking solution (1:100) for 2 hours, and washed 3 times with PBS. Staining was visualized using Alexa Fluor 488 goat anti–rabbit (A11008) and Alexa Fluor 594 goat anti–mouse (A11005) (Invitrogen) secondary antibodies (1:1000) in dark condition for 1 hour. Nuclei were stained using DAPI (containing mounting solution), and stained cells were viewed under a confocal laser scanning microscope.

Western Blot Analysis

Chordoma cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₃, 1 µg/ml leupeptin, and 1 mM PMSF). The lysates were clarified by centrifugation at 12,000 g for 10 mins at 4°C, and protein content in the supernatant was measured by Bradford’s method. An aliquot (30–50 µg protein per lane) of the total protein was separated by 10% or 12% SDS-PAGE and blotted to nitrocellulose transfer membrane (0.2 µm; Amersham). The membrane was blocked with 5% nonfat skim milk in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.01% Tween 20) for 1 hour at RT, followed by overnight incubation with the primary antibodies at 4°C. After extensive washing with TBST, the membrane was re-probed with horseradish peroxidase–linked anti–rabbit immunoglobulin, at 1:1000 diluted in 5% nonfat skim milk in TBST, for 1 hour at RT. Immunoblots were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer’s protocol.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells were dissociated into single cells and were washed with PBS followed by stained with phycoerythrin-conjugated antibodies at a 4°C refrigerator for 30 mins. After washing with PBS twice, cells were analyzed on FACSCalibur (Becton Dickinson).

Soft-Agar Clonogenic Assays

For soft-agar clonogenic assay, single-cell suspensions (5 × 10³ cells/ml) were obtained in 2 × DMEM/F12 with serum (10%), resuspended in the same volume of 0.7% low-melting agar (final 0.35%), and poured onto 24-well plates coated at the bottom with agar (1:1 mixture of 2 × DMEM/F12 and 1% low-melting agar; final 0.5%). After 14 days of incubation, colonies in 5 random fields per well were counted under a microscope.

Ionizing Radiation Exposure

For measuring IR sensitivity, cells were seeded in 60-mm dishes (5 × 10⁴ cells) and were exposed to γ-rays from a ¹³⁷Cs γ-ray source (Atomic Energy of Canada, Korea Institute of Radiological and Medical Sciences) at a dose rate of 3.81 Gy/min.

Immunohistochemistry

The paraffin sections of xenograft tumor tissues were dewaxed in xylene for 20 mins, followed by sequential hydration in 100%, 95%, 90%, and 80% ethanol solutions. After rinsing with PBS, endogenous peroxidase activity was blocked by 3% hydrogen peroxide treatment for 30 mins. Primary antibodies against cytokeratin (1:1000, Biogenex) and CXCR4 (1:2000, Abcam) were applied to the sections overnight in a moisture chamber at 4°C. After rinsing with PBS, the sections were incubated with secondary antibody for 10 mins at RT, rinsed with PBS, and incubated with horseradish peroxidase–conjugated tertiary antibody for 10 mins at RT. After rinsed with PBS, sections were incubated with diaminobenzidine for 10 mins, counterstained with Meyer’s hematoxylin, dehydrated, and mounted.

In Vivo Tumor Formation

All animal protocols and studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee in the Korea Institute of Radiological and Medical Sciences. Nude mice (female, 15–18 g, 6 weeks old) were obtained from Nara Biotech Co. DTC cells (5 × 10⁴ cells) were mixed with Matrigel (1:1 mixture) and injected into the left flanks of the mice. Tumor size was measured every 2–3 days when the tumor volume reached 50 mm³.

Results

Primary Culture of Chordoma Cells

First, we tried to isolate chordoma cells from a recur-
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The aseptically delivered fresh chordoma specimen from the operating room was dissected using a scalpel and cultured in tissue culture flasks in DMEM/F12 media containing 10% FBS in a humidified chamber at 37°C with 5% CO₂ conditions. After 3 weeks of culture, chordoma cells were visualized under a phase-contrast microscope and were seen to form colonies with spikelike edges. This characteristic is not typical morphology of the reported chordoma cell line U-CH1, in which many vacuoles are present (Fig. 2A).¹⁸,²¹ MTS assays revealed that the established DTC cells exhibited similar proliferation as U-CH1 cells (Fig. 2B). FACS analysis indicated that there was no significant difference in the cell-cycle progression between the two cell lines (Fig. 2C). In soft-agar clonogenic assays, a surrogate method for measuring tumorigenic potential in vitro, DTC cells showed much higher potential for clonogenic activity than did the U-CH1 cells (Fig. 2D). Taken together, these data suggest that the primary-cultured DTC cell line has a similar proliferation capacity and higher in vitro tumorigenic potential than the conventional-type chordoma cell line, U-CH1.
Different Molecular Expression Patterns Between DTC and UCH-1 Cells

Next we examined molecular expression patterns of DTC cells and compared them to those of the U-CH1 cells. In the chordoma-related gene expression patterns, DTC cells showed higher expression of PDGFR-β and lower expression of PDGFR-α, EGFR, cytokeratin 8/18, CD44, and brachyury than U-CH1 cells (Fig. 3A). c-Met and CD24 expression were similar between the two cell lines. PTEN, a tumor suppressor protein, was expressed in DTC cells; however, as described in a previous study, the U-CH1 cells did not express PTEN. Reduced expression of cytokeratin 8/18 is an indication of the epithelial to mesenchymal transition (EMT), which was investigated in these cells. As shown in Fig. 3B, DTC cells showed significantly higher expression of EMT factors including N-cadherin, Slug, and Twist than did U-CH1 cells; Snail expression was only marginally lower in the DTC cells. E-cadherin expression was very weak in both cell lines; however, vimentin expression was higher in the U-CH1 cells than in the DTC cells. Intriguingly, DTC cells exhibited enhanced expression of stemness-related molecules including CD133, c-Myc, Oct4, Sirt1, FoxM1, and Musashi, whereas the expression of nestin, Sox2, and Notch1 was not enhanced (Fig. 3C). CXCR4 was slightly upregulated in DTC cells; however, Nanog expression was much lower than that in the U-CH1 cells (Fig. 3C). With regard to the activation of cellular signaling molecules, DTC cells exhibited enhanced expression of p-JNK and reduced expression of p-STAT3 (S727, Y705), p-Akt (S473, T308), p-ERK1/2, and p-mTOR (Fig. 3D). Immunohistochemical staining confirmed the expression pattern of the critical molecules in both cell lines (Fig. 4). Interestingly, PDGFR-β and CXCR4 stained blurry-like patterns in DTC cells, indicating the membranous and cytosolic staining of these molecules. FACS analysis was performed to examine the membrane expression of these molecules. The results indicated that DTC cells have a high level of cell surface expression of CXCR4, but not PDGFR-β (Fig. 5). In contrast, U-CH1 cells showed a higher level of CD24 and CD44—cell surface glycoproteins that are used for prognostic markers in chordoma—expression on their cell surfaces than DTC cells (Fig. 5). The molecular characteristics of various chordoma cell lines are summarized in Table 1.

Sensitivity of DTC Cells to Different Drugs and IR

We next compared the sensitivity to drugs and IR between DTC and U-CH1 cells. Treatment with paclitaxel, cisplatin, and etoposide reduced cell viability in a dose-dependent manner; however, the DTC cells were more resistant to these drugs than U-CH1 cells (Fig. 6). Because cell surface expression of CXCR4 is enhanced in DTC cells, we investigated the effect of the CXCR4 antagonist AMD3100 on the proliferation of DTC and U-CH1 cells. As shown in the lower panel of Fig. 6, AMD3100 suppressed the proliferation of both cell lines, but U-CH1 cells were more susceptible to this drug than the DTC cells.
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**FIG. 4.** Immunocytochemistry of DTC and U-CH1 cells with antibodies. Immunofluorescence staining was visualized by probing with Alexa Fluor 594 goat anti–mouse secondary antibody (green). Nuclei were visualized by DAPI staining (blue). Original magnification ×100. Figure is available in color online only.

**FIG. 5.** FACS analysis of DTC and U-CH1 cells for evaluating cell surface expression of the proteins with specific antibodies. Figure is available in color online only.
Another CXCR4 antagonist, AMD3465, also inhibited the proliferation of the DTC and U-CH1 cells but its activity was much weaker than AMD3100 (Fig. 6). Finally, to evaluate the IR sensitivity of both cell lines, we performed MTS-based assays and found that the DTC cells exhibited greater survival activity after IR exposure than the U-CH cells (Fig. 7). These data suggest that the DTC cells exhibited greater resistance to drugs and IR exposure than the U-CH1 cells.

In Vivo Tumor Formation of DTC Cells

Next, we examined the tumor formation of the DTC cells in vivo. Injection of $5 \times 10^5$ DTC cells in the flank region of mice was sufficient to induce tumor formation within 2 weeks of the injection in all 6 mice that were injected with the cells (Fig. 8A). An exponential growth pattern of xenograft tumor is shown in Fig. 8B. Histological examination indicated that mouse xenograft tumor exhibited a similar morphology to that of the patient’s tissue, with slightly decreased cytokeratin and CXCR4 expression (Fig. 8C). The data indicate that primary-cultured DTC cells would be useful to develop effective therapeutic approaches to treat patients with DTCs.

Discussion

Because of the limited availability of chordoma cell lines, it is critical to establish defined cell lines from various chordoma tissues. This advancement will allow for a

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**FIG. 6.** Effect of drugs on the cytotoxicity of DTC and U-CH1 cells. MTS assay of DTC and U-CH1 cells treated with various concentrations of drugs. *0.01 < p < 0.05; **0.005 < p < 0.01; ***0.001 < p < 0.005. Figure is available in color online only.
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possible therapeutic target to enhance the effectiveness of chemo- and radiotherapy of chordoma. In addition, the high tumorigenic potential of DTC cells in vivo and the fact that the pathological features of the xenograft tumor were similar to those of the patient tissue would make it possible to use these cells for developing suitable therapeutic strategies to treat patients with malignant chordoma.

Almost all chordomas express the notochord developmental transcription factor, brachyury, a crucial biomarker for chordoma. Along with brachyury, cytokeratin is also known as a combinational marker in chordoma. In addition, receptor tyrosine kinases, including PDGFR-α and -β, EGFR, and c-Met, are frequently expressed in chordomas, resulting in the activation of the downstream Akt and mTOR signaling pathways. Our Western blot and immunocytochemistry analyses indicated that the DTC cells also expressed brachyury, albeit at a much lower level than what was observed in U-CH1 cells. With regard to cytokeratins, the DTC cells showed significantly lower expression of cytokeratins 8 and 18 compared with the U-CH1 cells. DTC's reduced expression of cytokeratins and its

Fig. 7. Effect of IR on the cytotoxicity of DTC and U-CH1 cells. MTS assays of DTC and U-CH1 cells after 4 days of IR at various doses. *0.01 < p < 0.05. Figure is available in color online only.

Fig. 8. In vivo tumor formation of DTC cells. A: Representative picture of the mouse (upper). Arrow indicates the site of the tumor. Tissue specimens isolated from mice (lower). B: Growth pattern of xenograft tumor. C: H & E and immunostaining of tissue specimens from a human patient with DTC (upper) and the mouse xenograft of DTC cells (lower). Both tumors showed similar morphology with slightly decreased cytokeratin and CXCR4 expression in the xenograft tumor. Original magnification ×400. Figure is available in color online only.
morphological characteristics are reminiscent of the EMT features. Indeed, the DTC cells showed enhanced expression of EMT-associated proteins including N-cadherin, Slug, and Twist. They also exhibited lower PDGF-α and higher PDGF-β expressions than the U-CH1 cells. In line with these results, the phosphorylation of signaling molecules including p-ERK, p-Akt, p-mTOR, and p-STAT3, but not p-JNK, was much weaker in the DTC cells than in the U-CH1 cells. The DTC cells did not express EGFR and CD44, as determined by Western blot and FACS analyses. c-Met expression was similar in both cell lines. Interestingly, although total expression of CD24 was nearly the same in both cell lines, a cell surface level of CD24 was only detected in U-CH1 cells. Further study will be needed to define the meaning and mechanism of cell surface localization of CD24 in chordoma cells. Collectively, our data indicate that the established DTC cell line shows different expression pattern of chordoma markers compared with the conventional-type chordoma cell line and tissues.

One of the most interesting expression patterns of the DTC cells was their higher surface expression of CXCR4 compared with the U-CH1 cells, although the total expression of CXCR4 was indistinguishable between the two cell lines. Treatment with the CXCR4-specific inhibitor AMD3100 significantly suppressed the growth of both DTC and U-CH1 cells. Intriguingly, the U-CH1 cells were more sensitive to AMD3100 treatment than were the DTC cells. Because CXCR4 is known as a cancer stem cell marker in various cancers, we compared stem cell marker expression patterns between the two cell lines, finding that the DTC cells expressed higher levels of stem cell markers, including CD133, c-Myc, Oct4, and Musashi, than the U-CH1 cells. However, Notch1 and Sox2 expression was similar in both cell lines, and Nanog was only expressed in the U-CH1 cells. Therefore, these data suggest that CXCR4 signaling along with enhanced stemness would be critical for the growth of DTC and U-CH1 cells. Accordingly, CXCR4 might be a good therapeutic target for treating malignant chordoma. In future studies we will further investigate the involvement of CXCR4 signaling in the stem-like cell characteristics of chordoma cells.

Conclusions

In the present study, we established a chordoma cell line from a patient with recurrent DTC. Compared with the U-CH1 cells, the established DTC cells exhibited lower expression of chordoma markers, brachyury and cytokeratins; higher expression of EMT- and stem cell–related markers; and higher surface expression of CXCR4. It has been established that the DTC cells are capable of producing tumors in nude mice. Moreover, the major differences in DTC cell line compared with previously established chordoma cell lines are that it is the first cell line raised from DTC and the cells exhibit high cell surface expression of CXCR4, which can be a molecular target for therapy in this type of tumor. Collectively, our data provide evidence indicating that the established DTC cells could be used to study the biology of DTCs in vitro and in vivo and to develop appropriate therapeutic targets in malignant chordoma.

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References


Disclosures
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