Chapter 6

Screening for potential targets for therapy in mesenchymal, clear-cell and dedifferentiated chondrosarcoma reveals Bcl-2 family members and TGFbeta as potential targets

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Abstract

Mesenchymal - , clear cell - , and dedifferentiated chondrosarcoma are extremely rare and together constitute 10-15% of all chondrosarcomas. Their poor prognosis and lack of efficacious treatment emphasizes the need to elucidate the pathways playing a pivotal role in these tumors.
We constructed tissue microarrays containing 42 dedifferentiated - , 23 clear cell - , and 23 mesenchymal chondrosarcomas and performed immunohistochemistry to study the expression of growth plate-signaling molecules, and molecules shown to be involved in conventional chondrosarcoma. We observed high expression of SOX9 and FGFR3, as well as aberrant cellular localization of heparan sulfate proteoglycans, in all subtypes. We found TGFbeta signaling through pSMAD2 and PAI1 to be highly active in all chondrosarcoma subtypes suggesting TGFbeta inhibitors to be a possible therapeutic strategy in rare chondrosarcoma subtypes. Like in conventional chondrosarcoma, antiapoptotic proteins (Bcl-2, and / or Bcl-xl) were highly expressed in all subtypes. Inhibition with the BH-3 mimetic ABT-737 rendered dedifferentiated chondrosarcoma cell lines sensitive to doxorubicin or cisplatin. We here show that antiapoptotic proteins may play an important role in chemoresistance, suggesting a promising role for targeting Bcl-2 family members in chondrosarcoma treatment irrespective of subtype.

Introduction

Chondrosarcoma of bone is a malignant tumor characterized by the formation of cartilage. It mainly affects adults in the third to sixth decades of life. In addition to conventional chondrosarcoma, several rare subtypes are recognized with distinct histological and clinical features. These subtypes include clear cell chondrosarcoma, mesenchymal chondrosarcoma, and dedifferentiated chondrosarcoma. Together they constitute 10-15% of all chondrosarcomas. Previously, it was suggested that the distinct chondrosarcoma subtypes show striking histological similarities with cartilaginous cells of the growth plate in various states of differentiation (1). This model is supported by results from expression analysis of extracellular matrix genes (2).

Clear cell chondrosarcoma (2%) is a low-grade malignant tumor, which rarely metastasizes, but commonly recurs after curettage. About 15% of the patients die as a result of the disease. The disease is characterized by tumor cells with clear, empty cytoplasm, resembling the hypertrophic cells of the growth plate (1). In addition, expression of collagen type X and osteonectin further supports the resemblance to chondrocytes in the hypertrophic state (3).

Mesenchymal chondrosarcoma (2% of all CS) is a highly malignant lesion which occurs in bone as well as in soft tissue of relatively young patients. The tumor consists of differentiated cartilage mixed with undifferentiated small round cells and usually follows an aggressive course with a high rate of distant metastases

and a 5-year overall survival of 55% (4). Histologically the undifferentiated cells resemble the resting cells of the growth plate (1) and Aigner et al confirmed the premesenchymal chondroprogenitor origin of these cells by studying cell differentiation and matrix gene expression (2;5).

Dedifferentiated chondrosarcoma (10%) is a tumor containing two clearly defined components: a high-grade non-cartilaginous anaplastic sarcoma juxtaposed to a usually low-grade well-differentiated cartilage tumor, with a sharply defined junction between the two components (6). It has a poor prognosis and no targets for therapy have been reported to date (7).

Nearly all chondrosarcomas arise in bones formed by endochondral ossification. Endochondral ossification occurs in the growth plate, in which four zones can be distinguished: the resting zone, the proliferating zone, the transition zone, and the hypertrophic zone (8). With the elucidation of the *EXT* genes being involved in the development of multiple as well as solitary osteochondromas (benign cartilaginous tumors at the surface of the bone), parallels between the normal growth plate and cartilage tumors became obvious since they shared a strong morphological resemblance (for review see: (9)). The exostosins are involved in heparan sulfate chain elongation on heparan sulfate proteoglycans (HSPGs). HSPGs include syndecan, perlecan and the splice variant of CD44 including variable exon 3 (CD44v3) and are crucial for facilitating signaling of FGF, Wnt, BMP, TGFbeta and indian hedgehog (IHH), all of which are important for chondrocyte proliferation and differentiation in the normal growth plate (reviewed in 10). The process of chondrocyte proliferation and differentiation is tightly regulated by a paracrine feedback loop involving both IHH/ parathyroid hormone like hormone (PTHLH) and fibroblast growth factor (FGF) signaling (11). Wnt signaling promotes chondrocyte differentiation in a SOX9 dependent manner (12). TGFbeta signaling can regulate PTHLH expression independently of IHH (13). Multiple studies have confirmed the importance of these pathways in conventional central and peripheral chondrosarcoma (14;15). In contrast, because of the rarity of clear cell, dedifferentiated and mesenchymal CS, little information is available on the role of these pathways in rare chondrosarcoma subtypes.

In this study, we therefore investigated the expression of the master transcription regulator for chondrogenic differentiation (SOX9), HS and HSPGs (10E4, syndecan 2, 3 and 4, CD44 variable exon 3 and NDST2), and proteins involved in PTHLH (PTHLH, PTHR1 and BCL-2), FGF (FGF18 and FGFR3), WNT (beta catenin), BMP (pSMAD1) and TGFbeta (pSMAD2 and PAI1) pathways as well as the expression of COX2 and KIT based on possible therapeutic consequences, in 42 dedifferentiated chondrosarcomas, 23 clear cell chondrosarcomas, and 23 mesenchymal chondrosarcomas. Based on the results we further investigated the role of Bcl-2 in chemoresistance of two dedifferentiated chondrosarcoma cell lines.

Materials and Methods

Tumor tissue

For this study, 42 dedifferentiated chondrosarcomas, 23 mesenchymal chondrosarcomas, and 23 clear cell chondrosarcomas were collected within the EuroBoNeT consortium, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumors. In total, formalin-fixed paraffin-embedded (FFPE) specimens from 88 patients were collected from the archives of the Department of Pathology, LUMC, The Netherlands (n=13), Nuffield Department of Orthopaedic Surgery, University of Oxford, UK (n= 6), The Royal National Orthopaedic Hospital, Middlesex, UK (n=22), Laboratory of Oncologic Research, ROI, Italy (n=30), Department of Pathology, RH, Denmark (n=10), and Department of Pathology, Medizinische Universität Graz, Austria (n=7). All specimens in this study were handled according to the ethical guidelines described in "Code for Proper Secondary Use of Human Tissue in The Netherlands" of the Dutch Federation of Medical Scientific Societies. Tumors were selected based on accepted clinicopathological and radiological criteria (16). All were primary tumors except for three clear cell chondrosarcomas and six mesenchymal chondrosarcomas, from which only material derived from the recurrent tumor was available. Histology was reviewed by two experienced bone tumor pathologists (JVMGB and PCWH). Clinicopathological data are shown in Table 6.1. Histological grading of the cartilaginous component of dedifferentiated CS was performed according to Evans (17).

Tissue microarray (TMA) construction

TMAs containing 2mm cores of all samples in triplicate were prepared using a TMA Master (3DHISTECH Ltd, Budapest, Hungary). From the dedifferentiated chondrosarcomas we included both the cartilaginous and the anaplastic components. From the mesenchymal chondrosarcomas we selected areas with the undifferentiated small cells as well as areas with cartilaginous differentiation. Normal non-decalcified liver, kidney, and tonsil samples were included on the TMAs for orientation purposes and as internal positive controls.

Immunohistochemistry (IHC)

Immunohistochemical reactions were performed according to standard laboratory methods (18) and visualized using DAB+ Substrate Chromogen System (Dako, Heverlee, Belgium). Details of the primary antibodies used for immunohistochemistry are described in Table 6.2. The negative controls were tissue sections incubated in PBS/BSA 1% without primary specific antibodies. All TMAs were scored by two observers independently (DM and JVMGB) both of whom were unaware of the clinicopathological data. Discrepancies were discussed to reach consensus. Staining intensity (0 = absent, 1 = weak, 2 = moderate, 3 = strong) and extent of the staining (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75%-100%) were assessed. These two measures were added to the sum

score, which was used in all the analyses. For dedifferentiated chondrosarcoma, the well-differentiated and the dedifferentiated component were scored separately. Likewise, for mesenchymal chondrosarcoma both the cartilaginous areas and the small cell component were evaluated separately. Tumors were divided into two groups, having low (mean sum score of <2.5) or high (mean sum score of ≥2.5) protein expression.

Table 6.1 Clinicopathological data of 88 formalin-fixed paraffin-embedded rare chondrosarcomas

	DDCS	MCS	CCS
Total number of tumors	42	23	23
Male	21	8	17
Female	21	15	6
Median age yrs (range)	66 (26-85)	29.5 (15-70)	43 (20-79)
Median follow up (range)*	11 (1-216)	40 (7-204)	57 (1-408)

*follow-up available for 34 dedifferentiated chondrosarcoma, 18 mesenchymal, and 20 clear cell, chondrosarcoma patients
DDCS: dedifferentiated chondrosarcoma, MCS: mesenchymal chondrosarcoma, CCS: clear cell chondrosarcoma

Statistical analysis

Kaplan Meyer analyses were performed using Breslow Generalized Wilcoxon for statistical significance. Survival analyses were performed for metastasis-free survival per subtype. Cox regression analysis was carried out with clinical outcome (metastasis-free survival) as the independent variable. Correlation between expression and grade and individual stainings were evaluated using Pearson chi-squared test for independent variables. Values of $p \leq 0.05$ for asymptomatic 2 sided testing were considered significant. Spearman rank correlation coefficients were calculated for correlations between protein expression patterns. Due to low patient number, loss of cores, and incomplete information on some cases, it was not possible to calculate Kaplan Meyer curves for each staining. The data was analyzed using SPSS version 17.0 software (Chicago, IL, USA).

Inhibition assay

Dedifferentiated chondrosarcoma cell lines L2975 (19) en NDCS-1 (20) were cultured in RPMI1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 1% penicillin/streptomycin (100U/mL) and 10% heat-inactivated Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. Identity of cell lines was confirmed using the PowerPlex® 1.2 system after completion of experiments (Promega Benelux BV, Leiden, The Netherlands). ABT-737 (Abbott Laboratories Inc, IL, USA) was dissolved in DMSO, and doxorubicin and cisplatin were obtained from the in-house hospital pharmacy in a 0.9% NaCl solution. For inhibition assays, the cell lines were plated in 96 well plates for viability assessment ($2x10^5$ cells/well) and allowed to grow and adhere overnight after which the respective drugs were added in their corresponding concentrations. Combination assays were performed as described (21). In short, over the course of 96hrs, cells were treated twice with ABT-737 with an intermittent treatment of cisplatin or doxorubicin. Dose response curves were established for each cell line using dosages ranging from 100nM to 1µM for doxorubicin and cisplatin and 100nM to 5µM for ABT-737, after which combination assays were performed using combinations of all dosages. Combination indices could not be calculated as IC50s were not reached for single treatments. All experiments were performed in triplicate and at least three times. Graphs show data from one representative experiment. Error bars indicate the standard deviation.

Immunoblotting

Immunoblotting using Bcl-2 (clone C 21 Santa Cruz, Heerhugowaard, the Netherlands) and Bcl-xl (clone 54H6, cell signaling, Leiden, the Netherlands) antibodies was performed as previously described (22), using 20µg of each sample.

Table 6.2. Procedures and details of the primary antibodies used for immunohistochemistry

antibody	Manufacturer	dilution	antigen retrieval	Blocking	localisation	pos. control
PTHLH	Oncogene	1:200	trypsin 30 min	-	cytoplasmic	skin
PTHR1	Upstate	1:400	citrate	-	cytoplasmic	skin
Bcl-2	Dako	1:1000	citrate	-	cytoplasmic	Tonsil
Bcl-xl	Cell Signaling	1:400	citrate	-	cytoplasmic	Prostate
FGFR3	Sigma	1:4000	citrate	-	cytoplasmic	umbilical cord
FGF18	Sigma (Atlas)	1:4000	citrate	-	cytoplasmic	tonsil
SOX9	Atlas	1:100	citrate	Milk	nuclear	testis
pSMAD1	Cell signaling	1:100	citrate	Milk	nuclear	colon
pSMAD2	Cell signaling	1:50	citrate	NGS	nuclear	Kidney
CTNB1	Transduction Biosciences	1:2000	citrate	-	cytoplasmic	Skin
SDC2	Lifespan biosciences	1:200	-	-	cytoplasmic	growth plate
SDC3	Proteintech.Group Inc.	1:200	citrate	NGS	cytoplasmic	colon carcinoma
SDC4	Atlas	1:1000	citrate	-	cytoplasmic	placenta
NDST1	Abcam	1:800	Tris-EDTA	-	cytoplasmic	Ileum
PAI-1	American Diagnostics	1:200	-	-	cytoplasmic	cervix carcinoma
CD44v3	Novocastra	1:200	citrate	-	cytoplasmic	tonsil
10 E4	Seikagaku corporation	1:400	Heparitinase buffer	NGS	cytoplasmic	skin
PTGS2	Nuclilab	1:100	citrate	NGS	cytoplasmic	colon carcinoma
KIT	Dako	1:2000	-	-	cytoplasmic	GIST

Results

Histological analysis of dedifferentiated chondrosarcoma
For the 42 dedifferentiated chondrosarcomas, the anaplastic component demonstrated an undifferentiated sarcoma in 30 cases, of which 23 showed spindle-cell morphology. Nine cases showed osteosarcomatous differentiation in the dedifferentiated component. From three tumors the dedifferentiated part was not available. The cartilaginous component demonstrated grade I morphology in 19 cases, grade II in eight and grade III in four cases. From 11 tumors no cartilaginous component was available.

Wnt and SOX9
Nuclear CTNB1 expression as a read-out for canonical Wnt-signaling was absent in all tumors. Variable intensity of cytoplasmic staining was, however, observed in all tumor subtypes (table 6.3). The master transcriptional regulator for chondrogenic differentiation SOX9 (23) was expressed in a large portion of all three subtypes. A trend of slightly higher expression in the cartilaginous parts of the tumors was observed (fig 6.1A, B, table 6.3). In the cartilaginous cells of dedifferentiated chondrosarcoma Spearman's rank correlation revealed an association ($p < 0.05$) between protein expression of SOX9 and SDC2 ($r_s = 0.70$), SDC3 ($r_s = 0.71$), and SDC4 ($r_s = 0.44$).

Heparan sulfate proteoglycan expression
Heparan sulfate as demonstrated by immunoreactivity for 10E4 was variable. The anaplastic component of dedifferentiated chondrosarcoma as well as the small cell component of mesenchymal chondrosarcoma demonstrated more extensive and more intense expression as compared to their cartilaginous components. The expression in clear cell chondrosarcoma was limited (fig 6.1C). NDST1 (N-deacetylase/N-sulfotransferase), an enzyme that can interact with EXT1 and EXT2 during heparan sulfate chain formation (24), was highly expressed in dedifferentiated chondrosarcoma, and in the small cell component of mesenchymal chondrosarcoma. In the cartilaginous components of mesenchymal chondrosarcoma and in clear cell chondrosarcomas high NDST1 expression was observed in approximately half of the tumors (fig 6.1D, table 6.3). The expression of the heparan sulfate proteoglycans syndecan 2, -3, and -4 and of CD44 variable exon 3 was also variable in all 3 tumor subtypes (fig 6.1E, F, G, H). Expression of syndecans 2, -3, and -4 was higher in the dedifferentiated parts of dedifferentiated chondrosarcomas than in the cartilaginous parts and the other tumor types.

FGF signaling
The expression of FGFR3 was strong and extensive in most tumors of all three subtypes (fig 6.2A, B). The expression of FGF18, the ligand for FGFR3 in the normal growth plate, was more variable (fig 6.1I).

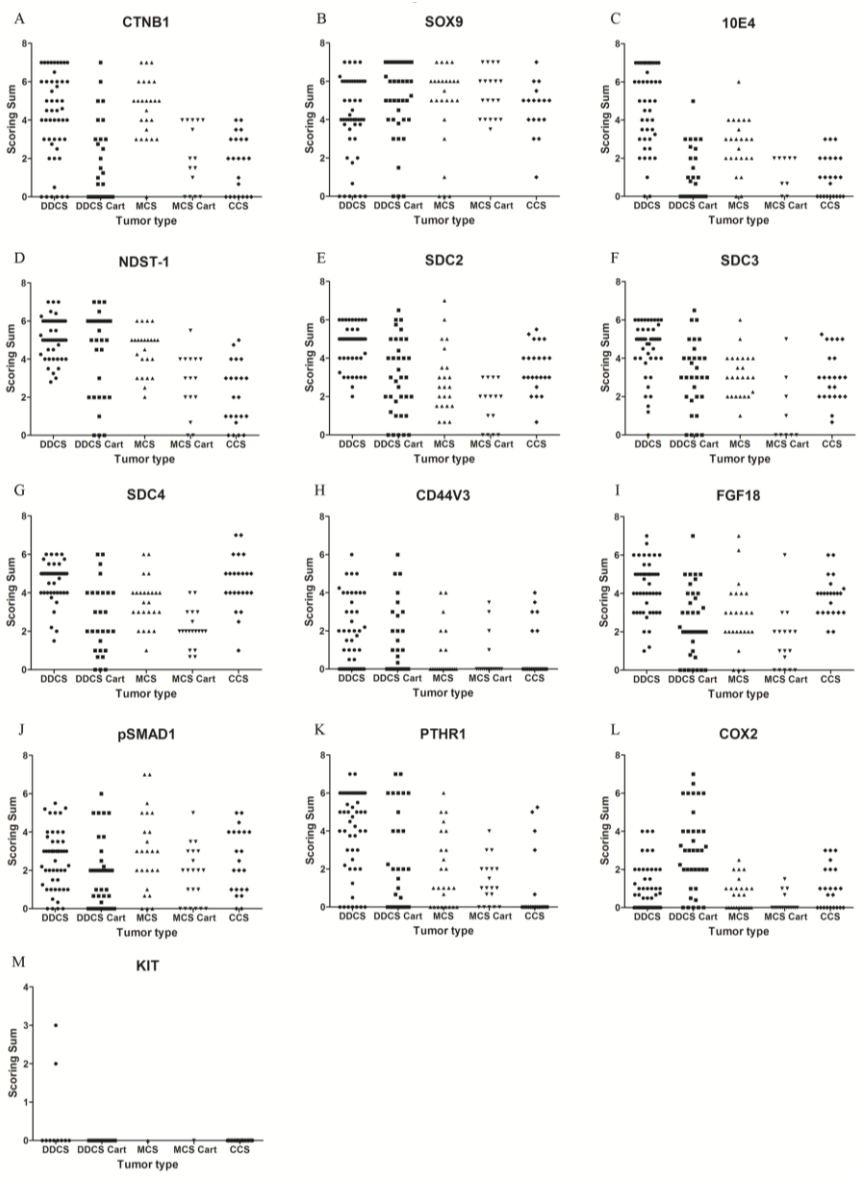


Figure 6.1: Dot plots representing sum scores for all immuno stainings in all tumor tissues included on the TMA, grouped by tumor type.
A: CTNB1. **B**: SOX9. **C**: 10e4. **D**: NDST-1. **E**: Syndecan 2 (SDC2). **F**: Syndecan 3 (SDC3). **G**: Syndecan 4 (SDC4). **H**: CD44v3. **I**: FGF-18. **J**: p-SMAD1. **K**: PTHR1. **L**: COX-2. and **M**: KIT. DDCS, anaplastic component of dedifferentiated chondrosarcoma; DDCS cart, the cartilaginous component of dedifferentiated chondrosarcoma; MCS, the small cell component of mesenchymal chondrosarcoma, MCS cart, the cartilaginous component of mesenchymal chondrosarcoma; CCS, clear cell chondrosarcoma.

TGFbeta/BMP signaling
Nuclear pSMAD1 expression, which indicates active BMP signaling, was moderate in all three subtypes (fig 6.1J). TGFbeta signaling, as evidenced by PAI-1 and nuclear pSMAD2 staining, was rather high in all three subtypes (fig 6.2C-E, table 6.3). Expression of pSMAD2 showed some variation, ranging from high expression in half of the mesenchymal chondrosarcomas in the small-cell component, to almost all clear cell chondrosarcomas. In clear cell chondrosarcoma, PAI-1 expression was positively correlated with both pSMAD2 ($r_s = 0.71$; $p < 0.001$) and pSMAD1 ($r_s = 0.60$; $p = 0.009$). We also demonstrated a positive correlation between PAI-1 and pSMAD2 expression in dedifferentiated chondrosarcoma ($r_s = 0.45$; $p = 0.01$), and high pSMAD2 protein expression was significantly associated with longer metastasis-free survival (HR = 0.38, p=0.048) (fig 6.2F).

PTHLH signaling
Parathyroid-hormone signaling was assessed using PTHLH and PTHR1 (25). PTHLH was high in dedifferentiated chondrosarcoma and clear cell chondrosarcoma (fig 6.3A, B), whereas, PTHR1 expression was found to be low in most of the clear cell chondrosarcomas and mesenchymal chondrosarcomas. In dedifferentiated chondrosarcoma PTHR1 expression was low in the cartilaginous component with higher expression in the anaplastic component (fig 6.1K).

Bcl-2 and Bcl-xl signaling and chemoresistance
Bcl-2 was assessed as the downstream signaling molecule of PTHLH in the growth plate (26). A positive correlation between PTHR1 expression and Bcl-2 was observed in clear cell chondrosarcoma ($r_s = 0.47$; $p = 0.04$). Since anti-apoptotic proteins were shown to play an important role in chemoresistance of conventional chondrosarcoma (21), we additionally evaluated the expression of the Bcl-2 family member Bcl-xl. Both clear cell and mesenchymal chondrosarcoma showed high expression of both Bcl-2 (fig 6.4C, D) and Bcl-xl (fig 6.4F). In contrast, in dedifferentiated chondrosarcoma, high expression of mainly Bcl-xl was found (fig 6.3E, F). L2975 and NDCS-1 are dedifferentiated chondrosarcoma cell lines showing strong expression of Bcl-xl and Bcl-2 (fig 6.4G). As L2975 showed only 40% reduction in cell viability after 1µM doxorubicin and 10% reduction in cell viability after 1µM cisplatin and no reduction in cell viability could be achieved in NDCS-1 after treatment with either doxorubicin or cisplatin (fig 6.3A, B), we continued to investigate the effect of inhibition of Bcl-2 family members using the BH-3 mimetic ABT-737. Cells were treated with ABT-737 prior to and after doxorubicin or cisplatin addition as we previously showed that this was the most effective course of combination treatment (21). Interestingly, even though single treatment with 5µM ABT-737 did not result in a reduction of cell viability in either cell line (fig 6.3C), at concentrations as low as 100nM, inhibition of Bcl-2 family members was sensitizing the cells to both doxorubicin and cisplatin (fig 6.4H).

Table 6.3 Percentage of tumors showing high expression (mean sum score of ≥2.5) of proteins per subtype as determined by immunohistochemistry

	Dedifferentiated CS		**Clear cell CS**	**Mesenchymal CS**	
	dedifferentiated areas	cartilaginous areas		Small cells	Cartilaginous areas
Bcl-2	10/38 (26%)	1/25 (4%)	19/22 (86%)	22/23 (96%)	10/17 (59%)
Bcl-xl	36/39 (92%)	10/32 (31%)	13/17 (76%)	8/12 (67%)	3/8 (38%)
PTHLH	30/37 (81%)	25/27 (93%)	11/12 (92%)	4/7 (57%)	1/5 (20%)
PTHR1	28/38 (74%)	8/25 (32%)	4/22 (18%)	9/23 (39%)	3/17 (18%)
SOX9	31/39 (79%)	26/30 (87%)	16/17 (94%)	19/23 (83%)	18/18 (100%)
NDST1	38/38 (100%)	22/31 (71%)	9/21 (43%)	21/22 (95%)	8/14 (57%)
10 E 4	32/38 (84%)	7/32 (22%)	3/22 (14%)	1/17 (6%)	13/23 (58%)
CD44V3	14/36 (39%)	6/23 (26%)	4/21 (19%)	7/23 30%)	4/18 (22%)
SDC2	36/37 (97%)	18/31 (58%)	19/22 (86%)	13/22 (59%)	4/14 (29%)
SDC3	31/36 (86%)	19/28 (68%)	14/22 (64%)	15/23 (65%)	2/9 (22%)
SDC4	35/38 (92%)	10/23 (43%)	21/22 (95%)	18/23 (78%)	6/17 (35%)
pSMAD1	19/39 (49%)	8/31 (26%)	10/21 (48%)	13/23 (57%)	7/20 (35%)
pSMAD2	29/39 (74%)	22/33 (67%)	21/22 (95%)	9/17 (53%)	15/23 (65%)
PAI1	38/38 (100%)	26/28 (93%)	20/21 (95%)	21/21 (100%)	13/14 (93%)
CTNB1	31/37 (84%)	9/30 (30%)	7/21 (33%)	22/23 (96%)	6/15 (40%)
FGF18	34/38 (89%)	18/30 (60%)	20/22 (91%)	12/23 (52%)	3/16 (19%)
FGFR3	40/40 (100%)	26/31 (84%)	21/21 (100%)	21/23 (91%)	17/18 (94%)
PTGS2	2/39 (5%)	17/32 (53%)	4/22 (18%)	1/23 (4%)	0/17 (0%)
KIT	2/37 (5%)	0/26 (0%)	0/21 (0%)	2/23 (9%)	0/19 (0%)

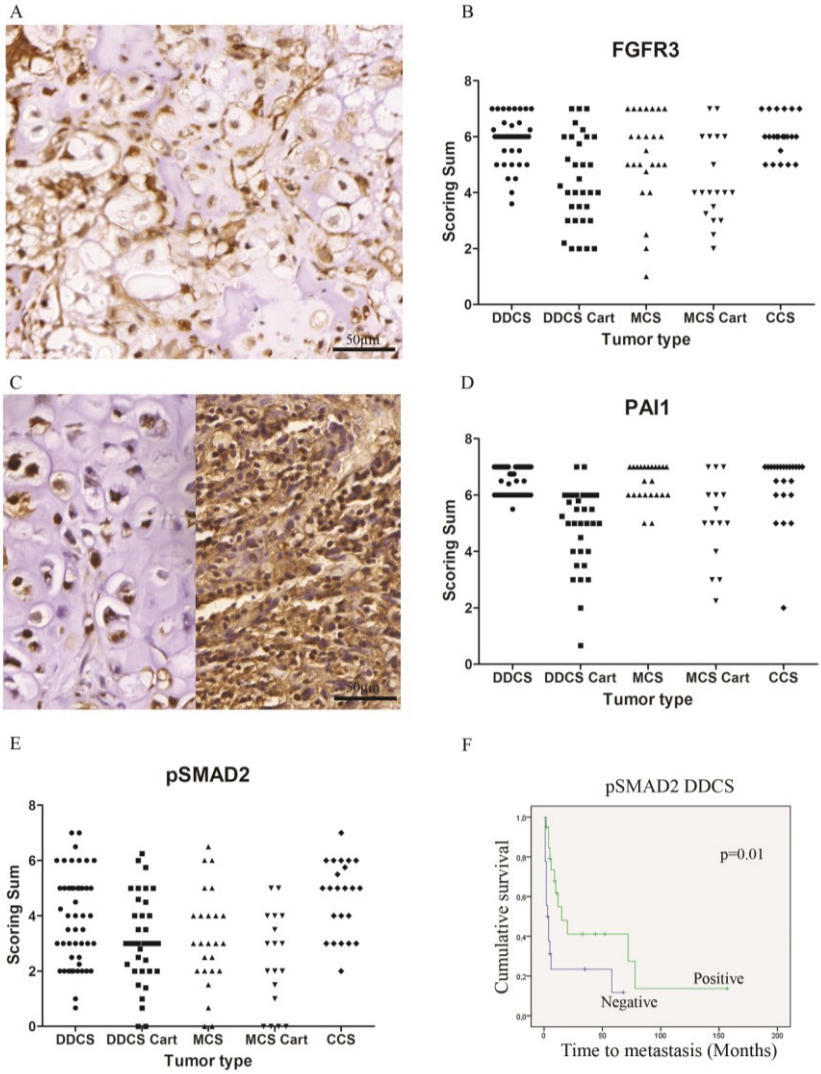


Figure 6.2: Active FGF signaling and TGFbeta signaling through pSMAD2 and PAI1 in all rare subtypes of chondrosarcoma.

A: High FGFR3 expression in CCS. **B**: Sum scores of FGFR3 in all tumor tissues included on TMA, by tumor type. **C**: High level of PAI1 expression in cartiilaginous component (left panel) and anaplastic component (right panel) of dedifferentiated chondrosarcoma. **D,E**: Sum scores of PAI1 (**D**) and pSMAD2 (**E**) in all tumor tissues included on TMA, by tumor type. **F**: Kaplan Meier of pSMAD2 staining in dedifferentiated chondrosarcoma (DDCS) shows positive association between pSMAD2 expression and metastasis free survival (p=0.01).

Possible therapeutic targets PTGS2 and KIT
In contrast to central chondrosarcomas (27), PTGS2 (COX-2) expression was rather low in the rare chondrosarcoma subtypes. Only in the dedifferentiated component of dedifferentiated chondrosarcoma was high expression observed in approximately half of the tumors. Expression of the tyrosine kinase receptor KIT was low to absent in all tumor subtypes (fig 6.1L, M, table 6.3).

Discussion
To identify possible therapeutic targets in rare chondrosarcoma subtypes, we investigated expression of signaling pathways that play pivotal roles in the normal growth plate and in conventional chondrosarcoma. The chondrosarcoma subtypes that are the subject of our study are rare and an extensive study of possible therapeutic targets in these tumors has not previously been carried out. Through the EuroBoNeT consortium we were able to collect paraffin blocks of a relatively large series enabling us to systematically analyze the activity of growth plate signaling pathways. Not only did we observe differences between the subtypes, but also between the cartilaginous cells and anaplastic or small cells in dedifferentiated and mesenchymal chondrosarcoma, respectively. Heparan sulfate proteoglycan and PTHLH expression were noted to vary between tumor types and cell types (fig 6.1). All heparan sulfate proteoglycans studied, including SDC2-4 and CD44v3, as well as 10E4 and NDST1, were expressed in the cytoplasm of the cells and sometimes to a lesser extent on the membrane. Aberrant cellular localization of these proteins is a known phenomenon in osteochondromas and chondrosarcomas (15;28;29).
SOX9, TGFbeta, and FGFR3 signaling was highly active in all chondrosarcoma subtypes. Whereas activating FGFR3 mutations stimulate proliferation in certain types of cancer, they cause several forms of dwarfism-associated chondrodysplasias in humans and mice, demonstrating an inhibitory effect in bone (30). In this study, we demonstrated high FGFR3 expression in central dedifferentiated -, mesenchymal - , and clear cell chondrosarcoma. Previously, we demonstrated high FGFR3 in peripheral dedifferentiated chondrosarcomas, but rather low expression in secondary peripheral chondrosarcomas (31). In 2007, Oji et al demonstrated high FGFR3 mRNA and protein in rat chondrosarcoma cells *in vitro*. Stimulation of the FGFR3 receptor with an FGFR3 agonist reduced the proliferative rate of the cells. However, the addition of a polyclonal antibody for FGFR3 did not reverse this effect, suggesting a deregulation in the FGFR3 signaling pathway (32). Based on the literature, overexpression of FGFR3 in chondrosarcoma would be expected to inhibit IHH and thereby PTHLH (25). However, our study showed PTHLH to be highly expressed in most of the tumors, indicating pathway activity independent of FGFR3, as was also found for conventional chondrosarcoma (14;18).

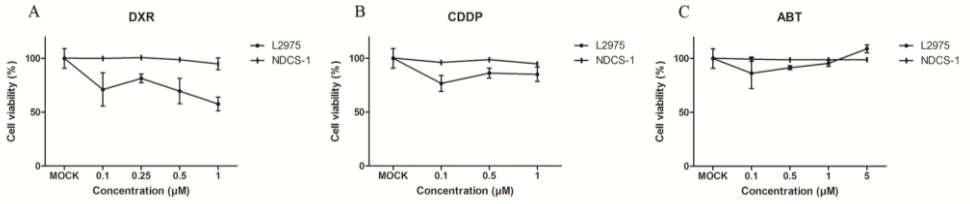
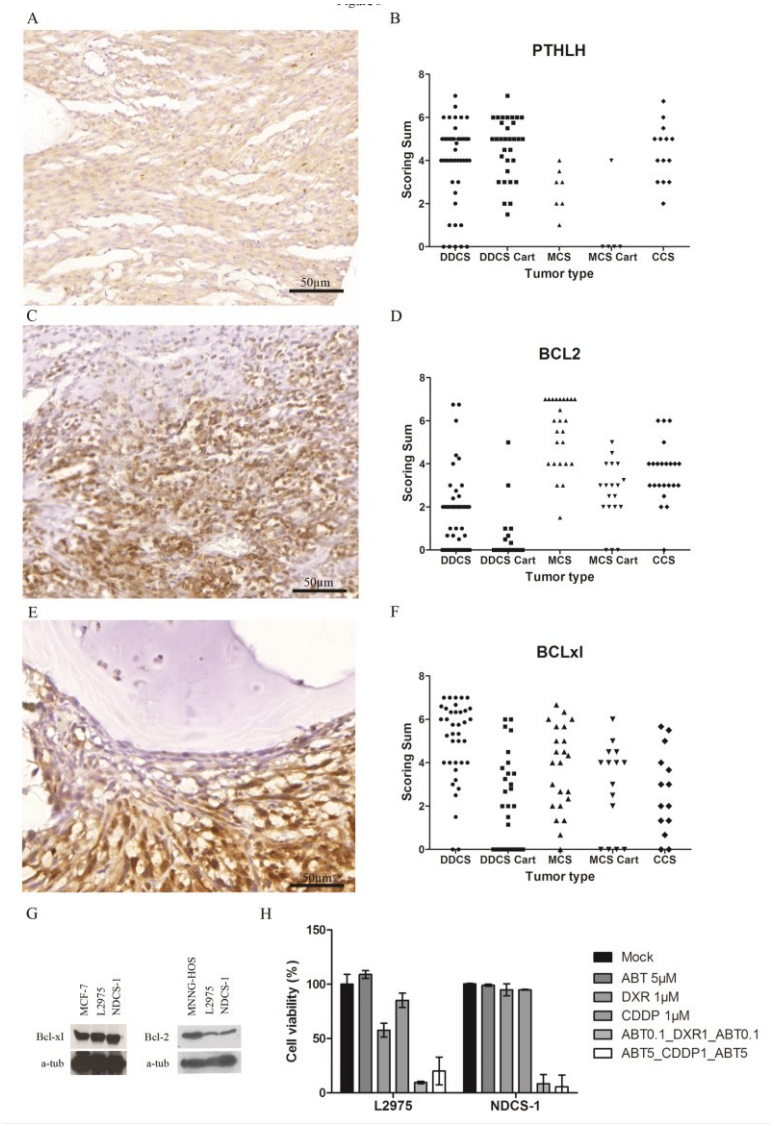


Figure 6.3: Dose response curves for doxorubicin (A) cisplatin (B) and ABT-737 (C). A, B: L2975 shows 40% reduction in cell viability after 1µM doxorubicin (DXR) and 10% reduction in cell viability after 1µM cisplatin (CDDP), whereas NDCS-1 shows no reduction in cell viability after either doxorubicin or cisplatin. **C:** Neither cell line shows a response to ABT-737 treatment alone.

In accordance with previous findings in central chondrosarcoma (18) we found PTHR1 expression to be higher in the anaplastic cells of dedifferentiated and mesenchymal chondrosarcomas as compared to the cartilaginous cells in these tumors and in clear cell chondrosarcomas, suggesting a role for PTHLH signaling in the fast proliferation of these tumors. Despite the low PTHR1 expression in clear cell and mesenchymal chondrosarcoma, we observed high expression of Bcl-2, indicating that this might be induced via a different signaling pathway, such as an apoptosis directed pathway. We previously found absence of Bcl-2 in peripheral dedifferentiated chondrosarcomas (31). We here show that Bcl-2 expression was generally high in mesenchymal and clear cell chondrosarcoma, whereas dedifferentiated chondrosarcoma rather shows high expression of Bcl-xl. In conventional central chondrosarcoma, we previously showed high Bcl-2 expression (18) and recently showed that conventional central chondrosarcoma cells with high expression of Bcl-2 family members could be sensitized to doxorubicin and cisplatin by inhibition of Bcl-2 family members using the BH-3 mimetic ABT-737 indicating an important role for Bcl-2 family members in chemoresistance of conventional chondrosarcoma (21). We now also show high Bcl-2 and Bcl-xl expression in the two dedifferentiated chondrosarcoma cell lines and a reversal of chemoresistance during combination treatment with ABT-737 and conventional chemotherapeutics. As a BH-3 mimetic ABT-737 has also been shown to inhibit Bcl-xl (33). Our results indicate an important role for Bcl-2 and Bcl-xl in the chemoresistance of dedifferentiated chondrosarcoma cells. The high Bcl-2 and Bcl-xl expression in mesenchymal and clear cell chondrosarcoma suggests that also in these subtypes Bcl-2 family members contribute to chemoresistance and that patients might benefit from a treatment combining Bcl-2 family inhibitors and chemotherapy. At the time of preparing this manuscript, no cell lines of these subtypes are available to further test this.

Figure 6.4: Bcl-2 as a possible target in rare chondrosarcoma subtypes. **A**: PTHLH expression in dedifferentiated chondrosarcoma. **C**: High Bcl-2 expression in mesenchymal chondrosarcoma. **E**: Bcl-xl expression in dedifferentiated chondrosarcoma, showing high expression in dedifferentiated component, with few positive cells in cartilage component. **B**, **D**, **F**: Sum scores of PTHLH (**B**), Bcl-2 (**D**), and Bcl-xl (**F**) in all tumor tissues included on the TMA, by tumor type. **G**: Western blotting shows strong expression of Bcl-2 and Bcl-xl in dedifferentiated chondrosarcoma cell lines L2975 and NDCS-1. **H**: Reversal of chemoresistance using the BH3 mimetic ABT-737 in dedifferentiated chondrosarcoma cell lines L2975 and NDCS1. Both cell lines show resistance to doxorubicin (DXR) and cisplatin (CDDP) alone, and were not responsive to ABT-737 as a single agent. Combination of ABT-737 with doxorubicin or cisplatin, however, showed strong inhibition of cell viability suggesting a role for Bcl-2 family members in chemoresistance of dedifferentiated chondrosarcoma (concentrations are in µM).



To investigate BMP and TGFbeta signaling, we evaluated the expression of pSMAD1, and pSMAD2 and PAI-1, respectively. BMP signaling, as indicated by pSMAD1 expression, was variable in all three subtypes. pSMAD1 expression was slightly higher in the dedifferentiated and small cell components of dedifferentiated and mesenchymal chondrosarcoma, respectively. In the growth plate, pSMAD1 plays a role in early condensation, correlating with the phenotypic characteristics of these components. TGFbeta signaling, as indicated by expression of pSMAD2 and PAI-1, was highly active in all chondrosarcoma subtypes investigated. High expression of PAI-1 was observed previously in the anaplastic components of conventional and peripheral dedifferentiated chondrosarcomas (31;34). In the cartilaginous components of peripheral dedifferentiated chondrosarcomas a prognostic value has been described for PAI-1 expression (31). In our study, high pSMAD2 in the anaplastic component of dedifferentiated chondrosarcoma was related to longer metastasis-free survival.

TGFbeta is known to have a dual role in cancer. Not only does it promote tumor growth, invasion and metastasis, it is also described to prevent malignant progression in the surrounding environment of an oncogenic process (35). Since TGFbeta signaling was active in a subset of dedifferentiated - and mesenchymal chondrosarcomas, and in almost all clear cell chondrosarcomas, TGFbeta might be a promising therapeutic target. The most useful agents in blocking TGFbeta signaling are likely TGFbeta-targeting monoclonal antibodies like fresolimumab and tyrosine kinase inhibitors (36). In dedifferentiated and mesenchymal chondrosarcomas, PAI-1 expression was higher than pSMAD2 expression indicating that PAI-1 may also be under the influence of other signaling pathways, like the EGFR signaling pathway (37). Further studies will be needed to determine the involvement of the EGFR pathway and the value of EGFR-targeting treatment in these subtypes.

As in conventional chondrosarcoma (38), KIT expression was absent in rare chondrosarcoma subtypes. In addition, the absence of nuclear CTNB1 indicated that canonical Wnt signaling is not important in these tumor types. Recently our group demonstrated beneficial effects of celecoxib treatment in central chondrosarcomas (27). However, whereas 65% of the conventional chondrosarcomas express PTGS2, expression was absent in most of the rare chondrosarcoma subtypes making beneficial effects of celecoxib unlikely.

In summary, we observed both common and distinct protein expression patterns in three rare chondrosarcoma subtypes. High pSMAD2 and PAI-1 expression emphasize the importance of TGFbeta signaling and suggest that TGFbeta inhibitors might be a promising therapeutic option for patients with rare chondrosarcoma subtypes. In addition, our results suggest an important role for the Bcl-2 family members Bcl-2 and Bcl-xl in chemoresistance of the rare chondrosarcoma subtypes. Similar to conventional chondrosarcoma, the chemoresistance of dedifferentiated chondrosarcoma *in vitro* could be overcome

using inhibition of Bcl-2 family members, repairing the apoptotic machinery rendering the cells sensitive to chemotherapy. This suggests that the combination of BH-3 mimetics with conventional chemotherapeutic agents is a promising therapeutic strategy for metastatic or inoperable chondrosarcoma, irrespective of the histological subtype.

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