Towards new therapeutic strategies in chondrosarcoma

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Towards new therapeutic strategies in chondrosarcoma

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1. General Introduction

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Based on Encyclopedia of Cancer, Springer Berlin Heidelberg 2008. ISBN 978-3-540-36847-2. DOI 10.1007/978-3-540-47648-1_1119

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1.1 Chondrosarcoma

Definition

Chondrosarcoma of bone is a malignant hyaline cartilage forming tumour (Figure 1.1). The term chondrosarcoma describes a heterogeneous group of leasions with diverse morphologic features and clinical behaviour. Apart from conventional central and peripheral chondrosarcoma constituting the largest subgroup (~85%) this encompasses rare subtypes such as clear cell chondrosarcoma (~1%), mesenchymal chondrosarcoma (~2%), juxtacortical chondrosarcoma (~2%) and dedifferentiated chondrosarcoma (~10%) as well[1]. In this thesis the use of the term chondrosarcoma is confined to conventional chondrosarcoma.

Characteristics

The incidence of conventional chondrosarcoma is about 1:50 000[1]. The incidence in males and females is almost equal, and the mean age of diagnosis is 30 to 60 years. Chondrosarcomas are mostly found in bones that elongate by endochondral ossification with the most common sites being the pelvis followed by the proximal femur, proximal humerus, distal femur and ribs. When comparing histologically the different cartilaginous tumours to the growth plate, parallels between normal and neoplastic chondrocyte growth and differentiation become evident. Resting (primitive, mesenchymal stem-cell like) chondrocytes are found in mesenchymal chondrosarcoma[2], while clear cell chondrosarcoma consists mainly of hypertrophic chondrocytes[3]. Osteochondroma, a benign cartilaginous tumour at the surface of bone, recapitulates all differentiation levels of the growth plate[4]. In contrast, enchondroma, a benign cartilaginous tumour in the medullar cavity of bone, and conventional peripheral and central chondrosarcoma mostly contain proliferating chondrocytes, lying in small lacunae[5,6]. The more rarely occurring dedifferentiated chondrosarcoma is thought to arise from conventional chondrosarcoma in which tumour cells transdifferentiate towards a more spindle-cell phenotype[7]. In addition, the rare subtype juxtacortical chondrosarcoma is recognised, which also contains proliferating chondrocytes[6]. This specific diagnostic term is used as a result of its typical clinicoradiological presentation and its in general relatively favourable prognosis as compared to conventional chondrosarcoma.

There is a clinical as well as a morphological spectrum of cartilaginous tumours. Central chondrosarcoma is the most common subtype (>85%) of conventional chondrosarcoma[8]. Malignant transformation of an enchondroma to a central chondrosarcoma is estimated to be < 1%. However, since in 40% of central chondrosarcomas remnants of a pre-existing enchondroma are found, there is considerable debate whether these tumours are secondary to enchondroma or arise mostly *de novo*[8]. The

frequency of malignant transformation is significantly higher (15-30%) in patients with multiple enchondromas in the context of the extremely rare non-hereditary disorder Ollier disease[9]. Conventional chondrosarcoma at the surface of bone (secondary peripheral chondrosarcoma) per definition develops within a pre-existing osteochondroma[6]. Secondary peripheral chondrosarcomas constitute up to 15% of conventional chondrosarcomas in referral centers[8]. Multiple osteochondromas (MO), previously known as hereditary multiple exostoses (HME), is an autosomal dominant disorder and malignant transformation occurs in 1-3% of the cases of MO[10-13]. In addition, chondrosarcomas may biologically progress: up to 13% of recurrent chondrosarcomas exhibit a higher grade of malignancy than the original neoplasm, with an adverse prognosis[14,15].

Diagnosis
Benign cartilaginous tumours are asymptomatic, and are often found by incidence at radiology made for other reasons[8]. In contrast, malignant tumours almost always produce symptoms such as local swelling and pain. The distinction between enchondroma or osteochondroma and low-grade conventional chondrosarcoma is difficult, both at the radiological level (in case of central chondrosarcoma) and the histological level (for both subtypes)[16,17]. Diagnosis should be made in a multidisciplinary setting, based on clinical, radiological and histological aspects. Dynamic MRI has been proven to

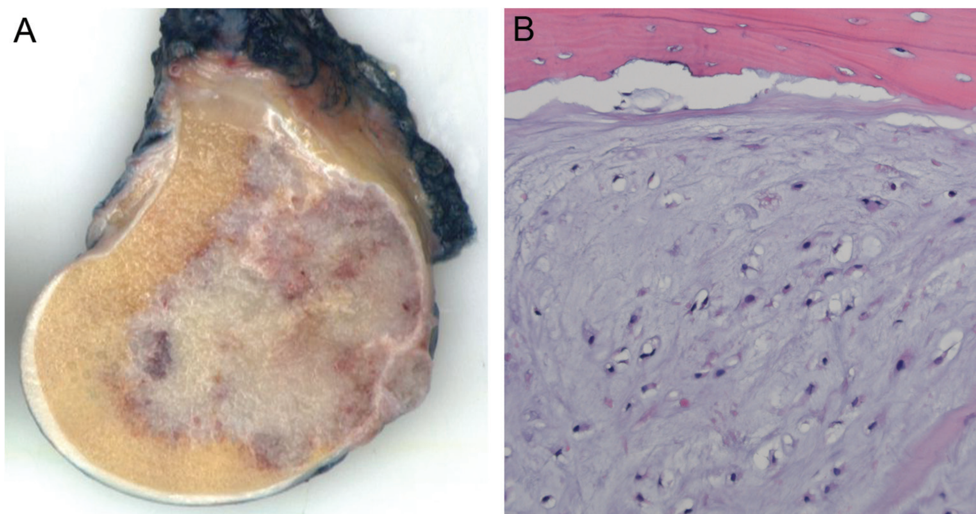

Figure 1.1 Central chondrosarcoma. (A) Gross specimen of central chondrosarcoma of distal femur. (B) Microscopic image of grade II chondrosarcoma (Hematoxylin and Eosin staining). Moderate cellularity Tumour cells are lying in a chondroid matrix, with moderate cellularity. Pre-existing lamellar bone (top).

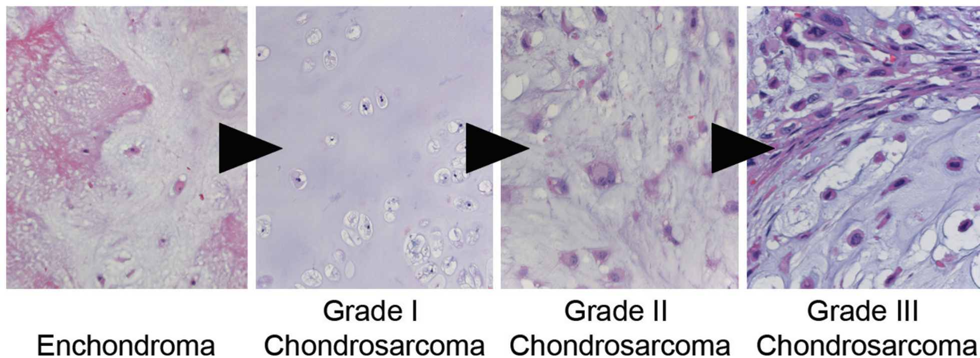

Figure 1.2 Histological spectrum of central cartilaginous tumours. Enchondroma is hypocellular and a large amount of cartilaginous matrix is present. Foci of calcification (left) are common. Cellularity in grade I chondrosarcoma is also low, cytonuclear atypia is limited and a large amount of hyaline extracellular matrix is present. In addition, binucleated cells are seen, while mitosis are absent. Grade II chondrosarcoma shows increased cellularity and a diminished amount of matrix which becomes more mucomyxoid. Cytonuclear atypia is found more often and mitosis may be present. High cellularity and abundant cutonuclear atypia are found in grade III chondrosarcoma. Note that progression to a higher grade occurs only in 13% of the tumours after (incomplete) surgical resection[19].

be informative in distinguishing benign from malignant cartilaginous tumours[18]. Histologically, the distinction between enchondroma and low-grade conventional central chondrosarcoma is mainly based on growth patterns and cytomorphological features[16,19,20]. Encasement (new shells of reactive bone, formed at the periphery of cartilage nodules), is a feature of benign tumours, while entrapment (permeation of tumour around pre-existing lamellar bone), points to a faster growing process and thus malignancy[16].

Histologically, chondrosarcomas are divided in three grades of malignancy based primarily on cellularity, nuclear size and chromasia, mitoses and the composition of the matrix[14] (Figure 1.2). Grade I tumours are moderately cellular and nuclei are uniformly sized and hyperchromatic. Grade II tumours are more cellular and nuclei are atypically shaped, hyperchromatic and larger, and mitoses can be found. At the end of the spectrum, grade III tumours are hypercellular, with nuclear pleomorphism, and mitoses can be frequent. In addition, the extracellular matrix of grade III tumours becomes more mucoid/myxoid compared to the abundant chondroid matrix seen in grade I tumours and their vascularity is increased. Differences in 5 year survival and the occurrence of metastases show the clinical importance of histological grading[14]. While grade I and II tumours rarely metastasise (respectively 0 and 10%), grade III tumours do so in 71% of the cases. 5 Year survival is lowest in patients with grade III tumours (29%) compared to 64% in grade II tumours and 83% in grade I chondrosarcomas[14,15].

Therapy

A correct diagnosis is essential for therapeutic decision making[21]. Surgery is the only option for curative treatment since chondrosarcomas are highly resistant to conventional chemotherapy and radiotherapy. Therefore, development of targeted therapy for chondrosarcoma would mean a major advance in chondrosarcoma therapy. Studies regarding the mechanism underlying resistance are sparse[22]. While for benign leasions a wait-and-see policy is justified, malignant tumours require more aggressive treatment. Grade I chondrosarcomas are prone to local recurrence but almost never metastasize[14]. Therefore, there is a trend in sarcoma centers to treat them by curettage with margin improvement by phenol[23] or cryosurgery[24]. In contrast, high-grade tumours are usually treated by often mutilating wide en bloc resection or even amputation, since these often metastasize, being lethal in the majority of patients.

Genetics

Although histologically similar, central and peripheral chondrosarcoma have been shown to be genetically, and thereby molecularly, different entities. In Multiple Osteochondromas germline mutations have been identified in the EXT tumour suppressor genes, located on chromosomes 8q24 (EXT1) and 11p11-12 (EXT2)[25-28]. These EXT genes encode glycosyltransferases involved in heparan sulphate biosynthesis[29]. In MO, germline mutations in EXT1 or EXT2 with loss of the remaining wild-type allele is found. Recently, in solitary osteochondromas somatic homozygous deletions of EXT1 have been demonstrated[30]. In both hereditary and solitary osteochondromas mRNA expression of EXT1 or EXT2 is decreased[31]. This probably results in intracellular accumulation of heparan sulphate proteoglycans (HSPGs), since the Syndecan2 and the CD44v3 core proteins were shown to aberrantly localize in the Golgi apparatus in solitary and hereditary osteochondroma and peripheral chondrosarcoma[31]. The EXT1 homologue in *Drosophila* (tout velu, ttv) is required for IHH diffusion to its receptor that signals to PTHLH and thereby controls chondrocyte proliferation[32]. In contrast to the growth plate, in osteochondroma IHH signalling has become cell autonomous, probably overcoming the diffusion problems caused by defective HSPGs due to EXT inactivation.

Additional genetic alterations are thought to be required for malignant transformation of osteochondroma towards low-grade secondary peripheral chondrosarcoma. These additional alterations presumably cause chromosomal instability, since peripheral chondrosarcomas are shown to be aneuploid with DNA indices ranging from 0.56 to 2.01[33]. At the protein level, progression from osteochondroma towards low-grade peripheral chondrosarcoma is characterised by a re-activation of PTHLH signalling[34]. Its downstream target, BCL-2, can be used as a diagnostic marker in those cases in which it is hard to distinguish between benign and malignant

cases, with osteochondromas being negative in 95% (specificity) and chondrosarcomas scoring positive in 57% (sensitivity)[35]. This re-activation of PTHLH is hypothesised to be caused by increased TGF-beta signalling, since IHH signalling has been shown to be downregulated in peripheral chondrosarcoma[36].

Despite the increasing number of genetical studies including peripheral and central chondrosarcomas as separate subgroups, no specific genetic aberrations for the more common central chondrosarcoma have been identified as yet. Mutations in EXT1 and EXT2 have not been reported, and reports on IHH signalling on proliferation in central chondrosarcoma are still inconclusive. A positive relation between histological grade and the degree of karyotypic complexity and aneuploidy was found[33]. Near-diploidy and limited loss of heterozygosity are typical of low-grade central chondrosarcomas rather than of peripheral chondrosarcomas pointing to an oncogenic mechanism with few alterations, sufficient for oncogenesis[33]. Multiple studies report alterations at chromosomal bands 9p21 and 12q13-15[37-41]. Genetic loss at the 9p21 locus as found by cytogenetics, loss of heterozygosity analysis and comparative genomic hybridisation suggest an important role for the CDKN2A/INK4a locus. Loss of protein expression of the tumour suppressor gene p16, encoded by this locus, was found to be associated with increased histological grade in central chondrosarcoma, and thereby to be important for tumour progression[42].
Rearrangements in the 12q13-14 region have been frequently reported in sarcomas. Several genes in this region have been indicated to be of importance for tumourigenesis, such as SAS (sarcoma amplified sequence), CDK4 (cyclin dependent kinase 4) and GLI (glioma associated oncogene homologue). Also two other often implicated genes in sarcomas, HMGA2 (high mobility group AT-hook 2) and MDM2 (murine double minute 2), are located just outside the 12q13-14 region. Moreover, the progression from low-grade to high-grade central chondrosarcoma is characterised by p53 alterations[33]. Despite the large number of studies involving central chondrosarcomas, the exact underlying molecular mechanism is still largely unknown.

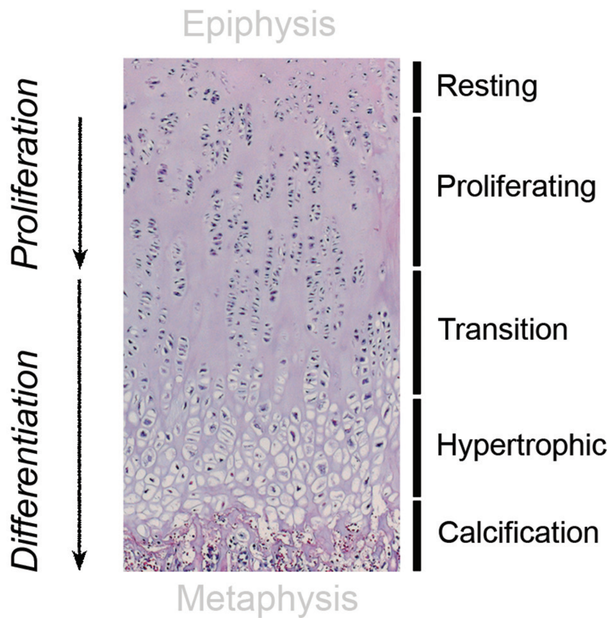

Figure 1.3 Organisation of the human growth plate.Hematoxylin and Eosin staining of the epiphyseal growth plate is shown. The resting zone contains stem-cell like chondrocytes. These cells start proliferating upon a yet unknown stimulus, thereby initiating longitudinal growth of the bone. The cells in the lower part of the resting zone enter the proliferative zone and ensemble on orderly, longitudinal collumns. These chondrocytes stop proliferating at a certain timepoint and differentiate into hypertrophic chondrocytes in the transition zone. Finally, the hypertrophic chondrocytes undergo apoptosis, allowing ingrowth of vessels and invasion of osteoblasts depositing bone. This leaves a scaffold for new bone formation.

1.2 Clinical problems in chondrosarcoma management

In this thesis three clinical problems are addressed:

1. The difficult histological distinction between benign and low-grade malignant cartilaginous tumours
2. The lack of prognostic markers superior to subjective histological grading
3. Chemo- and radiotherapy resistance of chondrosarcoma

1.2.1 Histological distinction between benign and low-grade malignant cartilaginous tumours

The distinction between enchondroma and low-grade chondrosarcoma is considered one of the most difficult subjects in surgical pathology. Currently, diagnostic parameters are lacking, both at the histological[16,19,20] and radiological[43-45] level. However, the distinction is important since enchondromas are normally expectatively followed. Surgical treatment of enchondromas is only applied in case of recurrent fracture, unacceptable swelling or functional loss. In these cases intraleasional surgery is applied, also known as curettage, in which the tumour is removed without aiming for tumour free margins. The benefit of this surgical technique is that the environment of the tumour, the bone, and thereby its function, is unaffected. In contrast, low-grade chondrosarcomas are more prone to recur after intraleasional surgery. In addition, they demonstrate a more aggressive behaviour in 13% of the local recurrences[14,15]. Therefore, in case of low-grade chondrosarcoma, intraleasional surgery is combined with local application of phenol[23] or cryosurgery to improve surgical margins[21,24].

Enchondroma and low-grade chondrosarcoma are part of a continuous spectrum (Figure 1.2) that, due to the lack of molecular parameters, is rather artificially separated and subjected to a large interobserver variability[17]. It would be of great help to make use of objective histological parameters, to identify those tumours that are prone to local recurrence and need a more aggressive therapy.

1.2.2 Lack of prognostic markers

At the opposite end of the spectrum of cartilaginous tumours, a similar diagnostic problem occurs. Chondrosarcomas are divided in three histological grades, according to the criteria proposed by Evans in 1977 (Figure 1.2). The 10 years survival of chondrosarcoma patients decreases gradually along the spectrum. Whereas 83% of the patients with a grade I chondrosarcoma are still alive after 10 years, this is 64% in case of grade II chondrosarcoma and only 29% for grade III chondrosarcoma. Whereas grade I chondrosarcoma almost never metastasise to distant organs, grade II chondrosarcoma metastasises in 10% of the cases and grade III chondrosarcoma in 71%[14,15].

This division of malignant tumours in three histological grades is based on the cellularity, nuclear atypia, the muco-myxoid changes and the increased vascularisation of the tumours (Table 1.1). However, also here a great interobserver variability is experienced and the need for objective parameters is high. Although many studies have been attempting to unravel molecular events underlying chondrosarcoma development and progression, no better predictors of outcome than histological grade have been found so far. The criteria presently used are summarised in table 1.1.

1.2.3 Challenges in chondrosarcoma treatment

Chondrosarcomas are notorious for their resistance to conventional chemo- and radiotherapy, leaving surgery the only treatment option. Therefore, there is nothing with curative intention to offer to patients with tumours at inoperable locations or metastatic disease. Little is known about the mechanisms of resistance of chondrosarcoma. It has been speculated that the expression of P-glycoprotein[22,46,47] is the culprit for chemotherapeutic resistance of chondrosarcoma. P-glycoprotein is the product of multiple drug resistance gene (MDR-1)[48]. P-glycoprotein is an ATP driven membranous pump, which removes a wide spectrum of cytotoxic drugs from tumour cells. Many studies have demonstrated chemotherapeutic resistance, an increased metastasis rate and poorer prognosis in tumours expressing P-glycoprotein, a.o. in osteosarcoma[49].

Secondly, cytostatic drugs are most effective in destroying cells which are fast dividing. Chondrosarcomas have a slow growth rate, as compared to other solid tumours, which suggests that chemotherapeutic agents might not be working efficiently on the tumour cells. The third problem

in attacking chondrosarcoma tumour cells may be the accessibility of the cells. Low-grade chondrosarcoma cells are surrounded by a firm, avascular cartilaginous matrix. One can imagine that this matrix protects the cells against chemotherapy. In this respect, systemic treatment of high-grade chondrosarcoma would be facilitated by their high vascularity, through which the drugs can be delivered to the tumour cells. Another mechanism through which the resistance of chondrosarcoma might be explained is the overexpression of anti-apoptotic protein BCL2, which inhibits the apoptotic machinery. This could also be an explanation for radiotherapy resistance of chondrosarcoma. In addition, for radiotherapy to be effective the formation of free radicals is essential. Cartilage however, is known to be highly hypoxic, which prevents the formation of free radicals[50].

Treatment attempts using conventional treatment modalities
Treatment of conventional chondrosarcoma with conventional chemo- and

Enchondroma
Encasement of pre-existing host bone
Low cellularity
Atypia seldom found
Grade I
Entrapment of pre-existing bone by permeative cartilage
Exclusive presence or marked preponderance of small, densely-staining nuclei
Intercellular background varies from chondroid to myxoid (with transitional areas being present in many tumours)
Calcification and bone formation are frequent, though not exclusive
Multiple nuclei within one lacuna are often frequent
Small number of larger, pleomorphic nuclei in isolated areas is not considered to indicate a higher grade as long as cellularity and mitotic activity are absent
Grade II
Significant proportion of the nuclei are at least of moderate size
Increased cellularity
Paler-staining nuclei with visible nuclear detail
Intercellular background is myxoid rather than chondroid
Finding of mitosis, but <2 per 10 high power fields
Grade III
Pleiomorphic/anaplastic nuclei
Cellularity may be so dense that the appearance is that of a spindle cell sarcoma with no appreciable chondroid or myxoid matrix
Increased vascularity
Presence of 2 or more mitoses per 10 high power fields

Table 1.1 Histological criteria within the cartilaginous tumour spectrum Criteria are site, syndrome and age dependent

radiotherapy has not led to satisfactory results, until now. However, some small studies had good results with combining two conventional drugs, or treatment modalities (listed in table 1.2). In 1999 a large retrospective study on 227 chondrosarcomas was published, which showed that adjunctive chemotherapy and/or radiation therapy after an intralaesional resection, for recurrent disease, or for distant metastasis did not appear to alter the outcome[51].

New treatment attempts in radiotherapy

Proton beam therapy has been used to increase the dose delivered to the tumour[52,53]. Preclinical and clinical studies are listed in table 1.2 and 1.3, respectively. Currently, a phase II trial is performed using proton beam therapy for skull base chondrosarcoma (Table 1.3). With this technique tumour cells can be attacked in near proximity of the brain, while the dosis to adjacent critical normal structures is minimised. The results of this study are expected in 2011 (http://clinicalresearch.nih.gov/).

Sensitisation for conventional treatment modalities

An important approach in overcoming resistance is to sensitise the chondrosarcoma cells to become more vulnerable to conventional chemo- or radiation therapy. One example is the inhibition of BCL2, which is expressed in peripheral and high-grade central chondrosarcomas and is controlling chondrocyte proliferation[34,35]. Overexpression of BCL-2 inhibits apoptosis and thereby could make the tumour cells insensitive to radiation- (and chemo) therapy. Restoration of the apoptotic pathway would then make the tumours vulnerable to therapy. Improvement in radiotherapy sensitivity of chondrosarcoma was found by silencing anti-apoptotic BCL-2, BCL-X and XIAP[54] (Table 1.4).

Also by restoration of p16, the inhibitor of the CDK4-Cyclin D1 complex, chondrosarcoma cells could be sensitised to radiation *in vitro.* This is discussed more in detail in **chapter 4** of this thesis[55]. Parch et al. used the telomerase activity inhibitor BIBR1532 to sensitise telomerase positive chondrosarcoma cells to paclitaxel, a conventional chemotherapeutic agent[56]. Telomeres are non-coding repetitive sequences that typically constitute the end of linear chromosomes. They protect the coding regions of the genome from degradation, but become shorter every cell division. Cancer cells are able to overcome their limited life span by activating telomerase. Martin et al. found telomerase activity in 7 out of 16 chondrosarcomas and hypothesised that this telomerase activity, together with the loss of cell cycle regulation, caused aggressiveness of chondrosarcoma cells *in vitro*[57]. However, our research group previously showed the absence of telomerase activity in 46 patient samples (9/10 enchondroma and 37 chondrosarcomas); only in one enchondroma weak telomerase activity was found[58].

Aim	Substance	Dose	Study	Subjects; base line characteristics	Response*	Median follow up	Ref
Chemotherapy	Gemcitabine and docetaxel	IV: Day 1&8 675 mg/m^2 Gem, Day 8 100 mg/m^2 doc	Phase I	1 CS; SD	SD	*n/a*	102
	Trofosfamide	Oral: 150 mg daily	Phase II	1 recurrent CS + lung metastasis; PD under doxorubicin	PR after 8 and 18 months	18 months	103
	MAC-321 (Taxane)	Oral: 25 to 75 mg/m^2 once every 21 days	Phase I	1 chondrosarcoma; progressiveness *nd*	SD after 12 cycles	252 days	104
	Ifosfamide and doxorubicin	3 days 2.5 mg/m^2 iso+ 20 mg/m^2 every 28 days	Case report	1 recurrent low grade CS of the cranial base, PD under radiotherapy	CR after 5 cycles	52 months	105
Radiotherapy	Carbon ion radiotherapy	7 x 3 Gy E per week, median total dose of 60 gray equivalents	Phase II	10 low-grade chondrosarcoma of the skull base, age < 21yr	SD, PR in 4 patients	49 months	106
	Photons and protons	Combination preop 20 Gy, surgical resection, and reduced-field high-dose (50,4 Gy) postop	Phase II	48 sarcoma patients of which 15 CS (31%)	5 years OS 53.8%, DFS 53.8%; and local control rate, 72%. Individual respons dependent on surgery margins	32 months	52
	Proton	63.2-68.0 Gray equivalents +/- resection	Phase II	4 chondrosarcomas, 10-20 yr; PD.	SD	36 months	53
Sensitising to chemotherapy	Hyperthermia 41.8-42.0°C+nitrosourea	8 days, 180 mg/m^2	Phase I	1 chondrosarcoma; PD under chemotherapy	SD	38 months	107
Sensitising to radiotherapy	Razoxane	125 mg twice daily + 60 Gy	Phase II	13 chondrosarcomas	SD in 7/12	22 months	108
New targets	VEGF-anti sense	IV: 200 mg/m^2, 5 days	Phase I	*n/d* (7 sarcomas)	SD in 1 CS	4 months	109
	LY293111 (diaryl ether carboxylic acid derivative with PPARgamma activity)	Oral: 200-800 mg 2x daily 21 days, 102 cycles	Phase I	*n/a* (6 sarcomas)	SD in 2 sarcomas	336 days	110

Table 1.2 Overview of clinical investigations on chondrosarcoma (CS) treatment *The best overall tumor response was categorizedby using the Response Evaluation Criteria in Solid Tumors (RECIST) from the NCI: *PD progressive disease, SD stable disease, PR partial remission, CR complete remission. Nd not documented.*

Another strategy to sensitise cells to conventional chemo- or radiotherapy, which is successfully applied in many carcinoma types, is hyperthermia. The whole body, or part of the body, is heated to 42 to 44 degrees, which makes the cancer cells more vulnerable to therapeutics. One study using hyperthermia has been described, including 1 chondrosarcoma (Table 1.2).

Targets for alternative treatment options

Many molecular events in chondrosarcoma progression have been ilucidated in the last years, which generated potential targets for therapy alternative or in addition to conventional chemo- and radiotherapy (reviewed in[59]). The most important being the finding of increased expression of PTHLH in high grade central chondrosarcomas suggesting a role for BCL2 inhibitors and metalloproteinases and cathepsin B suggesting a potential role for cathepsin inhibitors. Another target for chondrosarcoma therapy might be estrogen signalling, as estrogen receptors are found in chondrosarcoma by immunohistochemistry[60]. In addition, aromatase, the enzyme that mediates the formation of estrogen, is active in chondrosarcoma [60]. Therefore the use of anti-estrogen treatment, which has been established in breast cancer, might have a place in the treatment of chondrosarcoma. Antiangiogenic therapy combined with chemotherapy was shown to induce apoptosis in a xenograft chondrosarcoma model[61]. Also attemps using HDAC inhibitors, monoclonal antibodies to PTHLH and PPAR agonists looked promising (Table 1.4)

Apomab is a fully human monoclonal antibody directed against human death receptor 5 (DR5; TRAIL-R2) with potential proapoptotic and antineoplastic activities. Mimicking the natural ligand TRAIL (tumour necrosis factor-related apoptosis inducing ligand), apomab binds to DR5, which may directly activate the extrinsic apoptosis pathway[62]. DR5 is expressed in a broad range of cancers (reviewed in[63]). However, the clinical trial on Apomab in which ao. chondrosarcomas were enrolled, has recently been closed for chondrosarcomas since no effect was found (Table 1.3). Perifosine is an orally active alkyl-phosphocholine compound with potential antineoplastic activity. Instead of targeting the DNA, like the conventional chemotherapeutic agents, perifosine targets cellular membranes and modulates membrane permeability and mitogenic signal transduction, resulting in cell differentiation and inhibition of cell growth[64]. Dasatinib and imatinib mesylate are extensively discussed in chapter 5 of this thesis.

Category	Treatment (modality/drug)	Trial name	Tumours enrolled	Current status	Phase	Executer	Nr of patients	Start	End
NR	Proton beam	Evaluation of Proton Beam Therapy for Skull Base Chondrosarcoma	Skull Base Chondrosarcoma	R	Phase II	M.D. Anderson Cancer Center	70	Apr-07	Apr-11
NT	Apomab	Efficacy and Safety of Single-Agent Apomab in Patients With Advanced Chondrosarcoma	Chondrosarcoma	CL	Phase II	Genentech	90	Jun-07	May-11
NR	Proton beam	Proton Beam Radiation Therapy for Chordomas and/or Chondrosarcomas of the Base of Skull	Skull Base Chondrosarcoma, Chordoma	R	Phase II	University of Florida	100	Oct-06	Oct-56
NT	Perifosine	Trial of Perifosine in Patients With Chemo-Insensitive Sarcoma	Chondrosarcomas, Alveolar Soft Part Sarcomas, Extra Skeletal Myxoid Chondrosarcomas	A/NR	Phase II	AOI Pharma, Inc.	111	Nov-06	Dec-08
CC	Pemetrexed disodium	Pemetrexed for Advanced Chondrosarcomas	Chondrosarcoma	A/NR	Phase II	NCI	75	Sep-05	-
CC	Gemcitabine hydrochloride and docetaxel	Gemcitabine hydrochloride and docetaxel in treating patients with recurrent osteosarcoma, Ewing's sarcoma or unresectable or locally recurrent chondrosarcoma	Ewing's Sarcoma, Osteosarcoma, Unresectable Or Locally Recurrent Chondrosarcoma	R	Phase II	NCI	120	Oct-06	Jul-09
CR	Charged Particle Radiation Therapy	Charged Particle RT for Chordomas and Chondrosarcomas of the Base of Skull or Cervical spine	Chordomas and Chondrosarcomas of the Base of Skull or Cervical spine	A/NR	Phase I/II	Massachusetts General Hospital	274	Jun-99	Jan-06
NT	Dasatinib	Dasatinib in Advanced Sarcomas	Various incl Chordoma, Osteosarcoma and Chondrosarcoma	R	Phase II	Bristol-Myers Squibb	502	May-07	Dec-13
NT	Imatinib mesylate	Imatinib Mesylate in Patients With Life Threatening Malignant Rare Diseases	Various incl chondrosarcoma	C	Phase II	Novartis	191	Feb-01	Sep-07

Table 1.3 Current clinical trials in which chondrosarcoma patients are enrolled Source: http://clinicalresearch.nih.gov/ Category CR: Conventional radiation, NR: New radiation modality, CC:Conventional chemotherapeutics, NT:New therapeutic targets, Current status R: Recruiting, A/NR: Active, not recruiting, C: Completed, CL: closed for chondrosarcomas

1.3 Finding alternatives to conventional anti-cancer therapy in chondrosarcoma: aim and outline of the thesis

Before addressing the approaches to new therapeutic treatment of chondrosarcoma that are presented in this thesis, the sometimes problematic distinction between benign and low-grade malignant cartilaginous tumours was assessed, within the Eurobonet consortium, an European Commission granted network of excellence to study the biology and pathology of bone tumours. In **Chapter 2,** the interobserver variability in the histological grading of cartilaginous tumours, between 18 specialised pathologists is investigated. Subsequently, a second set of 57 cartilaginous tumours, were studied to find an optimal set of parameters to differentiate enchondroma from low-grade chondrosarcoma. A algorithm based on five parameters is proposed that may improve reliability of the diagnosis of cartilagious tumours.

Four different approaches to new therapeutic treatment of chondrosarcoma are presented in this thesis.

1.3.1 Normal growth regulators (Morphogens/HSPGs) in analogy to peripheral tumours

Based on knowledge we have on genetic aberrations in the EXT genes in peripheral chondrosarcomas and Multiple Osteochondromas we investigated the role of these genes and their downstream Indian Hedgehog pathway in central chondrosarcomas.

Central cartilaginous tumours mostly arise in bones that elongate via endochondral ossification. The growth plate plays a pivotal role during this process. Therefore, studying the signalling pathways implicated in the normal growth process might elucidate the development of cartilaginous tumours. The growth plate is a cartilaginous structure entrapped between the epiphysis and metaphysis at the end of the bone. It functions as a scaffold and is replaced by bone in a coordinated fashion[65,66]. Different morphological zones of chondrocytes at different stages can be distinguished (Figure 1.3)[67]. The resting zone is located in the part of the growth plate most proximal to the epiphysis. Upon a yet unknown stimulus, the resting chondrocytes enter the proliferative zone. The flat proliferating chondrocytes assemble in orderly, longitudinal columns and start producing extracellular matrix proteins (e.g. collagen type II). Eventually these chondrocytes loose their proliferative capacity and start to differentiate into hyperthrophic chondrocytes. These hypertrophic chondrocytes become larger and obtain a more rounded appearance. Now, also a different type of collagen is produced, collagen type X. Finally, the extracellular matrix around the hypertrophic chondrocytes is calcified and the chondrocytes will undergo apoptosis (programmed cell death). The calcified matrix is resorbed by osteoclasts and osteoblasts enter

Aim	Substance	Action	In vitro/ in vivo	Subjects	Response	Ref
Sensitizing to chemotherapy	BIBR1532	Telomerase activity inhibitor	In vitro	SW1353 and CAL-78	Increased paclitaxel sensitivity	56
	TRAIL	Sensitize to doxyrubicin	In vitro	HTB-94		89
Sensitizing to radiation	Viral transduction	Restoration of p16	In vitro	p16 negative CS cell line (n=2)	Increased radiation sensitivity	55
	siRNA	Silencing of BCL2, BCLX and XIAP	In vitro	SW1353	Increased radiation sensitivity	54
Alternative treatment option	Depsipeptide	Histone deacetylase inhibitior	In vitro	SW1353, OUMS27, RCS	Growth inhibition	90
	Suberoylanilide hydroxamic acid (SAHA)	Histone deacetylase inhibitior	In vitro/in vivo	SW1353, OUMS27, RCS	Apoptosis in SW1353, autophagy-associated cell death in OUMS27 and RCS	91
	Anti-CD44 antibody	binding of CD44	In vitro	SW1353	Apoptosis in SW1353	92
	SU6668	VEGFR2, PDGFRbeta, FGFR1 inhibitor	In vivo	SW1353 in SCID mice	Inhibition of angiogenis and growth	80
	15d-PGJ2	PPAR gamma agonist	In vitro	OUMS27	Growth inhibition of CS cells by BAX/BCL-X and p21 upregulation	93
	Cyclopamine	IHH antogonist	In vitro	Explant organ culture	Decreased cell proliferation	75
	Triparanol	IHH antogonist	In vivo	CS xenograft (n=12)	Reduced tumour size	75
	Monoclonal antibody to PTHLH	PTHLH inhibition	In vitro	HTB-94	Increased apoptosis	94
	Antisense RNA for MMP-1	MMP-1 inhibition	In vitro	JJ012	Decreased invasiveness	95
	Exemestane	Aromatase inhibitor	In vitro	Primary culture	Growth inhibition	60
	2-Methoxyestradiol	Estrogen metabolite	In vitro	JJ012	Cytotoxity in chondrosarcoma cells: increased Bax, Cytochrome C, and Caspase-3 and Bax/Bcl-2 ratio	96
	Alendronate	Bisphosphonate	In vitro	JJ012	Inhibitory effect on invasion and migration of JJ012 via MMP-2	97
	Minodronate	Bisphosphonate	In vitro	SW1353, OUMS27	Cell cycle dysregulation in both, apoptosis in SW1353	98
Immunotherapy	IFN gamma	Increase of HLA-1 presentation	In vitro	FS	MAGE specific cytolytic T-lymphocyte lysis of FS cells	99
	Herpes simplex virus type 1 thymidine kinase (HSV-TK)/ganciclovir (GCV) suicide gene therapy		In vitro	SW1353	Cytotoxity	100
	Herpes simplex virus type 1 thymidine kinase (HSV-TK)	Gancyclovir	In vitro/in vivo	Nude mice	Decreased growth	101

the area to form trabecular bone[65].

Heparan sulphate proteoglycans (HSPGs) are extracellular matrix proteins which are important for signal transduction in the growth plate. HSPGs are crucial for the gradient formation by which long distance diffusion of Indian hedgehog (IHH), decapentaplegic and wingless signal to their receptors as demonstrated in *Drosphila Melanogaster*[32,68-70]. The human homologues for these morphogens are Indian and sonic hedgehog, TGFβ/BMP and WNT, respectively[71]. Indian Hedgehog (IHH) orchestrates chondrocyte proliferation and differentiation in the human growth plate. IHH signals to its receptor patched (PTCH), which subsequently releases its inhibition on intracellular smoothened (SMO), resulting in the translocation of GLI transcription factors to the nucleus (Figure 1.4). Here, PTHLH is transcribed together with PTCH and GLI, guaranteeing the preservation of this signalling cascade[72]. PTHLH

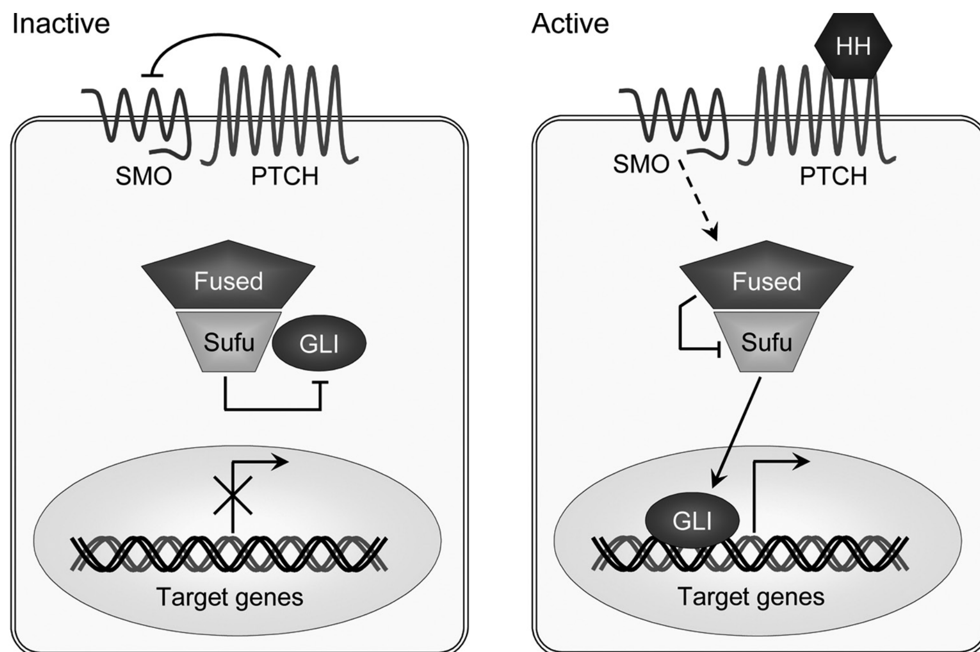

Figure 1.4 Hedgehog signalling. Left: In the absence of the ligand, hedgehog (HH), signalling is inactive. The transmembrane receptor Patched (PTCH) inhibits another transmembrane protein Smoothened (SMO). This prevents the transcription factor GLI to enter the nucleus through interactions with cytoplasmic proteins, including Fused and Supressor of fused (Sufu). Right: HH signalling is initiated upon binding of the ligand, e.g. Indian Hedgehog, to PTCH. This results in the release of SMO by PTCH, thereby activating a cascade that leads to the translocation of GLI to the nucleus. There it activates transcription of targets genes, amongst which are also PTCH and GLI itself. Active HH signalling leads to activation of PTHLH in the human growth plate and thereby controls longitudinal growth of the bones. Adapted from Pasca di Magliano et al. 2003;*Nat Rev Cancer*:903-11.

left page **Table 1.4 Preclinical investigation on chondrosarcoma treatment**

signalling inhibits chondrocyte differentiation and consequently controls longitudinal growth[73,74].
HSPGs are formed in the golgi apparatus of the chondrocytes. Elongation of the heparan sulphate side chains that are linked to the proteoglycan protein cores occurs by the hetero-oligomeric EXT1/EXT2 complex, a type II transmembrane glycoprotein. This complex is formed by the protein products of *EXT1* and *EXT2 genes.* While it is evident that inactivation of the EXT genes is the driving force for the development of benign peripheral cartilaginous tumours[26-28,30,59], in the far more common central chondrosarcomas the role of EXT and its downstream targets has not been systematically studied so far. Since the ultimate goal of the PTHLH pathway is controlling chondrocyte proliferation, interfering with this pathway might inhibit chondrosarcoma growth. As described previously, some promising results were found by using IHH blocking agents as triparanol and cyclopamine[75]. In **Chapter 3** *EXT1 and EXT2* are evaluated in central chondrosarcoma at the DNA (mutational screening, arrayCGH) and mRNA level. Localisation of HSPGs (CD44v3 and SDC2) in the chondrosarcoma tumour cells was studied. Morphogens signalling WNT (β-catenin) and TGFB (PAI-1 and phosphorylated Smad2) were studied by immunohistochemistry, while IHH signalling was studied by qPCR. The possible role of cyclopamine in chondrosarcoma treatment was studied *in vitro*.

1.3.2 Cell cycle regulation

The second hypothesis driven approach was based on (the loss of) cell cycle regulation in chondrosarcoma. 2q13 amplifications and 9p21 deletions suggest an important role for cell cycle regulators in the progression of chondrosarcoma. **Chapter 4** describes the investigation of the pRb and p53 pathways in chondrosarcomas and their potential targets for therapy of high grade chondrosarcomas. The role of *CDKN2A/p16* and *CDK4* in chondrosarcoma cell survival and proliferation is investigated *in vitro* using lentiviral constructs overexpressing CDKN2A/p16 and inhibiting CDK4. CDK4 controls progression through the cell cycle by forming a complex with CyclinD1, which regulates the transit of the cell through the G1 restriction point. CDKN2A/p16 is the inhibitor of this CDK4-CyclinD1 complex. The CDKN2A/p16 tumour suppressor gene, located in this region, was shown to be important for chondrosarcoma progression, since inactivation was restricted to high-grade chondrosarcoma[37,42]. Defects in the cell cycle pathway are found at high rates in almost all types of human cancer[76,77]. In breast cancer cells, CDK inhibitors were effective in treating tumours that overexpress the CDK4-cyclin D1 complex or that have lost CDKN2A/p16 function[78]. Therefore we hypothesise that the inhibition of CDK4 and the re-expression of p16 might be of therapeutic value in chondrosarcoma. Subsequently, in this chapter the expression of *CDK4*, *MDM2,* and *c-MYC* at the mRNA and protein level in a large series of central chondrosarcomas

was investigated as potential progression markers, to look for prognostic markers.

1.3.3 Kinome profiling

In **chapter 5** an array approach is used to search for new treatment options for chondrosarcoma. Large scale kinase analysis, also referred to as kinomics, was applied using the Pepchip. Kinases, alternatively known as a phosphotransferases, are enzymes that phosphorylate tyrosine/serine or threonine residues on other proteins. Phosphorylation means the addition of one extra phosphate group causing the donor protein to be either activated or inactivated (Figure 1.5). The opposite action is executed by phosphatases, which remove a phosphate group from a protein (dephosphorylation).
Many enzymes and receptors are switched “on” or “off” by phosphorylation and dephosphorylation, by kinases and phospatases (Figure 1.5). Thereby kinases play a major role in signalling cascades that determine cell cycle entry, cell survival and differentiation fate. Kinases are excellent targets for anti-cancer therapy because of their switch function; their regulation is reversible, rapid (merely in seconds) and does not require new protein synthesis (reviewed in[79]). Thereby kinases have a large advantage over conventional chemotherapeutics that work less targeted and thereby cause much more damage in the patient. Up to 518 different kinases have been identified in humans.

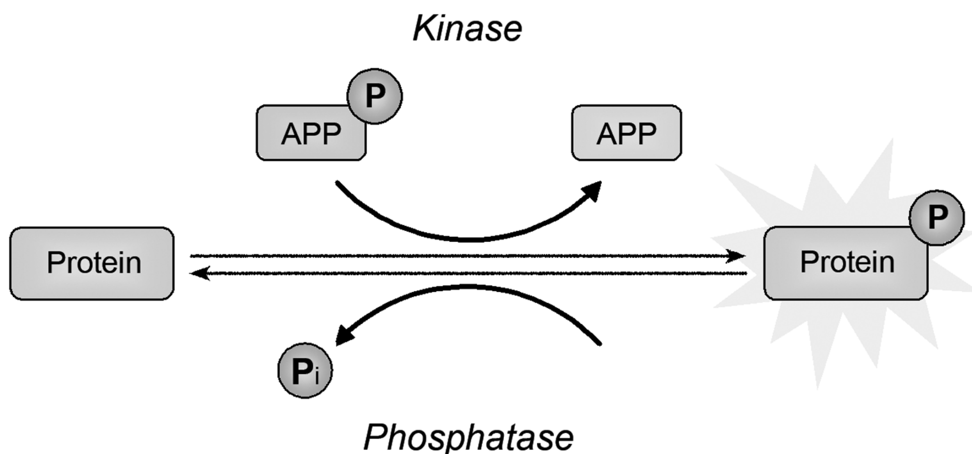

Figure 1.5 Kinases are able to tranfer a phosphate group to a donor protein (phosphorylation). This protein is thereby either activated or inactivated. Phosphatases exert the opposite; they remove a phosphate group from a protein (dephosphorylation). These actions provide a molecular switch and are important in many cellular processes, i.e. transcriptional activation, stimulation of cell division and apoptosis.

Until now, little is known about the use of kinase inhibitors in chondrosarcoma treatment. Klenke et al. showed that SU6668, which inhibits tyrosine kinases Flk-1/KDR, PDGFRbeta and FGFR1, is able to repress chondrosarcoma growth via antiangiogenesis in an *in vivo* model using severe combined immunodeficient (SCID) mice[80] (Table 1.4). Another study reported the prolonged cell survival of chondrosarcoma cell line JJ012 upon activation of AKT by Tenascin-C, an extracellular matrix protein[81]. This suggests an important role for the AKT-kinase in chondrosarcoma survival.

Since our results pointed to susceptibility of chondrosarcoma cell lines to dasatinib and imatinib, both drugs were tested *in vitro*.

1.3.4 COX-2 inhibition

Based on the finding of COX-2 protein expression in central and peripheral cartilage forming tumours we hypothesised a potential role for the use of selective COX-2 inhibitors, which was the subject of our studies described in **chapter 6**.

Both COX enzyme isoforms, COX-1 and COX-2, are responsible for the production of prostaglandins, tromboxane and leukotriens[82]. Whereas COX-1 is constitutively expressed under physiologic conditions, COX-2 is induced by cytokines, growth factors and free radicals, which render this molecule a suitable target for (anti-cancer) therapy. A protective effect of non-steroidal anti-inflammatory drugs (NSAIDs) has been suggested against development and growth of colorectal cancer. Celecoxib and rofecoxib, both selective COX-2 inhibitors, were shown to reduce the number and size of colorectal polyps in the adjuvant treatment of Familiar Adenomatous Polyposis (FAP) patients[83,84]. NSAIDs interfere with the cyclooxygenase pathway by blocking the attachment site for arachidonic acid (AA) on the COX enzyme (Figure 1.6).

Tumour specific COX-2 positivity has been extensively described for various malignancies; i.e. colorectal carcinoma with 80% positive tumours[85] and breast cancer (reviewed in[86]). Endo et al. reported high COX-2 expression in a substantial amount of chondrosarcoma (16/72), which was associated with histological grade and poor prognosis[87]. Another study showed 13/24 chondrosarcomas to express COX-2 by western blot analysis, whereas 8 enchondromas were negative[88]. In **chapter 6** COX-2 mRNA levels are evaluated in a large series of chondrosarcoma patients. The effects of COX-2 inhibition at COX-2 protein expression, PGLE2 levels and cell proliferation in 4 high-grade chondrosarcoma cell lines was investigated *in vitro*. Moreover, a chondrosarcoma xenograft model of immunoincompetent nude mice was used to study the effects of (prophylactic) treatment with the specific COX-2 inhibitor celecoxib.

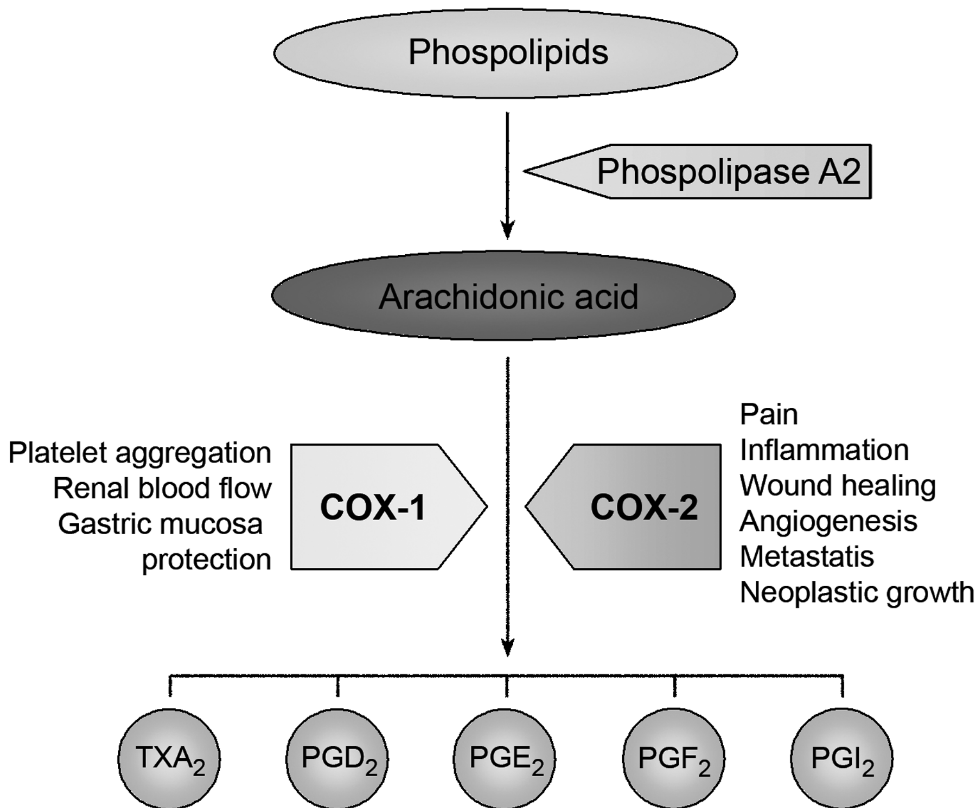

Figure 1.6 COX-1 is consitutively expressed in different cell types and is considered to be mainly associated with the production of prostaglandins (PGD2, PGE2, PGF2), tromboxane (TXA) and leukotriens under normal physiologic conditions. In contrast COX-2 is induced by cytokines, growth factors and free radicals and is expressed in inflammatory cells.

State of the art as described in this introduction and aims of the thesis are summarised in figure 1.7 for peripheral cartilaginous tumours and in figure 1.8 for central cartilaginous tumours.

The results of all chapters will be summarised in **chapter 7**, together with implications for further research.

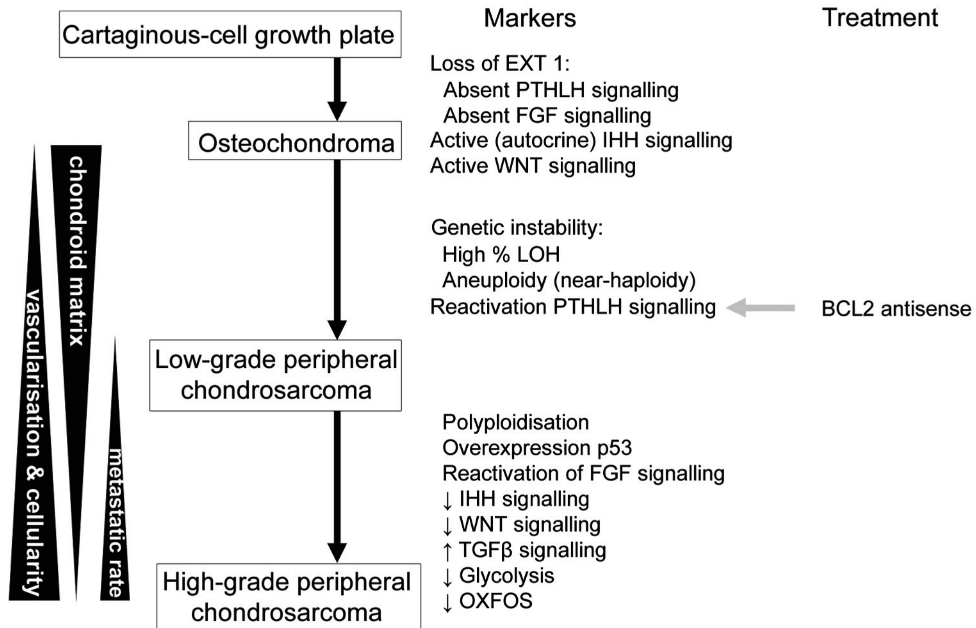

Figure 1.7 State of the art and implications for potential therapeutic strategies in peripheral chondrosarcoma. A multistep model of the progression of osteochondroma towards secondary peripheral chondrosarcoma is shown. Results from previous studies are summarised. Arrow shows a candidate for potential therapeutic strategy.

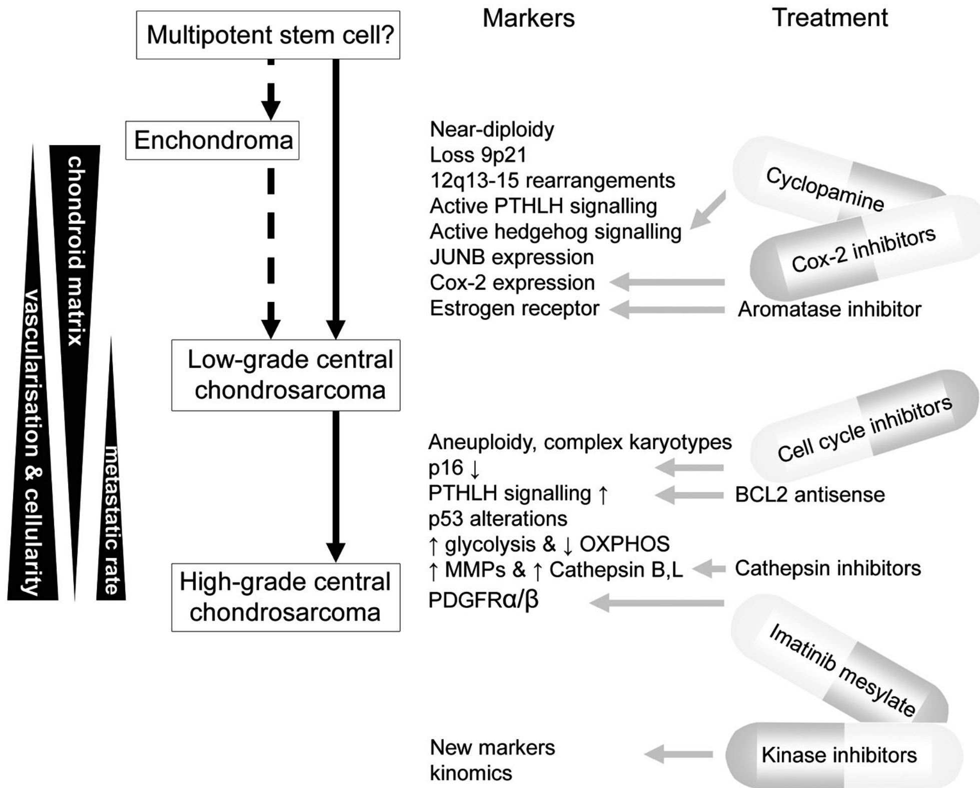

Figure 1.8 State of the art and implications for potential therapeutic strategies in central chondrosarcoma. A multistep model of the progression of central chondrosarcoma is shown. Results from relevant previous studies are summarised. Arrows indicate possibilities for targeted treatment. Strategies investigated in this thesis are represented in capsules. MMP: matrix metalloproteinase, OXPHOS: oxidative phosphorylation.

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2. Assessment of interobserver variability and histological parameters to improve reliability in classification and grading of central cartilaginous tumours.

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Abstract
The distinction between benign and malignant cartilaginous tumours of bone is one of the most difficult subjects in surgical pathology. The grading of chondrosarcoma also seems to vary considerably among pathologists. However, clinical management differs. The purpose of this study was 1) to investigate interobserver variability in histological diagnosis and grading of central cartilaginous tumours and 2) to assess the diagnostic value of defined histological parameters in differentiating enchondroma and central grade I chondrosarcoma. The interobserver variability was assessed using a set of 16 cases evaluated by 18 specialised pathologists. Subsequently, 20 enchondromas and 37 central grade I chondrosarcomas diagnosed in a multidisciplinary team with full clinical, radiological and pathological data available with 10 years of follow-up were collected**.** Cytological and tissue-architectural features were assessed to find an optimal set of parameters to differentiate enchondroma from central grade I chondrosarcoma. We demonstrate considerable variation in the histological assessment of cartilaginous tumours (weighted kappa = 0.78). The distinction between enchondroma and grade I chondrosarcoma was shown to be the most disconcordant (kappa coefficient = 0.54), and also the differentiation between grade I and grade II chondrosarcoma was subjected to variation (kappa coefficient = 0.80). The application of a combination of 5 parameters (high cellularity, presence of host bone entrapment, open chromatin, mucoid matrix quality and age above 45 years) allowed optimal differentiation between enchondromas and central grade I chondrosarcomas. With a classification tree based on 2 parameters (mucoid matrix degeneration more then 20% and/or host bone entrapment present), 54 of the 57 (94.7%) cases were assessed correctly (sensitivity 95% and specificity 95%). Our study confirms the low reliability of the diagnosis and grading of central chondrosarcoma. However these classifications guide therapeutic decision making in daily practice. Therefore we propose a classification model that, combined with a tailored radiological assessment, may improve reliability of the diagnosis of cartilaginous tumours.

Introduction

Cartilaginous tumours are the most common primary tumours of bone[1,2]. Enchondroma and its malignant counterpart central chondrosarcoma account for 10% (range 3% -17%) and 13% (range 8% -17%) of bone tumours, respectively[3-5]. The incidence of chondrosarcoma is 1:50,000, with a nearly equal age distribution between males and females. Central and peripheral chondrosarcomas are recognised, the latter developing secondary to a pre-existent osteochondroma. Although central and peripheral chondrosarcomas are similar at the cytomorphological level, these two types of chondrosarcomas have been shown to arise through distinct genetic pathways[6]. On the molecular level, expression of certain molecules i.e. BCL-2 and PTHLH have been found to be indicators of malignant progression in peripheral chondrosarcomas[7,8]. For the differential diagnosis central chondrosarcoma versus enchondroma these parameters are lacking, stressing the need of better defining parameters at the light microscopical level.

Surgical treatment is the only curative treatment for patients with chondrosarcoma[9], since these tumours do not respond well to radiotherapy and/or chemotherapy[10]. En-bloc resection is the only curative treatment[4,9,11], frequently leading to considerable morbidity and demanding reconstruction. However following recent trends, grade I chondrosarcoma may be treated by curettage, eventually followed by vigorous local adjuvant therapy i.e. cryosurgery or phenolisation[12,13]. However, little follow-up data is available as yet to advocate it as a proven save alternative. For enchondromas a wait-and-see approach or intraleasional treatment is permissible[4,9,11]. As a result, the preoperative differentiation between enchondromas and central grade I chondrosarcomas remains important.

The preoperative assessment of these leasions is based upon clinico-radiological and histopathological evaluation of a biopsy specimen. Clinical symptoms and radiographic features are of help in differentiating between enchondroma and central grade I chondrosarcoma, but both lack specificity[11,14,15]. Previous studies have shown that conventional radiology is not reliable in differentiating benign from low-grade malignant tumours in this differential diagnosis, amongst others hampered by the absence of objective and reproducible criteria[11]. However, studies by means of dynamic contrast enhanced MR-imaging have improved the sensitivity in this specific differential diagnosis[16,17].

Several histopathological features are used to differentiate central grade I chondrosarcoma from enchondroma, but this differentiation remains a diagnostic challenge, often requiring expert opinion.

In the present study, reliability and reproducibility of current histopathological methods were assessed between 18 expert pathologists. The level of agreement was assessed by the evaluation of 16 slides

accompanied by conventional radiology, magnetic resonance images or computed tomography scans. Subsequently, we evaluated the diagnostic value of individual morphologic criteria in differentiating enchondroma from central grade I chondrosarcomas and investigated their diagnostic power using a set of 57 cases, of which 10 years of follow up was available.

Materials and Methods

Cases to study interobserver variability

To investigate the interobserver variability between expert pathologists, 16 central cartilaginous tumours were selected (Table 2.1). There was consensus in advance that grading would be performed according to the criteria originally described by Evans et al[18]. Patient age and location of the tumour were provided, together with pre-operative radiographs, magnetic resonance images or computed tomography scans. One tumour occurred in the context of enchondromatosis (Ollier disease). All specimens were handled according to the ethical guidelines described in "Code for

Slide number	Age	Location
1	48	Proximal femur
2	22	Distal femur (tumour related to Ollier disease)
3	40	Femur
4	54	Proximal humerus
5	44	Humerus
6	39	Tibia
7	37	Proximal humerus
8	22	Proximal humerus
9	62	Proximal femur
10	29	Proximal phalanx, dig 3
11	40	Proximal tibia
12	39	Medial femur condyl
13	37	Femur
14	68	Rib
15	54	Proximal fibula
16	58	Distal femur

Table 2.1 Patients to study interobserver variability. Information as provided to the pathologists.

Proper Secondary Use of Human Tissue in The Netherlands" of the Dutch Federations of Medical Scientific Societies. Eighteen musculoskeletal pathologists participated in the study. Sixteen of the pathologists were involved in EuroBoNeT, an European Commission granted Network of Excellence for studying the pathology and genetics of bone tumours and two were USA expert pathologists.

Cases to study histological criteria

Seventy-four sequential patients admitted to the Leiden University Medical Center between 1984 and 1990 with either enchondroma or central grade I chondrosarcoma were retrieved from our database. This database consists of radiological documentation, histological slides as well as at least 10 years of detailed follow-up including adequate radiological follow-up documentation. Fifteen out of 74 patients were excluded, because their tumour was localised in the phalanges. Phalangeal chondrosarcomas have been shown to have a protracted clinical course with almost no metastases despite ominous histology[19]. Clinical details are given in table 2.2. Two cases were excluded because there was too little histological material available to make a reliable diagnosis. This leaves 57 patients, with a mean and median age of 46 years (range: 2-88 years).

Histopathology

The criteria used were those noted to be of diagnostic value in the literature[14,15,18,20]. The histological criteria used and the scoring system are documented in table 2.3 and illustrated in figure 2.1. The histological slides of the biopsies of all 57 patients were subsequently scored by two investigators (D.E. and P.C.W.H.). Localisation of the tumour, age of the patient and clinical symptoms (swelling, pain or fracture) were provided.
As a gold standard for a benign diagnosis (enchondroma) following biopsy, a clinico-radiological follow up of at least 10 years was a prerequisite and the diagnosis was obtained in consensus after review by a panel of clinicians, radiologists and pathologists, established in the Netherlands Committee on Bone Tumours. For grade I chondrosarcomas the diagnosis obtained from the resected specimen as discussed in the aforementioned panel was used as confirmation.

Statistical Analysis

Interobserver variability

Intra class correlations were calculated to assess agreement between the 18 pathologists. These intra class correlations are equivalent to weighted kappa coefficients with quadratic weights and we refer to them in the text as weighted kappa's. Kappa coefficients measure the percentage of agreement among observers adjusted for the degree of agreement that would be expected by chance alone.

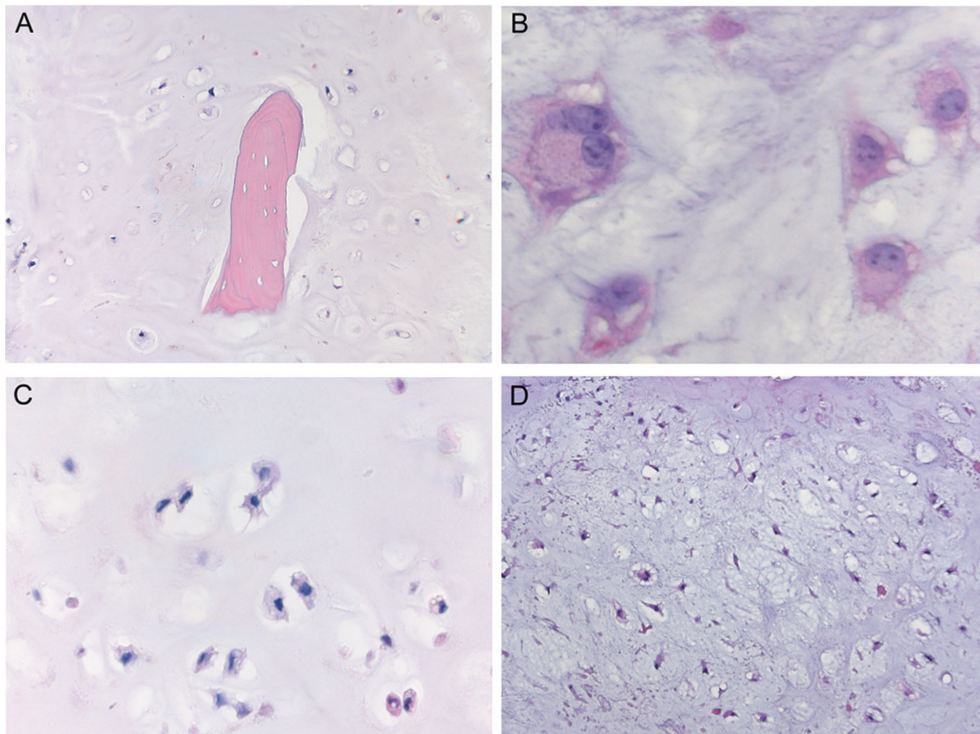


Figure 2.1 Light-micrographs displaying the morphologic parameters shown to be of diagnostic value (A) Presence of host bone entrapment, magnification 20x. (B) Open chromatin, magnification 63x. (C) High cellularity, magnification 40x. (D) Mucoid matrix quality, magnification 10x.

Histological criteria

Cross tables were made and sensitivity, specificity and positive predictive values for enchondroma or low-grade chondrosarcoma were computed for different diagnostic parameters. The Chi-square test was used to compare percentages between the two groups. A p-value less than or equal to 0.05 was determined as significant. Classification trees[21] and forward stepwise logistic regression with likelihood ratio test (LR) were used to select the optimal combination of cytological and histological criteria to differentiate enchondroma from central grade I chondrosarcoma. Logistic regression was also used to assess the value of the matrix quality in differentiating enchondroma from central grade I chondrosarcoma.

Results

Assessment of interobserver variability

The most discordant results were found in the distinction between enchondroma and grade I chondrosarcoma (kappa coefficient = 0.54). Results for all cases are shown in figure 2.2. The weighted kappa for the total dataset was 0.78. From the ten cases that were considered to be benign by one or more pathologists only one showed complete consensus (case 10). Light-micrograph and radiograph corresponding to slide 4, a tumour with considerable interobserver variability, is shown in figure 2.3. Furthermore, the distinction between enchodroma and grade I, versus grade II and III resulted in a kappa coefficient of 0.80. Particularly the cases 1, 6 and 9 show a lot of variation (figure 2.2). Distinction between grade II and grade III chondrosarcoma seems to be less problematic, according to figure 2.2.

Assessment of histological criteria to distinguish enchondroma vs grade I chondrosarcoma

Clinical details of the 57 cases are documented in table 2.2. Spontaneous pain alone proved to be of some diagnostic value in this differential diagnosis (35% of the enchondromas versus 62% of the central grade I chondrosarcomas, p-value=0.05). Also the presence of a combination of clinical symptoms (Spontaneous pain, presence of a mass, fracture) pointed to malignancy, although the p-value did not reach significance (p-value=0.08).

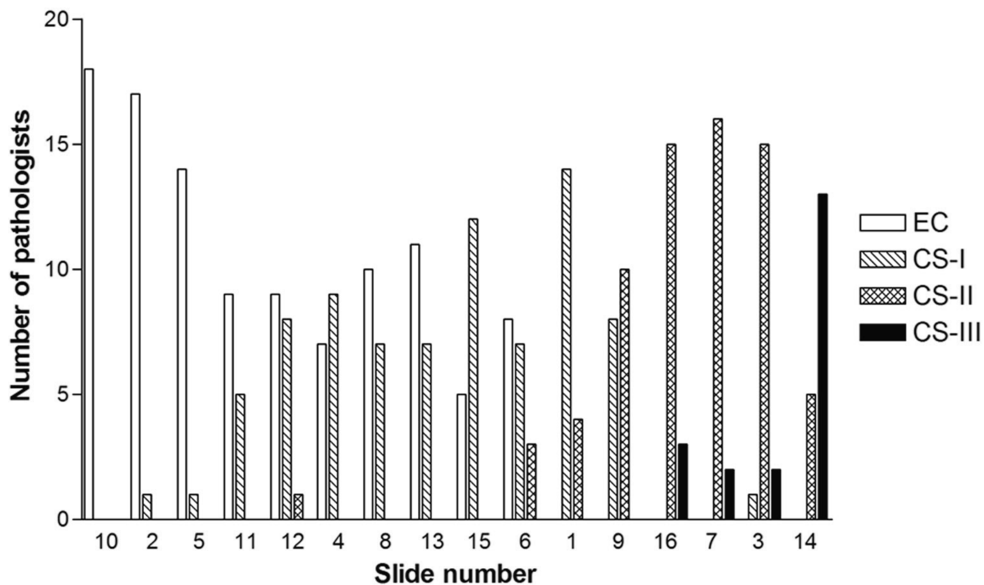


Figure 2.2 Diagnoses of the 18 pathologists are given per slide. Slides are arranged from merely benign interpretation (left) to malignant interpretation (right).

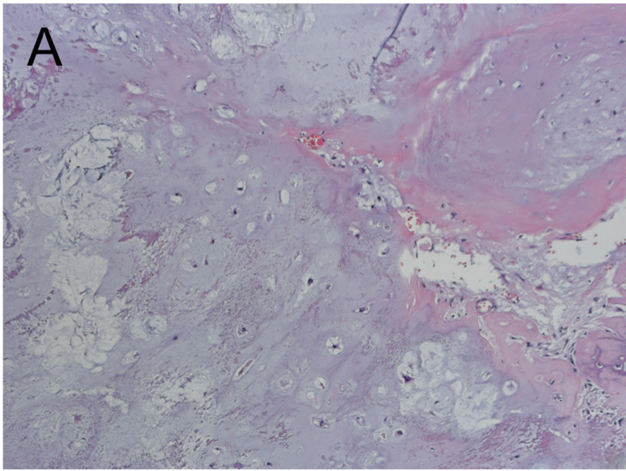
	Enchondromas (n=20)		Chondrosarcomas (n=37)		P-value	PPV(%) (CCS I)
	No.	%	No.	%		
Spontaneous pain (without pathologic fracture)	7	35	23	62	0.050*	
Mass (without pathologic fracture)	3	15	5	14	0.877	
Fracture (with or without mass)	2	10	2	5	0.517	
No clinical symptoms	8	40	7	19	0.085	
Acromion			1	2.7		
Femur	5	25	12	32.4		
Fibula			3	8.1		
Humerus	9	45	3	8.1		
Ileum			4	10.8		
Metacarpal bone	2	10	1	2.7		
Metatarsal bone			1	2.7		
Radius	1	5				
Rib			4	10.8		
Scaphoid	1	5				
Scapula			1	2.7		
Spine	1	5	2	5.4		
Sternum	1	5	2	5.4		
Tibia			2	5.4		
Ulna			1	2.7		
Age (years)						
=< 45	15	75.0	13	35.1	0.004*	46.4
>45	5	25.0	24	64.9		81.8
Median (range)	38 (2-69)			55 (11-88)		

Table 2.2 Clinical details, localisation and age at presentation of the 57 patients to study histological criteria. *Statistically significant

Slight differences in the anatomical localisation of enchondromas and central grade I chondrosarcomas were observed in our patient selection (Table 2.2). This difference however did not reach statistical significance. Age frequencies for patients below 45 year versus above 45 year are given.
A cut-off value of 45 years was chosen because it was found to distinguish best between the groups in our data set (details not shown). Table 2.3 shows the frequencies of several cytopathological and architectural parameters, used to differentiate between central grade I chondrosarcoma and enchondroma. The parameters with the most discriminating strength were host bone entrapment as shown in figure 2.1A (positive predicting value (PPV)=97.0%), marked nuclear pleomorphism (Figure 2.1B) (PPV=88.2%), high cellularity (Figure 2.1C) (PPV=89.8%) and irregular distribution of cells (PPV=85.4%) The p-value of these individual parameters was less than 0.001.
Table 2.4 shows the differences in matrix quality between enchondromas and grade I chondrosarcomas. All 22 tumours with mucoid matrix degeneration

	Enchondromas (n=20)		Chondrosarcomas (n=37)		P-value	PPV (%) (CCS I)
	No.	%	No.	%		
Cytology						
Binucleated cells (No)						
=< 2	14	70.0	6	16.2	0.000*	30.0
> 2	6	30.0	31	83.8		83.8
Nuclear polymorphism						
Slight	16	80.0	7	18.9	0.000*	30.4
Marked	4	20.0	30	81.1		88.2
Condensed nuclei						
Present	5	25.0	26	70.3	0.001*	83.9
Absent	15	75.0	11	29.7		42.3
Open chromatin						
Present	8	40.0	29	78.4	0.004*	78.4
Absent	12	60.0	8	21.6		40.0
Mitosis						
Present	0	0.0	1	2.7	0.458	100.0
Absent	20	100.0	36	97.3		64.3
Histology						
Distribution of cells						
Regularly	14	70.0	2	5.4	0.000*	12.5
Irregularly	6	30.0	35	94.6		85.4
Cellularity						
Moderate	17	85.0	11	29.7	0.000*	39.3
High	3	15.0	26	70.3		89.7
Secondary bone formation						
Present	16	80.0	32	86.5	0.522	66.7
Absent	4	20.0	5	13.5		55.6
Calcification						
Present	18	90.0	33	89.2	0.924	64.7
Absent	2	10.0	4	10.8		66.7
Encasement						
Present	11	55.0	20	54.1	0.189	64.5
Absent	3	15.0	12	32.4		80.0
Unobservable	6	30.0	5	13.5		
Host bone entrapment						
Present	1	5.0	32	86.5	0.000*	97.0
Absent	16	80.0	3	8.1		15.8
Unobservable	3	15.0	2	5.4		
Cortical extension						
Present	0	0.0	16	43.2	0.001*	100.0
Absent	9	45.0	6	16.2		40.0
Unobservable	11	55.0	15	40.5		

Table 2.3 Cytological and architectural parameters scored on biopsy specimens of the 57 patients, with either enchondroma or grade I central chondrosarcoma. *Statistically significant



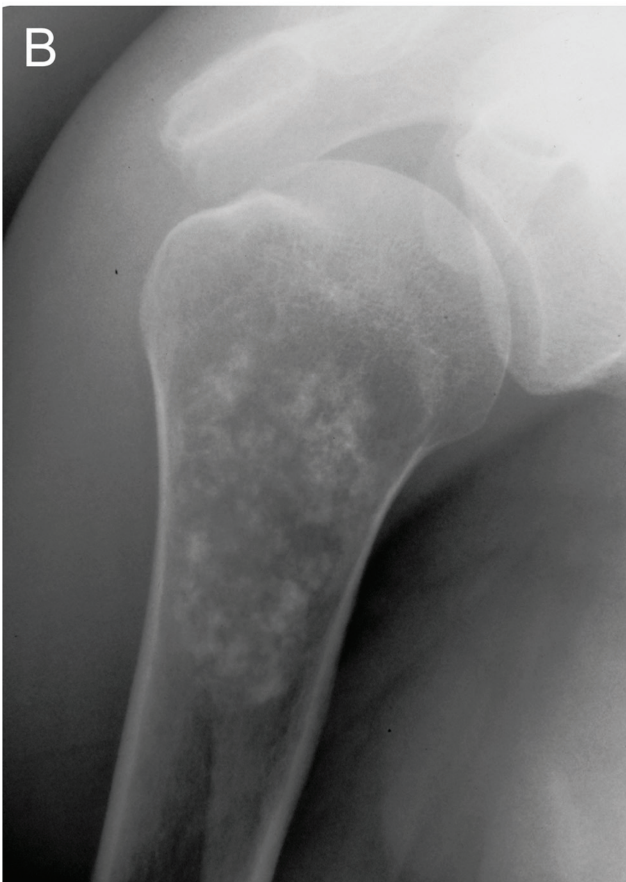


Figure 2.3: Light-micrograph, magnification 10x (A) and radiograph (B) corresponding to slide 4, which was interpreted as a benign leasion by seven of the pathologists and as a malignant grade I chondrosarcoma by nine pathologists.

Matrix quality		Enchondromas (n=20)		Chondrosarcomas (n=37)		P-value
		No.	%	No.	%	
Chondroid	< 80%	0	0	16	43,2	0.000*
	=> 80%	20	100	21	56,8	
Mucoid	< 20%	20	100	15	40,6	0.000*
	=> 20%	0	0	22	59,4	
Myxoid	< 20%	20	100	25	67,6	0.002*
	=> 20%	0	0	12	32,4	

Table 2.4. Morphologic matrix quality of enchondromas and chondrosarcomas.
*Statistically significant in X^2-test: linear by linear association

greater than or equal to 20% were central grade I chondrosarcoma. The same was true for all 16 tumours with a matrix quality which consisted of less than 80% of chondroid structure and all 12 tumours with myxoid structure greater than or equal to 20%. Multiple logistic regression was used to asses the value of the matrix quality in differentiating enchondroma from central grade I chondrosarcoma. An increase of chondroid structure by 10% decreases the “chance” (odds) for chondrosarcomas by a factor of 0.8 (p=0.008). An increase in mucoid or myxoid structure of the matrix enhanced the “chance” (odds) of malignancy by a factor 7.18 (p=0.004) and 2.12 (p=0.315) respectively.
Multiple stepwise logistic regression method (LR), using both patient characteristics, the variables of table 2.3 and the matrix quality variables as possible predictors, showed that the most predictive combination of parameters for central grade I chondrosarcoma was high cellularity, presence of host bone entrapment, open chromatin, mucoid matrix degeneration and age above 45 years. These parameters together gave a perfect prediction in our series. Using classification trees, a simple classification rule was obtained in which 54 of the 57 (94.7%) cases were assessed correctly. If mucoid matrix degeneration greater than or equal to 20% and/or host bone entrapment was present, the tumour was statistically considered malignant. With this classification rule, 2 tumours were assessed incorrectly as enchondromas and 1 as a chondrosarcoma (sensitivity 95%, specificity 95%).
Table 2.5 shows the consensus diagnosis made after at least 10 years of clinico-radiological follow-up and used as gold standard compared to the diagnosis made on the biopsy assessed by the investigators in this study, using only the histological slides at the start of the follow-up period. Reviewing and scoring the biopsies without having access to radiological data, a correct diagnosis was made in 90% of enchondromas and 89% of chondrosarcomas.

		Consensus diagnosis after ten years of follow-up		Total
		Enchondromas	Chondrosarcomas	
Diagnosis of the actual histological evaluation*	Enchondromas	18	4	22
	Chondrosarcomas	2	33	35
Total		20	37	57

Table 2.5. Histopathological assessment compared with the consensus diagnosis after ten years of follow-up. *This diagnosis was made only aware of the localisation of the tumour, the age of the patient and some clinical symptoms (like swelling, pain or fracture).

Discussion

The distinction between benign and malignant central cartilaginous tumours of bone is regarded to be one of the most difficult subjects in surgical pathology. In addition, the grading of these neoplasms also seems to differ considerably among pathologists. Our study, assessing interobserver variability among 18 specialised bone tumour pathologists, confirmed that the diagnosis and histological grading of cartilaginous tumours is challenging. This is in concordance with the results of the American SLICED (Skeletal Leasions Interobserver Correlation among Expert Diagnosticians) study group[22]. We demonstrated that the largest variability was found in the distinction between enchondroma and grade I chondrosarcoma.

This distinction is important to guide surgical management. While an wait-and-see approach or intraleasional treatment is permissible for enchondromas[4,9,11], grade I tumours should be treated more aggressively using curettage with vigorous adjuvant therapy (cryosurgery or phenolisation)[12,13] or by en-bloc resection.

Histopathological features to distinguish benign from malignant cartilaginous tumours were advocated in the past[9,15,18,20], although the diagnostic value of individual morphologic criteria or how they are handled in clinical practice by different pathologists have never been investigated statistically. We therefore collected a second set of 57 central cartilaginous tumours to assess the value of histomorphological criteria and clinical parameters to investigate the diagnostic power of an optimal combination of these in predicting a correct diagnosis. The diagnosis of these tumours was substantiated by a multidisciplinary assessment in the Netherlands Committee on Bone Tumours and by 10 years follow-up.

Clinical symptoms are believed to be helpful in the evaluation of a patient presenting with a cartilaginous tumour. In this study, 35% of patients with

enchondromas and 62% of the patients with central grade I chondrosarcoma presented with spontaneous pain. This difference is significant, although presentation without pain does not exclude malignancy and should not delay additional analysis. Anatomical tumour localisation was not found to be of diagnostic value in the differential diagnosis in our selection. This was skewed by the exclusion of cases with phalangeal localisation in the group of cases to study histological criteria, because of their specific clinicopathologic features[19].

Our study demonstrates that the 4 most important histological parameters are host bone entrapment (PPV=97.0%), high cellularity (PPV=89.75%), marked nuclear pleomorphism (PPV=88.2%) and irregular distribution of cells (PPV=85.4%) ($p< 0.001$). The differential diagnosis can be assessed with a great degree of accuracy if the biopsy specimen contains a combination of presence of host bone entrapment, open chromatin, mucoid matrix degeneration and the patient's age is above 45 years.

A prerequisite for this diagnostic power is the necessity that all of these parameters can be assessed in a biopsy, which depends on quality and quantity of the biopsy specimen. The present series contained both open and closed biopsies. Previous studies showed that reliable results could be obtained by either open or closed (Trocart) biopsy in experienced hands[23]. Sampling errors however could give false predicting results when interpreted without detailed knowledge of the radiological presentation.

Using the simple classification rule proposed in this paper (mucoid matrix degeneration and/or host bone entrapment present), 3 of the 57 tumours were assessed incorrectly. To improve this, a more complex algorithm is needed: classify all tumours as malignant if host bone entrapment is present, unless the cellularity in the slide is low, the patient age is below 45 years and the mucoid matrix degeneration is less than 20%. With this more complicated classification rule only one tumour was assessed incorrectly as an enchondroma.

Without using the proposed algorithm we were able to correctly distinguish between enchondromas and chondrosarcomas in 90% (18/20) and 89% (33/35) of the cases, respectively (Table 2.5) using 13 cytological and morphologic features. However, this study shows that a combination of 5 (or at a minimum of 3) of these features improved this distinction.

Histological grading is currently the only predictor of clinical outcome to guide therapeutic strategy, since there are no biomarkers available so far, despite extensive literature[24]. Our study amongst 18 bone tumour pathologists also showed that histological grading of chondrosarcoma is subjected to a large interobserver variability. Therefore we would advocate using follow-up data instead of histological grading as a measurement for outcome in molecular studies searching for prognostic parameters of chondrosarcoma.

In institutes using intraleasional surgery with adjuvant therapy for grade I chondrosarcoma, the distinction with grade II chondrosarcoma is essential

to determine whether en-bloc resection is indicated.
In conclusion; we showed considerable interobserver variability, even amongst expert bone tumour pathologists, in the histological diagnosis of enchondroma and low grade chondrosarcoma. In daily practice, the primary differential diagnosis will be made based upon radiology. Localisation in the axial skeleton and size greater than 5 cm have been shown to be a reliable predictor for malignancy[11]. Even using dynamic contrast enhanced MR-imaging and an experienced bone tumour radiologist an absolute distinction between malignant and benign cannot be made on radiological grounds alone[11,25,26]. Therefore, when the radiological assessment of a benign versus a low- grade malignant central cartilaginous tumour remains uncertain, a biopsy needs to be performed and assessed by an experienced pathologist, addressing the question of malignancy using a combination of histopathological criteria (high cellularity, presence of host bone entrapment, open chromatin, mucoid matrix quality and age above 45 years). This combined multidisciplinary approach using defined criteria could optimise the diagnostic pathway leading to optimal therapeutic management.

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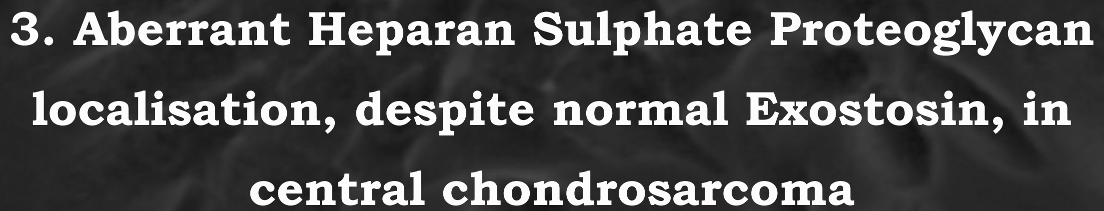

3. Aberrant Heparan Sulphate Proteoglycan localisation, despite normal Exostosin, in central chondrosarcoma

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Abstract
EXT1 and *EXT2* are involved in the formation of (multiple) osteochondromas, which can progress to secondary peripheral chondrosarcomas. Primary central chondrosarcoma occurs in the medullar cavity of bone and is the most common subtype. The role of EXT and its downstream targets in central chondrosarcomas is unknown.

The EXT1/EXT2 protein complex is involved in heparan sulphate proteoglycan (HSPG) biosynthesis, important for signal transduction of Indian Hedgehog (IHH), WNT and transforming growth factor beta (TGFB).

EXT1 and EXT2 were evaluated in central chondrosarcoma at the DNA (mutational screening, arrayCGH) and mRNA level. Immunohistochemistry was used to asses HSPGs (CD44v3 and SDC2), WNT (β-catenin) and TGFB (PAI-1 and phosphorylated Smad2) signalling, while IHH signalling was studied by qPCR and *in vitro*.

EXT1 and *EXT2* mRNA levels are normal in central chondrosarcoma. Genomic alterations are absent in these regions and in 30 other HSPG related genes. HSPGs are aberrantly located; CD44v3 in the Golgi and SDC2 in cytoplasm and nucleus, which was not caused by mutation. WNT signalling was negatively- and TGFB was positively correlated with increasing histological grade (p=0,038 and p=0,002). IHH signalling was active, and inhibition decreased cell viability in one of six cell cultures. Our data suggest that, despite normal EXT in central chondrosarcomas, HSPGs and HSPG dependent signalling are affected in both central and peripheral chondrosarcomas.

Introduction

The tumour suppressor genes EXT1 and EXT2 are known for their involvement in peripheral cartilaginous tumours. They cause the hereditary syndrome Multiple Osteochondromas (MO)(OMIM #133700), previously known as Hereditary Multiple Exostoses[1-3]. In MO patients, benign cartilage capped bony outgrowths (osteochondromas) develop at the surface of bones formed by endochondral ossification. Histologically, they bear a strong resemblance to the normal growth plate. 1-5% of osteochondromas progress to (secondary) peripheral chondrosarcoma.

While secondary peripheral chondrosarcoma is a rare chondrosarcoma subtype (less than 15% in tertiary referral centers), primary conventional central chondrosarcoma arising in the medullar cavity of bone is far more common (>80% of conventional chondrosarcoma)[4,5]. Enchondromas are benign cartilage tumours occurring in the center of the bone [4]. Enchondromas occur mostly as solitary leasions, although they may occur as multiple leasions in the context of non-hereditary enchondromatosis (Ollier disease) (OMIM #166000)[6].

The protein products of *EXT1* and *EXT2*, Exostosin 1 and Exostosin 2, are type II transmembrane glycoproteins that form a hetero-oligomeric complex, located in the Golgi membrane. This complex is responsible for elongation of heparan sulphate side chains that are linked to the proteoglycan protein cores, consequently forming heparan sulphate proteoglycans (HSPGs)[7,8]. HSPGs are involved in sequestering growth factors, anchorage to the extracellular matrix and in several growth signalling pathways[9]. Various HSPGs have been described e.g. syndecan (SDCs), perlecan and glypican families and CD44 isoforms containing HS bearing variable exon 3 (v3)[9,10]. In *Drosophila Melanogaster,* HSPGs were shown to be essential for gradient formation of the morphogens hedgehog, decapentaplegic and wingless[11]. The human homologues for these morphogens are Indian and Sonic hedgehog, TGFB / BMP and WNT, respectively[12]. Indian Hedgehog (IHH) orchestrates chondrocyte proliferation and differentiation in the human growth plate. IHH signals to its receptor patched (PTCH), which subsequently releases its inhibition on intracellular smoothened (SMO), resulting in the translocation of GLI transcription factors to the nucleus. Here, PTHLH is transcribed together with PTCH and GLI, guaranteeing the preservation of this signalling cascade[13]. PTHLH signalling inhibits chondrocyte differentiation and consequently controls longitudinal growth[14,15].

While it is evident that inactivation of the *EXT* genes is the driving force for the development of benign peripheral cartilaginous tumours[1-3,16,17], in the far more common central chondrosarcomas the role of EXT and its downstream targets has not been systematically studied so far.

We therefore investigated EXT and its downstream targets in central

chondrosarcoma. Results are compared to its rare peripheral counterpart for which EXT involvement is quite well characterised[1-3]. We studied *EXT* at the DNA and mRNA level and investigated the expression of HSPGs using immunohistochemistry. Activity of IHH, TGFB and WNT signalling, which require HSPGs for proper signalling, was also studied in a large series of central chondrosarcomas including six chondrosarcoma cell cultures.

Material and methods

Patient material

Enchondromas and conventional central chondrosarcomas were selected based on accepted clinicopathological[18] and radiological[19] criteria. Peripheral, juxtacortical, mesenchymal, dedifferentiated and clear-cell chondrosarcomas were excluded. In total, specimens of 110 patients were used for this study, including both formalin fixed paraffin embedded (n=95) and fresh frozen (n=34) tissue. Both paraffin and frozen series included enchondromatosis (Ollier disease) related tumours. Details are outlined in table 3.1. Histological grading was performed according to Evans et al[20]. All specimens were handled according to the ethical guidelines as described in “Code for Proper Secondary Use of Human Tissue in The Netherlands” of the Dutch Federation of Medical Scientific Societies.

	Enchondroma		**Chondrosarcoma**	
	FFPE	fresh frozen	FFPE	fresh frozen
Total number of tumours	**33**	**7***	**62**	**27**
Grade I	-	-	27	11
Grade II	-	-	25	7
Grade III	-	-	10	9
Male:Female	20:13	3:4	32:30	17:10
Enchondromatosis	6	5	6	7
Median age at diagnosis years (range)	37,8 (6,1-66,4)	18,0 (12,0-37,0)	50,0 (17,8-78,7)	40,0 (17,8-84,0)
Median follow up months (range)	65 (6-170)		87 (5-247)	

Table 3.1 Clinicopathological data of the 110 patients. FFPE: formalin fixed paraffin embedded. *All fresh frozen enchondromas were located in the phalanx.

Quantitative real time reverse transcriptase PCR (q-RT-PCR)
q-RT-PCR was used to study the expression level of *EXT1* and *EXT2* and the activity of IHH signalling. Of 34 cases fresh frozen tumour tissue was available for RNA isolation, performed as described previously[21]. Four growth plate samples, acquired from resections or biopsies for orthopedic clinical conditions not related to cartilaginous tumours, were used as controls. Results were compared to those of 28 peripheral tumours described previously[16]. Primers for *EXT1*, *EXT2, PTCH*, *GLI1* and *GLI2* were described previously[22]. Four control genes (*CYPA*, *CPSF6*, *SRPR* and *HNRPH1*) were selected to normalise the expression data, because of their invariable expression in chondrosarcoma[23]. Data of the cell cultures were normalised against *HPRT* and *GAPDH*. As a reference for normalisation and statistical analysis a calibration curve of a mixture consisting of 15 highly diverse cell lines[24] was included. Normalisation was performed using GENORM[25].

Genomic analysis:
Mutation analysis EXT1/2 and SDC2
Two tumours with decreased *EXT1* expression (L803 and L1689) were subjected to direct sequencing of the coding sequences of *EXT1* and *EXT2* and multiplex ligation-dependent probe amplification assay designed for *EXT1* and *EXT2* as described[26,27]. DNA was isolated from fresh frozen tumour tissue. DNA isolated from another set of seven tumours (four with nuclear and three with cytoplasmic SDC2 expression at IHC) and their corresponding normal lymphocytes were screened for mutations by direct sequencing analysis of the 5 exons of the coding region of the SDC2 gene. Primer sequences are listed in table 3.2.

	Sequence (5' - 3')	T_m	Product Size
SDC2-ex1F	TGTAAAACGACGGCCAGTGTACTCTGCTCCGGATTCGT	59.31	266
SDC2-ex1R	AACAGCTATGACCATGAGGGCTCCTCTCGTAGCTTCA	61.96	
SDC2-ex2F	TGTAAAACGACGGCCAGTCTCAACATCCTGACTCCCTTG	59.71	269
SDC2-ex2R	AACAGCTATGACCATGAATCCTGCCTGTCTCCTTGAA	59.8	
SDC2-ex3F	TGTAAAACGACGGCCAGTTCATGATTGCCATGCTCAGT	60.23	243
SDC2-ex3R	AACAGCTATGACCATGAGATAATGCAATGCAATGGAAA	58.5	
SDC2-ex4F	TGTAAAACGACGGCCAGTTTTCTTCTTTCCAACACATTTCC	59.51	270
SDC2-ex4R	AACAGCTATGACCATGAGTAGGCACCCTCCCACCT	59.92	
SDC2-ex5F	TGTAAAACGACGGCCAGTTGTCTGCAACCCTTGAATCTC	60.25	313
SDC2-ex5R	AACAGCTATGACCATGATGCAAAAGCTTTATTTGAAAAGTT	59.76	

Table 3.2 Primer sequences SDC2 mutation analysis

Array comparative genomic hybridisation (CGH)

L803 and L1689 were also analysed by array CGH using a high resolution 8q array containing a tiling bacterial artificial choromosome (BAC) clone set as described[16]. In addition, the seven tumours that were subjected to mutational screening for SDC2 were hybridised to a custom made HD-Agilent oligonucleotide array containing 4x44000 immobilised 60mer oligonucleotides. Genomic intervals containing 35 genomic regions of 37 genes selected on their function in cartilage biosynthesis (Supplementary table 3.1) were subjected to Agilent's eArray web tool (http://earray.chem.agilent.com/earray/) to generate oligonucleotide (60mer) microarray probe sequences. For each gene of interest probes were selected both for exonic and intronic areas with a spacing at 500-1000 base intervals, additional probes sequences were selected 5000 bps before and after targeted genes to include potential control elements, such as promoter regions. A total 37040 oligonucleotide probes were selected in genomic regions, additional probes till 44000 were added as internal control selected as a default from the array design options. Slides were printed and produced by Agilent Technologies using standard company protocols available as custom service. Array probes, layout, and access to purchase this microarray from Agilent array can be obtained from the responsible author (KS).

Test and reference samples were labeled, hybridised, washed, scanned and analysed following standard protocols provided by Agilent (Agilent Technologies, Amstelveen, The Netherlands).

Immunohistochemistry

For 95 tumours formalin-fixed, paraffin-embedded material was available (Table 3.1). All were stained for PAI-1 and a subset of 19 tumours for phosphorylated Smad2 to evaluate TGFB signalling[28,29]. In addition all were stained for β-catenin, to evaluate canonical WNT signalling[30], HSPG expression (SDC2 and CD44v3) was evaluated in a subset of 30 tumours. Subsets were representative for the total group of patients. Details of primary antibodies are described in table 3.3. PAI1, phosphorylated Smad2 and β-catenin immunohistochemistry was scored semi-quantitatively as described previously[31], by two observers (YMS, JVMG) independently. Both were blinded towards clinicopathological data. In brief, scores were given for β-catenin, PAI1 and phosphorylated Smad2 intensity (1 = weak, 2 = moderate, 3 = strong) and for percentage of positive cells (1 = 0-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75%-100% positive cells in the total tumour section). To avoid tumours with single positive cells being regarded as positive, cut off levels for statistical analysis were applied corresponding to general staining pattern (sum of score β-catenin ≥ 2 and PAI1 ≥ 3). SDC2 and CD44v3 were evaluated based on presence or absence and localisation of staining as described [23]. Specificity of the SDC2 antibody for membranous staining was tested using a tissue microarray (TMA) containing 79 soft tissue

Antigen	**Manufacturer**	**Mono/ poly clonal**	**Positive control**	**Staining**	**[AB]**	**AR***
CD44v3	Labvision	Mono	Tonsil	Membrane, cytoplasm	1:400	C
58K Golgi protein	Abcam Ltd	Poly	Tonsil	Perinuclear	1:100	C
Syndecan-2 (10H4)	G. David[51]	Mono	Growth plate	Membrane, ECM	35 ug/ ml	N
Heterochromatin protein-1 γ (2MOD-1G6-AS)	Euromedex	Mono	Any cell	Nuclear	1:5000	N
B-catenin	BD Transduction Laboratories	Mono	Skin	Nuclear	1:800	C
PAI-1	American diagnostica inc.	Mono	Mamma-carcinoma	Cytoplasm	1:350	N
PS2	P. ten Dijke[29]	Poly	Prostate	Nuclear	1:2000	C

Table 3.3 Antibodies used for IHC, WB and IF. [AB]; antibody concentration, AR; antigen retrieval, C; citrate, N; none. *Antigen retrieval was performed using citrate buffer at 98°C for 20 minutes.

tumours of 28 different entities, as was described previously[32].

Immunoblotting

To confirm the nuclear localisation of SDC2 as shown by immunohistochemistry two fresh central chondrosarcoma samples were digested overnight in dissociation medium containing 0.1% collagenase (Sigma, Zwijndrecht, The Netherlands) and 0.1% dispase (Life Technologies, Breda, The Netherlands) 100 IU/mL. Cell fractionation was performed using 2 different lysis buffers. To extract cytoplasmic proteins a buffer containing 10 mM HEPES pH7,9, 10 mM KCl, 0,1 mM EGTA, 0,1 mM EDTA, 1 mM DTT, 0,5 mM PMSF, 2 μg/ml Leupeptin, 1 μg/ml Pepstatin A and 2 μg/ml Aprotinin was used, followed by the addition of 10% NP-40. Subsequently, the extraction of nuclear proteins was performed by vigorously rocking the sample for 15 minutes in the presence of a buffer containing 20 mM HEPES pH7,9, 400mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml Leupeptin, 1 μg/ml Pepstatin A and 2 μg/ml Aprotinin. Protein concentrations were measured using a *DC* Protein Assay (Biorad, Hercules, CA, USA). 10 mg of each sample was run on SDS-PAGE. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Membranes were pre-incubated with 15% Skinned milk in Tris-buffered saline/Tween. After incubation with first and secondary

antibodies (Table 3.3), the membranes were developed with ECL™ Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and visualised by exposure to X-ray films (Hyperfilm ECL, Amersham Biosciences, Buckinghamsire, UK).

Confocal microscopy and immunofluorescence
To confirm localisation of CD44v3 in the Golgi apparatus, two central chondrosarcomas (with peri-nuclear CD44v3 expression) were selected for fluorescent double staining using CD44v3 and the 58K Golgi protein antibodies as described previously[22].

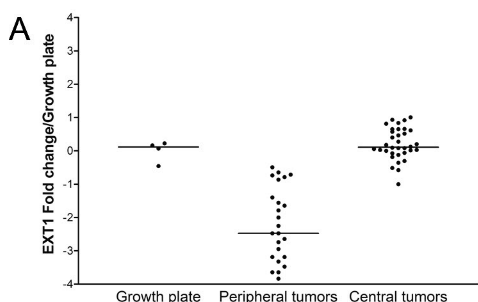
Hedgehog activity in vitro
Chondrosarcoma cell lines CH2879[33], C3842[34], OUMS27[35] and SW1353 (American Type Culture Collection, Manassas, VA (ATCC)), central chondrosarcoma primary cultures L784 and L869 and cyclopamine responsive pancreatic carcinoma cell line PANC1 (ATCC)[36] were used to analyse HH signalling. Chondrosarcoma cell lines were cultured in RPMI1640 and PANC1 in DMEM (both Gibco, Invitrogen Life-Technologies, Scotland, UK)) supplemented with 10% heat-inactivated fetal calf serum (Gibco) at 37°C in a humidified incubator with 95% air and 5% CO_2. Cartilaginous phenotype was confirmed by RT-PCR, showing mRNA expression of *collagens I*, *2B*, *3,* and *10*; *Aggrecan;* and *SOX9*[37].
For RNA analysis, 2,5 * 10^5 cells were seeded in a 6-wells plate. After 24 hours the medium was replaced with serum starved medium (0,05% FCS) containing either 10 µM cyclopamine (Toronto Research Chemicals, North York, Ontario, CA) or DMSO 0,1%. Cells were harvested after 24 hours and RNA was isolated using Trizol and microspin column (Qiagen, Hilden, Germany). Cell viability was assessed by WST-1 colorimetric assay (Roche Diagnostics GmbH, Penzberg, Germany), which measures mitochondrial activity. Cells were seeded into 96-well flat-bottom plates (1,5* 10^3 cells/well for SW1353 and PANC1 and 5,0* 10^3 cells for CH2879, C3842, OUMS27, L784 and L869). After 24 hours DMSO, cyclopamine at 5 µM and 10 µM and tomatidine (Toronto Research Chemicals), an inactive but structurally related compound[38], at 10 µM were added in the presence of 5% FCS, each condition in quadruplicate. After 3 days of treatment, the metabolic activity of the cells was measured on a Victor^3 Multilabel Counter 1420-042 (Perkin Elmer, MA, USA) at 450 nm.

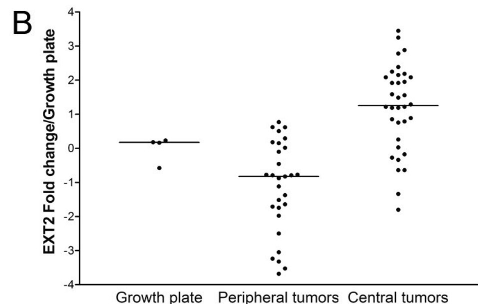
Statistical analysis
Correlations between histological grade and immunohistochemical results were calculated by Chi-square (X^2) test. Differences in cell viability *in vitro* were calculated by Students t-test. P-values <0.05 were considered significant.

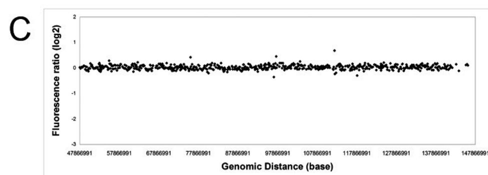
Results

Normal EXT1 and EXT2 in central chondrosarcoma

The expression of *EXT1* and *EXT2* mRNA in central cartilaginous tumours is not decreased as compared to growth plate samples, which is in contrast to the low *EXT1* expression levels previously found in peripheral tumours (Figure 3.1A and 3.1B) [22]. No correlation of *EXT1* or *EXT2* expression with histological grade was found (not shown). Direct mutation screening and MLPA for both *EXT1* and *EXT2* did not reveal any genetic aberrations (not shown). Comparative genomic hybridisation on a 8q BAC tiling array of the two central chondrosarcomas with the lowest expression of *EXT1* did not reveal any alterations on 8q24, the locus for *EXT*1. Results for L803 are shown in figure 3.1C, which are representative for L1689 (not shown). No other gains or losses in 8q were detected. Moreover, no losses were found in the *EXT1* and *EXT2* genes in the 7 chondrosarcomas hybridised at the oligonucleotide Agilent array.







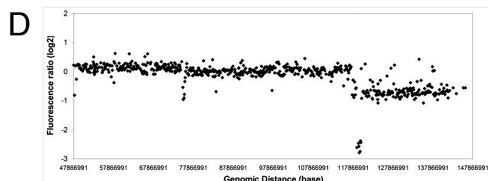


Figure 3.1 (A) EXT1 mRNA expression levels of central chondrosarcoma are comparable to growth plate levels while peripheral tumours (published previously[16]) show a three fold decrease. (B) EXT2 mRNA levels are slightly higher in central and slightly lower in peripheral chondrosarcoma, compared to growth plate. Expression levels of the tumours are log2 transformed and relative to the growth plate levels. (C) 8q tiling array CGH pattern of central chondrosarcoma L803 is shown. Genomic alterations are absent, despite low EXT1 expression at qPCR. L1689 showed similar results as L803 (data not shown). For comparison an osteochondroma with homozygous loss of multiple clones covering the EXT1 gene and hemizygous loss of a larger part of 8q, is shown in (D) (figure composed of data published previously[16]).

Aberrant location of HSPGs SDC2 and CD44v3 in central chondrosarcoma
Cytoplasmic staining of CD44v3 was found in 26 of 30 (87%) central tumours (7/8 EC and 14/22 CS). The staining was dot-like suggesting localisation in the Golgi apparatus (Figure 3.2A), which was confirmed using immunofluorescent confocal microscopy (Figure 3.2B). Central chondrosarcomas showed cytoplasmic staining of SDC2 in 28/29 (97%). Moreover, additional nuclear staining, shown in figure 3.2C, was found in half of the tumours (4/7 EC and 11/23 CS). The soft tissue tumour TMA did not show nuclear staining in any of the specimens (not shown). Control sections of tonsil showing membranous expression pattern of CD44v3 in the epithelial layer and normal growth plate showed SDC2 staining at the cell membrane and in the extracellular matrix surrounding hypertrophic chondrocytes (not shown). Nuclear localisation of SDC2 as found by IHC was verified in the nuclear component of the cell fractionation by immunoblotting (Figure 3.2D).

No aberrations in other genes important for HSPG formation
Since we found normal EXT in central chondrosarcomas, we searched for aberrations in other regulators of HSPG formation. Mutation screening of the five coding exons of the *SDC2* gene did not reveal any mutations in the seven tumours (not shown). Using the oligonucleotide tiling array covering 30 EXT, EXT-like and other genes involved in HSPG biosynthesis we did not find any specific genomic losses or gains in these seven tumours.

Decreased WNT signalling and increased TGFB signalling in high grade chondrosarcomas
Nuclear staining for β-catenin (Figure 3.3A) was found in 17% (5/30) of enchondromas, in 47% (11/23) of grade I and in 29% (7/24) of grade II and in 11% (1/9) grade III chondrosarcoma (Figure 3.3B). The activity of canonical WNT signalling was increased in grade I chondrosarcomas compared to enchondromas. However upon further increase in histological grade the activity decreased again (Pearson X^2 p=0,038). Expression of PAI-1, implicating active TGFB signalling (Figure 3.3C), was also associated with increased histological grade (Pearson X^2 p=0,002). All high grade tumours were positive (35/35), whereas the enchondromas and low grade chondrosarcoma were positive in 73% (24/33) and 74% (20/27), respectively (Figure 3.3D). Moreover, nuclear localisation of phosphorylated Smad2 was demonstrated in all of 6 enchondromas and 13 chondrosarcomas, suggesting active TGFB signalling (Not shown).

HH signalling in central chondrosarcoma in vivo and in vitro
By qPCR active HH signalling was shown in central chondrosarcoma, irrespective of histological grade, since *PTCH*, *GLI1* and *GLI2* were expressed at similar levels as in the growth plates (Figure 3.4A, B and C). Cyclopamine

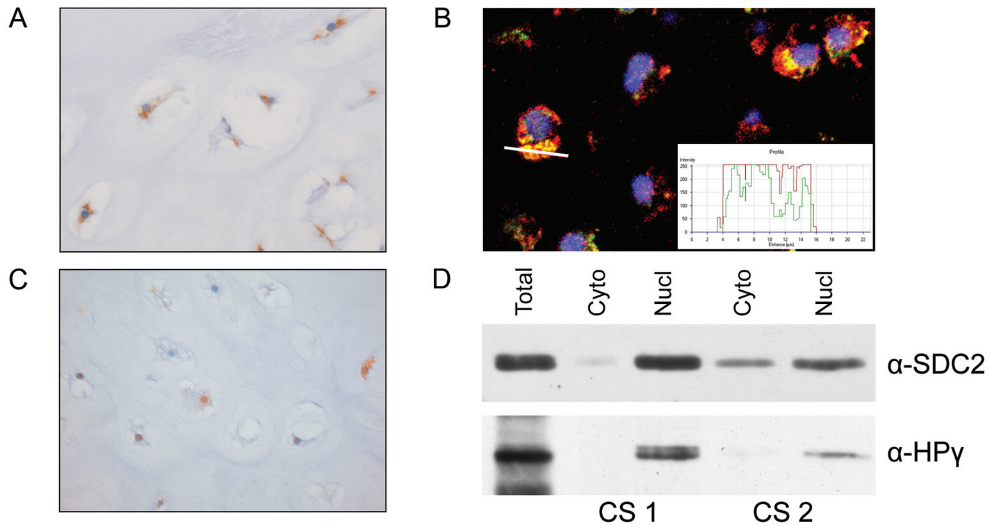


Figure 3.2 (A) Central chondrosarcoma showing cytoplasmic dot-like accumulation of CD44v3 at IHC (magnification 40x), suggestive for Golgi retention. This was confirmed by IF, using a Golgi specific marker, 58K protein, in green and CD44v3, in red, resulting in a yellow color when co-localisation occurs. The white line indicates the position where the staining profile (inset) has been taken (B). (C) Central chondrosarcoma showing nuclear staining of SDC2 (magnification 40x), which was found in 50% of chondrosarcomas in addition to cytoplasmic staining. (D) Nuclear localization of SDC2 was verified by immunoblotting in 2 chondrosarcomas. In the isolated nuclear fraction heterochromatin protein 1 gamma was present, verifying the procedure for separating the nuclear from the cytoplasmic staining.

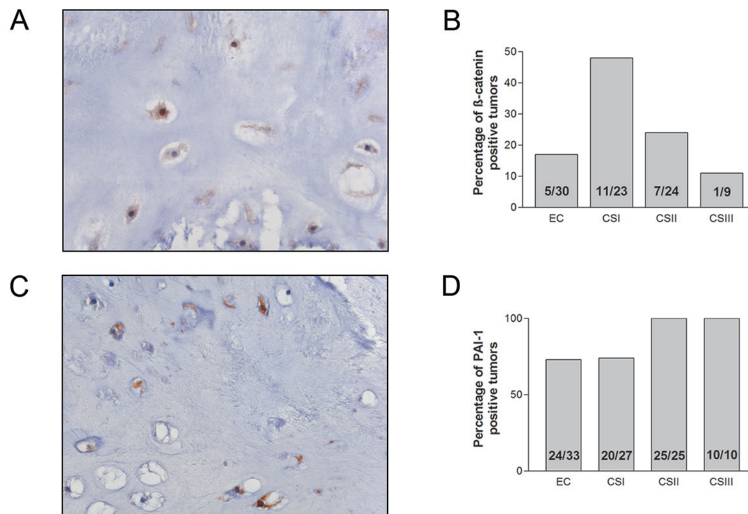


Figure 3.3. (A-B) The activity of canonical WNT signalling was increased in grade I chondrosarcomas compared to enchondromas. However upon further increase in histological grade the activity decreased again (Pearson X2 p=0,038). (C-D) PAI-1 expression, indicating active TGFB signalling, was found in the majority of central chondrosarcomas. PAI-1 expression was correlated to histological grade (X^2 test p=0,002). Magnification 40x.

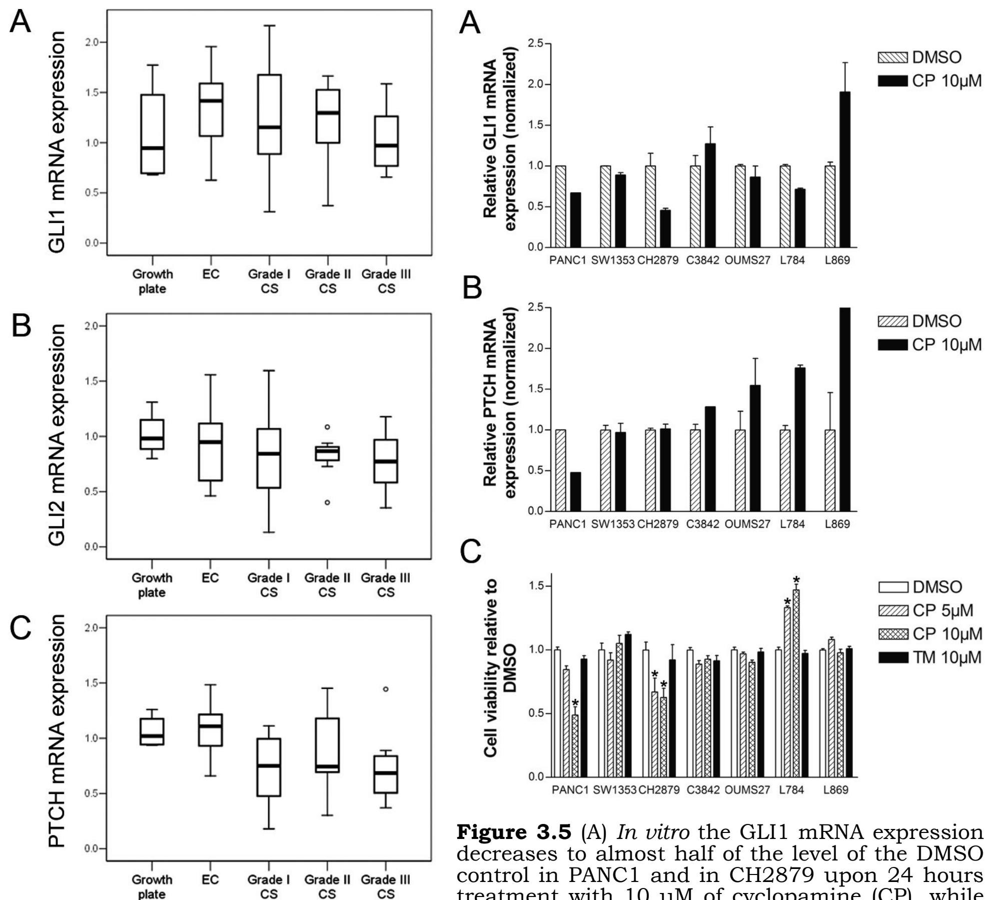


Figure 3.4. mRNA levels of GLI1 (A), GLI2 (B) and PTCH (C) were measured as a readout of IHH signalling. Levels of all three genes in chondrosarcomas were in the same range of growth plate samples and large variations within the groups were observed. No relation to histological grade was found. Expression levels are log10 transformed.

Figure 3.5 (A) *In vitro* the GLI1 mRNA expression decreases to almost half of the level of the DMSO control in PANC1 and in CH2879 upon 24 hours treatment with 10 µM of cyclopamine (CP), while this was not found in the other five chondrosarcoma cell cultures. (B) None of the chondrosarcoma cell cultures showed a negative effect on PTCH mRNA expression upon 24 hours 10 µM CP treatment. Both experiments were performed in duplo. (C) Both PANC1 and CH2879 demonstrated a decrease in cell viability measured by WST-1 assay, upon 72 hours of 5 µM and 10 µM CP treatment. An increase in viability was observed in L784, whereas the other four chondrosarcoma cell cultures did not show an effect of cyclopamine treatment. The black bars represent the inactive compound tomatidine (TM), which demonstrate the selective effect of CP. Data represent 3 individual experiments performed in quadruplicate.
* indicates Students t-test for paired data $p<0{,}05$.

treatment resulted in a profound decrease of *GLI1* mRNA expression combined with decreased cell viability in only one cell culture (CH2879), whereas all other cultures did not respond by decreased GLI1 levels or cell viability (Figure 3.5). In L869, an increase of GLI1 was observed while L784 showed increased cell viability. In all chondrosarcoma cell cultures *PTCH* levels were unchanged or even increased after cyclopamine treatment (Figure 3.5B). The viability of the tomatidine controls showed that the decrease in viability in PANC1 and CH2879 is not a toxic side effect of the cyclopamine compound.

Discussion

We present the first systematic evaluation of EXT and its downstream targets in central chondrosarcoma. We confirm that peripheral and central chondrosarcomas are clear distinct genetical entities also with respect to their *EXT* expression. Whereas inactivation of *EXT* is the driving force for the development of benign peripheral cartilage tumours[22], we demonstrate in central cartilage tumours the *EXT* genes to be normal both at the DNA as well as at the mRNA expression level. The absence of larger deletions in the *EXT1* region is in concordance with the previously reported absence of LOH[31] and karyotypic abberations[39] at 8q24 in central chondrosarcomas.

The EXT proteins are involved in the biosynthesis of heparan sulphate. Despite normal expression of the *EXT* genes in central tumours, heparan sulphate proteoglycans (CD44v3 and SDC2) unexpectedly accumulated in the cytoplasm. This is similar to what we previously described for peripheral tumours carrying *EXT* mutations[22], while in other tumours membranous expression of CD44v3 and SDC2 is described[40,41]. In addition, 50% of central chondrosarcomas also demonstrated nuclear SDC2 expression, which was not observed in peripheral chondrosarcomas, nor in 79 soft tissue sarcomas. In addition to the general function of SDC2 in modulating extracellular ligands, intracellular actions are implicated for SDC2. In osteosarcoma SDC2 has been shown to induce apoptosis[42,43]. We excluded mutations in the nuclear localisation signal (NLS), located at the first exon of the SDC2 gene, however other mechanisms like i.e. aberrant phosphorylation of the NLS or mutations in a kinase responsible for the phosphorylation of the NLS might explain the aberrant localisation of SDC2 in central chondrosarcoma.

In addition, it is tempting to speculate that, for example, other glycosyltransferases than EXT or the sulphotransferases or epimerases that function to complete the formation of heparan sulphate proteoglycans are affected in central chondrosarcoma. By using a custom designed oligonucleotide tiling array of 30 EXT, EXT-like and other genes involved in HSPG biosynthesis we excluded that neither heterozygous nor homozygous

losses, nor gains, are found in these genes. Nonetheless, we can not exclude that these HSPG biosynthesis related genes might be subjected to point mutations, or other copy number neutral alterations, i.e. inversion or methylation.

In *Drosophila*, HSPGs are essential for gradient formation of the morphogens hedgehog, decapentaplegic and wingless[11]. In human, the IHH pathway, through PTHLH signalling, is vital for chondrocyte proliferation and differentiation in the growth plate. We show hedgehog signalling to be active in central chondrosarcoma. We previously demonstrated PTHLH signalling, which is downstream of IHH, to be active in central chondrosarcomas as well[44,45]. In osteochondromas, IHH signalling is active, while activity decreases in peripheral chondrosarcoma with increasing histological grade[23]. Tiet et al. reported active IHH signalling in chondrosarcoma, but did not distinguish between peripheral en central tumours[46]. In a previous pilot series we reported lower levels of IHH signalling in central tumours as compared to the growth plate [45]. In the present larger series IHH signalling levels are comparable to growth plate samples. Moreover, in one of the cell lines, CH2879, an important role for hedgehog signalling in cell proliferation could be shown since inhibiting HH signalling using cyclopamine decreased cell viability. IHH signalling has been shown to be activated in many cancers such as medulloblastoma, basal cell carcinoma, small-cell lung cancer, breast cancer, and pancreatic cancer (reviewed in[47]). Therefore, targeting IHH signalling seems to be promising in cancer therapy[48]. Previously, Tiet et al. also showed that blocking HH signalling using either cyclopamine or triparanol, an inhibitor of 7-dehydrocholesterol reductase, reduced proliferation and tumour volume in 10 chondrosarcoma xenografts[46]. However, we observed an effect of IHH blockade using cyclopamine only in one of 6 chondrosarcoma cell cultures. Surprisingly, PTCH was found to be increased in those cultures that were resistant to cyclopamine, which might be induced by other pathways. These data suggest that despite aberrant cellular HSPG distribution, IHH signalling is active in central chondrosarcoma and is vital for cellular proliferation and therefore a putative therapeutic target in a small subset of them. Since HSPGs are thought to be important for the diffusion of HH to its receptor on target cells, it might be that in central chondrosarcoma these diffusion problems are overcome by cell autonomous (autocrine) HH signalling, as was previously also suggested for osteochondromas[49].

The activity of other signalling pathways dependent on HSPG was similar to the activity in peripheral chondrosarcoma[23]. Our results suggest that active canonical WNT signalling might be important for the transition from enchondroma towards low-grade central chondrosarcoma, however it is not crucial for progression towards higher grade (Figure 3.3). In contrast,

TGFB signalling was shown to increase with increasing histological grade by both the presence of PAI-1 and the finding of nuclear localisation of phosphorylated Smad2. This suggests that TGFB has a role in either rearrangement of extracellular matrix and/or vessel formation, which are the main characteristics of high grade chondrosarcoma[50].

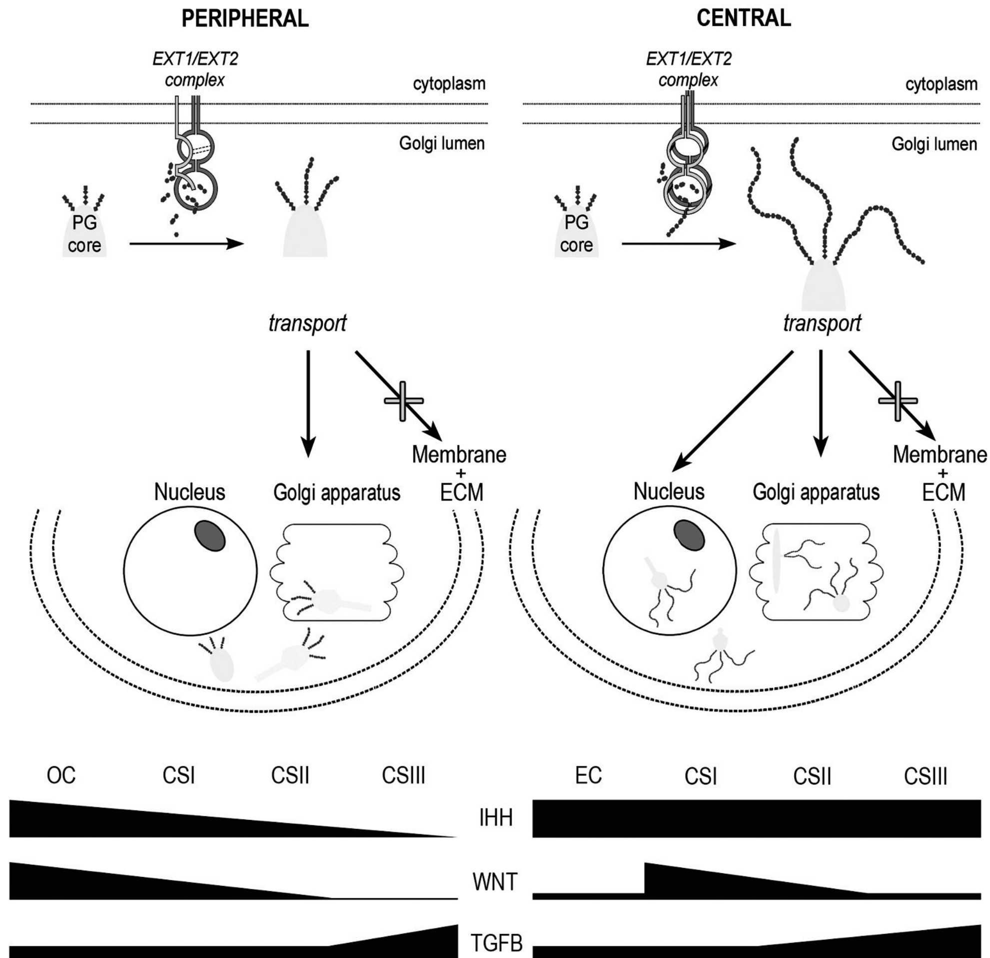


Figure 3.6 Overview of the EXT/HSPG pathway involved in both peripheral and central chondrosarcomas. Despite the absence of EXT alterations in central chondrosarcomas there is cytoplasmic retention of HSPGs, which is similar to peripheral chondrosarcomas and nuclear SDC2 localisation that was specific for central chondrosarcomas. In addition, while IHH signalling decreased with grade in peripheral chondrosarcomas, it is still active in high grade central chondrosarcomas.
Active canonical WNT signalling might be important for the transition from benign to malignant cartilaginous tumour type, however is not crucial for tumour progression, while TGFB is. Our results therefore suggest that a disturbed HSPG functioning is involved in the histogenesis of both central and peripheral chondrosarcoma, although different steps in HSPG biosynthesis seem affected. (Figure based on Hameetman et al.[22])

In conclusion, we present a systematic investigation of EXT and its downstream targets in central chondrosarcoma. We clearly show that the *EXT* genes are normal in central chondrosarcoma, with nevertheless aberrant localisation of HSPGs. Despite this, IHH signalling is active in central chondrosarcoma and is important for proliferation and therefore a potential therapeutic target in a small subset of central chondrosarcomas. The aberrant intracellular accumulation of HSPG in central chondrosarcoma which is similar to peripheral chondrosarcoma and the nuclear SDC2 localisation that is exclusively seen in central chondrosarcoma is difficult to explain. Nevertheless, our data, summarised in figure 3.6, suggest that a disturbed HSPG functioning is involved in the histogenesis of both central and peripheral chondrosarcoma, although different steps in HSPG biosynthesis seem affected.

Acknowledgements
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Supplementary table 3.1

Gene ID	Gene Name	Genome Location	Description(if known)
PAPST			
ENSG00000157593	SLC35B2	Chromosome 6: 44.33m	Adenosine 3'-phospho 5'-phosphosulfate transporter 1 (PAPS transporter 1) (Solute carrier family 35 member B2) (Putative MAPK-activating protein PM15) (Putative NF-kappa-B-activating protein 48). [Source:Uniprot/SWISSPROT;Acc:Q8TB61]
ENSG00000124786	SLC35B3	Chromosome 6: 8,358,300-8,380,793.	Adenosine 3'-phospho 5'-phosphosulfate transporter 2 (PAPS transporter 2)
Sulphate anion transporters: IPR001902 / ENSF00000000268			
ENSG00000145217	SLC26A1	Chromosome 4: 963.19k	Sulfate anion transporter 1 (SAT-1) (Solute carrier family 26 member 1). [Source:Uniprot/SWISSPROT;Acc:Q9H2B4]
ENSG00000155850	SLC26A2	Chromosome 5: 149.32m	Sulfate transporter (Diastrophic dysplasia protein) (Solute carrier family 26 member 2). [Source:Uniprot/SWISSPROT;Acc:P50443]
ENSG00000091138	SLC26A3	Chromosome 7: 107.19m	Chloride anion exchanger (Protein DRA) (Down-regulated in adenoma) (Solute carrier family 26 member 3). [Source:Uniprot/SWISSPROT;Acc:P40879]
ENSG00000091137	SLC26A4	Chromosome 7: 107.09m	Pendrin (Sodium-independent chloride/iodide transporter) (Solute carrier family 26 member 4). [Source:Uniprot/SWISSPROT;Acc:O43511]
ENSG00000170615	SLC26A5	Chromosome 7: 102.78m	Prestin (Solute carrier family 26 member 5). [Source:Uniprot/SWISSPROT;Acc:P58743]
ENSG00000181045	SLC26A11	Chromosome 17: 75.81m	solute carrier family 26, member 11 [Source:RefSeq_peptide;Acc:NP_775897]
Heparan sulfate N-deacetylase/N-sulfotransferases: ENSF00000001586 [EC:3.1.1.- 2.8.2.8]			
ENSG00000070614	NDST1	Chromosome 5: 149.87m	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 1 (EC 2.8.2.8) (Glucosaminyl N-deacetylase/N-sulfotransferase 1) (NDST- 1) ([Heparan sulfate]-glucosamine N-sulfotransferase 1) (HSNST 1) (N- heparan sulfate sulfotransferase 1) (N-HSST 1) [Inc [Source:Uniprot/SWISSPROT;Acc:P52848]
ENSG00000166507	NDST2	Chromosome 10: 75.23m	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 2 (EC 2.8.2.8) (Glucosaminyl N-deacetylase/N-sulfotransferase 2) (NDST- 2) (N-heparan sulfate sulfotransferase 2) (N-HSST 2) [Includes: Heparan sulfate N-deacetylase 2 (EC 3.-.-.-); Heparan sul [Source:Uniprot/SWISSPROT;Acc:P52849]

ENSG00000164100	NDST3	Chromosome 4: 119.18m	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 3 (EC 2.8.2.8) (Glucosaminyl N-deacetylase/N-sulfotransferase 3) (NDST- 3) (hNDST-3) (N-heparan sulfate sulfotransferase 3) (N-HSST 3) [Includes: Heparan sulfate N-deacetylase 3 (EC 3.-.-.-); H [Source:Uniprot/SWISSPROT;Acc:O95803]
ENSG00000138653	NDST4	Chromosome 4: 115.97m	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4 (EC 2.8.2.8) (Glucosaminyl N-deacetylase/N-sulfotransferase 4) (NDST- 4) (N-heparan sulfate sulfotransferase 4) (N-HSST 4) [Includes: Heparan sulfate N-deacetylase 4 (EC 3.-.-.-); Heparan sul [Source:Uniprot/ SWISSPROT;Acc:Q9H3R1]
Heparan sulfate 2-O-sulfotransferase: ENSF00000005941			
ENSG00000153936	HS2ST1	Chromosome 1: 87.15m	Heparan sulfate 2-O-sulfotransferase 1 (EC 2.8.2.-) (2-O- sulfotransferase) (2OST). [Source:Uniprot/SWISSPROT;Acc:Q7LGA3]
Heparan-sulfate 6-O-sulfotransferases: ENSF00000002453			
ENSG00000136720	HS6ST1	Chromosome 2: 128.74m	Heparan-sulfate 6-O-sulfotransferase 1 (EC 2.8.2.-) (HS6ST-1). [Source:Uniprot/SWISSPROT;Acc:O60243]
ENSG00000171004	HS6ST2	Chromosome X: 131.59m	Heparan-sulfate 6-O-sulfotransferase 2 (EC 2.8.2.-) (HS6ST-2). [Source:Uniprot/SWISSPROT;Acc:Q96MM7]
ENSG00000185352	HS6ST3	Chromosome 13: 95.54m	Heparan-sulfate 6-O-sulfotransferase 3 (EC 2.8.2.-) (HS6ST-3). [Source:Uniprot/SWISSPROT;Acc:Q8IZP7]
3'-phosphoadenosine 5'-phosphosulfate synthetases: IPR002650 and IPR002891 (two domains for two different activities)			
ENSG00000138801	PAPSS1	Chromosome 4: 108.75m	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 1 (PAPS synthetase 1) (PAPSS 1) (Sulfurylase kinase 1) (SK1) (SK 1) [Includes: Sulfate adenylyltransferase (EC 2.7.7.4) (Sulfate adenylate transferase) (SAT) (ATP-sulfurylase); Adenylyl-sulfate [Source:Uniprot/SWISSPROT;Acc:O43252]
ENSG00000198682	PAPSS2	Chromosome 10: 89.41m	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (PAPS synthetase 2) (PAPSS 2) (Sulfurylase kinase 2) (SK2) (SK 2) [Includes: Sulfate adenylyltransferase (EC 2.7.7.4) (Sulfate adenylate transferase) (SAT) (ATP-sulfurylase); Adenylyl-sulfate [Source:Uniprot/SWISSPROT;Acc:O95340]
Heparan sulfate glucosamine 3-O-sulfotransferases: ENSF00000001132			
ENSG00000002587	HS3ST1	Chromosome 4: 11.01m	Heparan sulfate glucosamine 3-O-sulfotransferase 1 precursor (EC 2.8.2.23) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 1) (Heparan sulfate 3-O-sulfotransferase 1) (h3-OST-1). [Source:Uniprot/ SWISSPROT;Acc:O14792]
ENSG00000122254	HS3ST2	Chromosome 16: 22.73m	Heparan sulfate glucosamine 3-O-sulfotransferase 2 (EC 2.8.2.29) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 2) (Heparan sulfate 3-O-sulfotransferase 2) (h3-OST-2). [Source:Uniprot/ SWISSPROT;Acc:Q9Y278]

ENSG00000153976	HS3ST3A1	Chromosome 17: 13.34m	Heparan sulfate glucosamine 3-O-sulfotransferase 3A1 (EC 2.8.2.30) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3A1) (Heparan sulfate 3-O-sulfotransferase 3A1) (h3-OST-3A). [Source:Uniprot/SWISSPROT;Acc:Q9Y663]
ENSG00000125430	HS3ST3B1	Chromosome 17: 14.15m	Heparan sulfate glucosamine 3-O-sulfotransferase 3B1 (EC 2.8.2.30) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3B1) (Heparan sulfate 3-O-sulfotransferase 3B1) (h3-OST-3B). [Source:Uniprot/SWISSPROT;Acc:Q9Y662]
ENSG00000182601	HS3ST4	Chromosome 16: 25.61m	Heparan sulfate glucosamine 3-O-sulfotransferase 4 (EC 2.8.2.23) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 4) (Heparan sulfate 3-O-sulfotransferase 4) (h3-OST-4). [Source:Uniprot/SWISSPROT;Acc:Q9Y661]
ENSG00000175818	HS3ST5	Chromosome 6: 114.49m	Heparan sulfate glucosamine 3-O-sulfotransferase 5 (EC 2.8.2.23) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 5) (Heparan sulfate 3-O-sulfotransferase 5) (h3-OST-5). [Source:Uniprot/SWISSPROT;Acc:Q8IZT8]
ENSG00000162040	HS3ST6	Chromosome 16: 1901.59k	Heparan sulfate glucosamine 3-O-sulfotransferase 6 (EC 2.8.2.23) (Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 6) (Heparan sulfate 3-O-sulfotransferase 6) (h3-OST-6). [Source:Uniprot/SWISSPROT;Acc:Q96QI5]
Plus the known EXT genes, (EXT1 EXT2, EXTL1, EXTL2, EXTL3)			

4. Central chondrosarcoma progression is associated with pRb pathway alterations; CDK4 downregulation and p16 overexpression inhibit cell growth *in vitro*

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Abstract

Chondrosarcomas are highly resistant to conventional radiation and chemotherapy and surgical removal is the only option for curative treatment. Consequently, there is nothing to offer patients with inoperable tumours and metastatic disease. The aim of this study is to investigate genes involved in cell cycle control: *CDK4, CDKN2A/p16, cyclin D1, p21, p53, MDM2* and *c-MYC,* which may point towards new therapeutic strategies. The pRb pathway was targeted using *CDKN2A/p16* overexpressing vectors and shRNA against *CDK4* in chondrosarcoma cell lines OUMS27, SW1353, and CH2879. Cell survival and proliferation were assessed. *CDK4, MDM2* and *c-MYC* expression levels were investigated by qPCR and immunohistochemistry (IHC) in 34 fresh frozen and 90 FFPE samples of enchondroma and chondrosarcoma patients. On a subset of 29 high grade chondrosarcomas IHC for cyclin D1, p21 and p53 was performed.

Overexpression of *CDKN2A/p16* and knock down of *CDK4* by shRNA in OUMS27, SW1353, and CH2879 resulted in a significant decrease in cell viability and proliferation and a decreased ability to form colonies *in vitro*. Expression of *CDK4* and *MDM2* was associated with high-grade chondrosarcoma both at the mRNA and protein level. Combining these results with the expression of *cyclin D1* and the previously shown loss of *CDKN2A/p16* expression show that the majority (96%; 28/29) of high-grade chondrosarcomas contain alterations in the pRb pathway.This suggests a role for the use of CDK4 inhibitors as a treatment of metastatic or inoperable high-grade chondrosarcoma.

Introduction

Chondrosarcoma of bone is a malignant cartilage-forming tumour which is notorious for its resistance to conventional chemo- and radiation therapy. The majority of tumours arise in the medullar cavity of bone and are designated primary central chondrosarcomas (80-85%)[1]. For <1% of chondrosarcomas, there is clinical evidence that they arose secondary to a pre-existing (benign) enchondroma[1,2]. Enchondromas occur mostly as solitary leasions, although they may occur as multiple leasions in the context of non-hereditary enchondromatosis (Ollier disease).

Chondrosarcomas are histologically divided into three grades, which is currently the only objective predictor of metastasis. While grade I tumours rarely metastasize and the 10 year survival rate is 83%, patients with grade III tumours develop metastatic disease in up to 71% of the cases and the 10 year survival rate decreases to 29%[3]. Marginal or intraleasional excision of tumours can result in local recurrence. Thirteen percent of recurrent chondrosarcomas are of a higher grade than the primary tumour[4]. Currently, surgical removal of the tumour is the only option for curative treatment. There is no treatment to offer patients with metastatic disease or inoperable tumours in the extremities or pelvis. Elucidating the molecular background of high-grade chondrosarcomas and the involved pathways that lead to tumour progression may help identify targets for future therapeutic strategies to improve clinical outcome.

In contrast to other solid tumours, central chondrosarcomas harbour relatively few consistent, numerical genomic alterations; however, amplification of 12q13[5,6] and deletion of 9p21[7-9] are two consistent genetic aberrations. Using array Comparative Genomic Hybridisation (CGH), we previously showed amplification of 12q13 in 6 of 21 (29%) central chondrosarcomas, which correlated with high histological grade[6], as was also suggested by others[9]. Several genes in this region are of importance for cell cycle control including *CDK4* and *MDM2*, players in the pRb and p53 pathway, respectively. Defects in these pathways are found at high rates in almost all types of human cancer[10,11]. Combining the array CGH results with those of our genome wide expression profiling experiments showed overexpression of the *CDK4* proto-oncogene in tumours with 12q13 amplification[6]. CDK4 controls progression through the cell cycle by regulating the transit of the cell through the G1 restriction point. This occurs by hyper-phosphorylation of pRb, leading to the release of E2F transcription factors. To accomplish this, CDK4 forms a complex with cyclin D1. This complex is tightly regulated by the inhibitory protein CDKN2A (CDKN2A/p16), which is encoded by the *INK4A-ARF* locus located on chromosome 9p21. Inhibition of the pRb-mediated cell cycle control through amplification of *cyclin D1* or *CDK4* and/or loss of expression of *CDKN2A/p16/INK4A* has been observed in many tumours[12]. Despite LOH of 13q14 has been found in a subset of chondrosarcoma[13,14], i.e. in 10/28 tumours by Yamaguchi et al.[5], pRb

mutations were not found[5]. Ropke et al. showed pRb expression in 16/17 chondrosarcomas by immunohistochemistry[14].
We and others previously demonstrated that loss of CDKN2A/p16 protein expression is correlated with increasing histological grade in central chondrosarcoma[7,15,16]. Cyclin D1 was previously shown to be expressed in 25 of 34 (73%) high-grade central chondrosarcomas[17].
In addition to *CDK4*, the 12q13 gene region harbours the *MDM2* gene which is frequently found to be co-amplified with CDK4[18]. The MDM2 gene encodes an E3 ubiquitin ligase involved in the degradation of p53 protein. The tumour suppressor protein p53 is activated upon various forms of stress, including aberrant mitogenic signalling, resulting in cell cycle arrest and/or the induction of apoptosis[11]. p53 mutations have been found in a subset of chondrosarcomas, and are mostly associated with aggressive behaviour (reviewed in Rozeman et al.[19]). Amplification of *MDM2* is frequently found in sarcomas (reviewed in Sandberg et al.[20]).
In addition to 12q13 and 9p21 alterations, Morrison et al. reported amplification of the oncogene *c-MYC* (8q24) in about 33% of high-grade chondrosarcomas[21]. However, these results could not be reproduced in other series[6]. C-MYC amongst others, drives cells into S phase[22]. Slight differences in *c-MYC* expression were reported between enchondromatosis-related and solitary chondrosarcomas[23].
The aim of our study was to investigate whether the pRb and p53 pathways harbour potential targets for therapy of inoperable or metastatic chondrosarcomas. Because 12q13 and 8q24 amplifications and 9p21 deletions suggest an important role for cell cycle regulators, especially those in the pRb and p53 pathways, we present the first *in vitro* evidence for an important role of *CDKN2A/p16* and *CDK4* in chondrosarcoma cell survival and proliferation. Subsequently, we validated the expression of *CDK4*, *MDM2,* and *c-MYC* at the mRNA and protein level in a large series of central chondrosarcomas.

Materials and Methods

Cell culture

Chondrosarcoma cell lines derived from chondrosarcoma grade II (SW1353, American Type Culture Collection, Manassas, VA), and chondrosarcoma grade III (CH2879[24] and OUMS27[25]) were cultured in RPMI 1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK). The breast carcinoma cell line MCF7 was grown in Dulbecco's modified Eagle medium. Media for both cell lines were supplemented with 10% heat-inactivated foetal calf serum (Gibco). Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. The cartilaginous phenotype was confirmed by RT-PCR, showing mRNA expression of *collagens I*, *2B*, *3,* and *10*; *Aggrecan;* and *SOX9*[26].

Overexpressing and short hairpin (sh) RNA lentiviral vectors
The *CDKN2A/p16*-expressing lentiviral vector (kindly provided by Dr. R. Hoeben, department of Molecular Cell Biology, Leiden University Medical Center) has been described previously[27]. To generate vectors expressing shRNA against *CDK4*, oligonucleotides (for sequences see supplementary table 4.1) were cloned into the pTER vector[28]. Subsequently, fragments containing the H1-promoter and cloned oligonucleotides were recloned into the lentiviral pRRL-CMV-GFP vector[29]. Production of lentiviruses by transfection into 293T cells has been described previously[29]. For infection of the chondrosarcoma cell lines, 10^5 cells were seeded into 6-cm dishes and allowed to attach overnight. Virus was quantitated by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation, NY). This value has been converted to an infectious titer using the approximation that 1 ng of p24 equals 2500 infectious units (multiplicity of infection (MOI)). To obtain overexpression, cells were infected with the *CDKN2A/p16*-expressing lentivirus with a MOI of 1. An empty vector was used as a negative control for infections. To obtain specific knock down, a mixture of three *CDK4*-shRNA-expressing lentiviral vectors was used (MOI 3); shRNA against murine *MDM4* was used as a control. Cells were transduced in the presence of 8 μg/ml polybrene (Sigma Aldrich, Zwijndrecht, The Netherlands). Microscopic evaluation of green fluorescent protein (GFP) expression three days post transduction showed 80-90% transduction efficiency for all conditions.

Immunoblotting
Proteins were extracted from cell cultures using Giordano lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, and 15% glycerol). Protein concentrations were measured using a Bradford assay (Bio-rad Laboratories, Hercules, CA, USA). 10 mg of total protein lysate from each sample was separated on SDS-PAGE. Lysates of normal human skin fibroblast cell line VH10, which was density-arrested and serum starved during two weeks, and subsequently reseeded in 20% serum, served as positive control for hyper-phosphorylated pRB (p-pRb). Two p16 negative melanoma cell lines were used as a control for loss of p16 staining in the chondrosarcoma cell lines. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). Equal protein loading was verified by α tubulin staining. The membranes were pre-incubated with blocking solution (10% Non-fat dry milk in Tris buffered saline pH 8.0, 0.2% Tween-20). After incubation with primary (Supplementary table 4.2) and secondary antibodies, the membranes were developed with Super Signal West Dura (Pierce Biotechnology, Rockford, IL, USA) and visualised by exposure to X-ray films or via the Chemigenius XE3 (Syngene, Cambridge, UK).

Proliferation assays
Cell counts were performed in duplicate using a Bürker chamber. A WST-1 colorimetric assay (Roche Diagnostics GmbH, Penzberg, Germany) was used to measure metabolic activity which represented the amount of viable cells. Briefly, cells were seeded into 96-well flat-bottom plates (1000 cells/well), each condition in quadruplicate. On days 3 and 6 post transduction, the metabolic activity of the cells was measured on a Victor^3 Multilabel Counter 1420-042 (Perkin Elmer, MA, USA) at 450 nm.

Clonogenic survival assay
Cells (1000, 5000 and 10000) were plated on 6-well plates. Cells were allowed to form colonies over a period of 14 days and subsequently fixed with methanol/acetic acid and stained using Giemsa.

Patient material
Conventional central chondrosarcomas were selected based on accepted clinicopathological and radiological criteria[1]. Peripheral-, juxtacortical-, mesenchymal-, dedifferentiated-, and clear-cell chondrosarcomas were excluded. In total, specimens from 105 patients were studied including 45 high-grade chondrosarcomas. The clinical details are outlined in table 4.1. Histological grading was performed according to Evans[3]. All specimens 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.

	Enchondromas		**Chondrosarcomas**	
	FFPE	fresh frozen	FFPE	fresh frozen
Total number of tumours	**20**	**7***	**70**	**27**
Grade I	-	-	25	11
Grade II	-	-	28*	7
Grade III	-	-	17*	9
Male	11	3	36	17
Female	9	4	34	10
Enchondromatosis	6	5	11	7
Median age at diagnosis years (range)	33.6 (11-66.4)	18 (12-37)	51.4 (17.8-84)	40 (17.8-84)
Median follow up months (range)	100 (6-221)		84 (5-247)	

Table 4.1 Clinicopathological data of the 105 enchondromas and chondrosarcomas. Abbreviations: FFPE Formalin fixed paraffin embedded. All fresh frozen enchondromas were located in the phalanx. * A subset of 29 FFPE high grade chondrosarcomas (grade II and III) was selected to study pRb and p53 pathway (Table 4.3).

Quantitative real time reverse transcriptase PCR (qPCR)
Fresh frozen tumour tissue was available for RNA isolation, performed as described previously, from 34 cases[30]. Growth plate samples (n=4) were used as controls. mRNA expression of *CDK4, MDM2,* and *c-MYC* (for primer sequences see supplementary table 4.3) were studied using quantitative RT-PCR, as previously described[31]. Four control genes (*CYPA, CPSF6, SRPR,* and *HNRPH1*) were selected because of their invariable expression in chondrosarcoma[31]. As a reference for normalisation and statistical analysis, a mixture of 15 cell lines[23] was included. Normalisation was performed using GENORM[32].

Immunohistochemistry (IHC)
Formalin-fixed, paraffin-embedded material from 90 tumours was used, including 45 high-grade (grade II and III) chondrosarcomas to study CDK4, MDM2 and c-MYC by IHC. Twenty-nine of these 45 high-grade chondrosarcomas were previously investigated for CDKN2A/p16[15] protein expression (Table 4.1 and 4.3) and 14 of these were negative for p16. To obtain a full overview of the pRb pathway in high grade chondrosarcomas, we further studied these 29 tumours for expression of other players in the pRb and p53 pathway by means of IHC for cyclin D1, p21 and p53. For 19 tumours, corresponding fresh frozen tissue was available (Table 4.1). Details of the primary antibodies used, are described in supplementary table 4.2. As negative controls, slides were incubated in PBS/BSA 1% without primary specific antibodies. An IHC protocol optimised for cartilaginous tissue was applied to avoid detachment of sections[13]. Antigen retrieval was performed using citrate buffer at 98°C for 20 minutes in a water bath. Slides were independently semi-quantitatively scored for nuclear staining, as described previously[13] by two observers (YS, JVMG). Both were blinded to the clinicopathological data. Scores were given for intensity (1 = weak, 2 = moderate, 3 = strong) and for the percentage of positive cells (1 = 0-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75%-100%). To avoid tumours with single positive cells being regarded as positive, cut off levels for statistical analysis were applied (sum of score CDK4 and p53 ≥4, and of cyclin D1, MDM2 and c-MYC ≥3. Tumours were regarded as negative for p21 with a sum of score ≤1, similar to the previous p16 staining[15]).

Statistical analysis
Normalised expression levels of different tumour groups were compared with growth plates using the Student's T-test or one-way ANOVA with Bonferroni correction, after $\log^{10}$ transformation. Correlation between immunohistochemical staining and histological grade was analysed using Pearson chi-square. Immunohistochemical data were correlated with follow

up by calculating the Kaplan-Meier curves and corresponding Log Rank tests. P-values <0.05 were considered significant.

Results

Functional analysis of the pRb pathway in vitro

Immunoblotting showed an absence of CDKN2A/p16 in all three chondrosarcoma cell lines, while pRb was mainly present in its inactive, hyper-phosphorylated form (Figure 4.1A). Overexpression of *CDKN2A/p16* caused a shift of hyper-phosphorylated pRb to hypo-phosphorylated pRb (4.1B), and a decrease in total pRb levels in SW1353, OUMS27 and CH2879. The relative number of cells decreased in SW1353, OUMS27, and CH2879 upon overexpression of *CDKN2A/p16* (p =0.035, 0.002, and

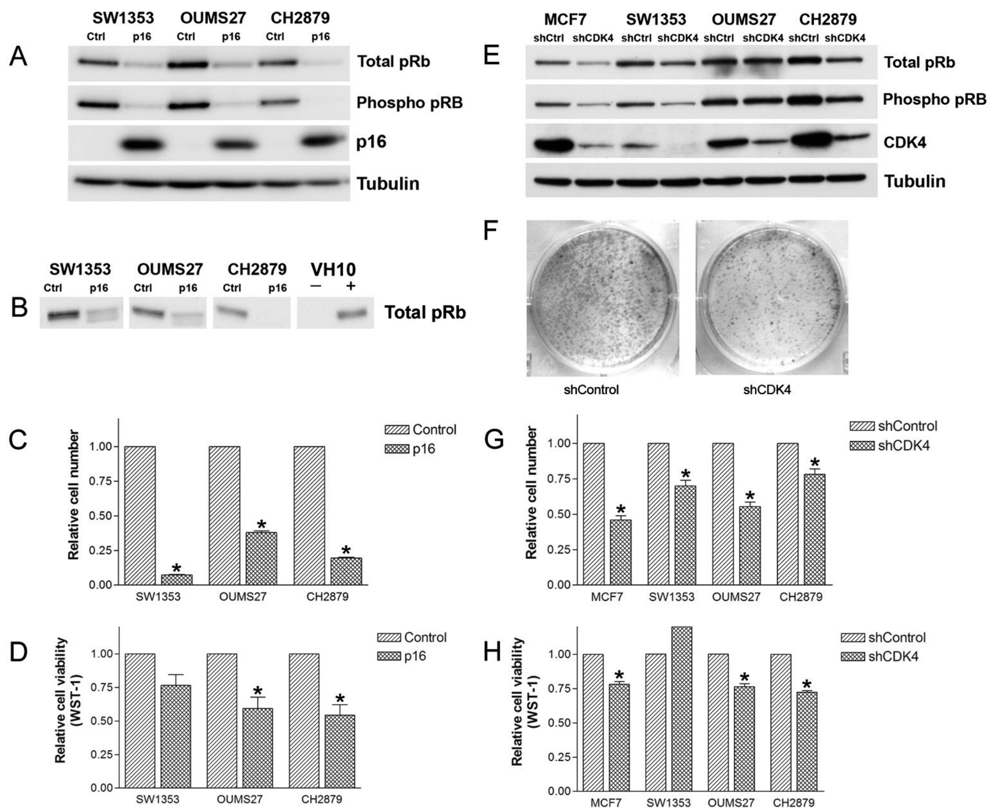

Figure 4.1 Immunoblot analysis of chondrosarcoma cell lines upon overexpression of CDKN2A/p16 (A) or knock-down of CDK4 (E). (B) Characteristic band shift of pRb on 7.5% gel in the chondrosarcoma cell lines SW1353 and OUMS27. VH10 that was density-arrested and serum starved and subsequently reseeded in 20% serum (+) served as a control for hyper-phosporylated pRb. (F) Decreased ability of OUMS27 to form colonies after knock-down of CDK4. Relative cell numbers (C, G) and cell viability (D, H) compared with controls are shown (both measured 6 days after treatment). * indicates significant differences.

0.014, respectively; figure 4.1C). In all cell lines, the WST-1 assay detected decreased metabolic activity, referred to as cell viability, to almost half of the metabolic activity of the controls for OUMS27 and CH2879 (p =0.003 and p=0.0455; figure 4.1D); this was less pronounced in SW1353 (p=0.059). All three cell lines showed high levels of *CDK4* mRNA expression (see supplementary figure 4.1). shRNA targeting *CDK4* in the chondrosarcoma cell lines and MCF7 was effective (Figure 4.1E). Decrease in hyper-phosphorylated pRb expression is indicative of cell cycle arrest and was found upon knock down of *CDK4* in all the cell lines, although to a lesser extent in OUMS27. Again, a reduction of total pRb with hypo-phosphorylation of existing pRb is observed. Enhanced degradation of pRb has been described previously after inhibition of *cyclin D1* in $p21^{WAF1}$ expressing lung cancer cells[33]. Interestingly, the CH2879 cells, in which the effect on pRb levels is most prominent (Figure 4.1E), show a relatively high level of $p21^{WAF1}$, correlating with the wild-type p53 status in these cells (data not shown). The number of cells in the *CDK4* shRNA transduced cells was significantly decreased (SW1353 p=0.002; OUMS27 p=0.003; CH2879 p=0.015; figure 4.1G). Metabolic activity was decreased in *CDK4* shRNA transduced OUMS27 and CH2879 cells (p=0.0002 and p<0.0001, respectively; figure 4.1H). In SW1353 an increase in metabolic activity was observed, analogous to the non-significant changes after overexpression of p16. SW1353 has a less cartilaginous appearance *in vitro*[34], despite its expression of typical cartilage mRNAs, and has a higher rate of proliferation than OUMS27 and CH2879, which may explain the divergent results in the WST-1 assay for this cell line. The capacity of all chondrosarcoma cell lines to form colonies *in vitro* was reduced upon knock down of CDK4 (E.g. OUMS27, figure 4.1F).

Expression of pRb and p53 components in clinical samples
Quantitative PCR
The increase in *CDK4* and *MDM2* mRNA expression correlates with increasing histological grade (Figures 4.2A and 4.2C) (Pearson R=0.684, p<0.0001 and R=0.508, p=0.007, respectively). Expression of *MDM2* was significantly higher in tumours demonstrating 12q13 amplification at array-CGH[6] than in tumours without amplification (Student's *t* test p=0.044, confidence interval [-0.83 -0.014], supplementary figure 4.2). *c-MYC* mRNA expression was not associated with histological grade (data not shown). In enchondromatosis related tumours, *c-MYC* mRNA expression was significantly higher than in solitary tumours (Student's *t* test p=0.011, confidence interval [-0.80; -0.116]; supplementary figure 4.3).

Immunohistochemistry
Results of CDK4, MDM2 and c-MYC staining on the series of 90 FFPE sections are shown in table 4.2. Nuclear expression of CDK4 and MDM2 protein, as illustrated by figures 4.2B en 4.2D, was correlated with

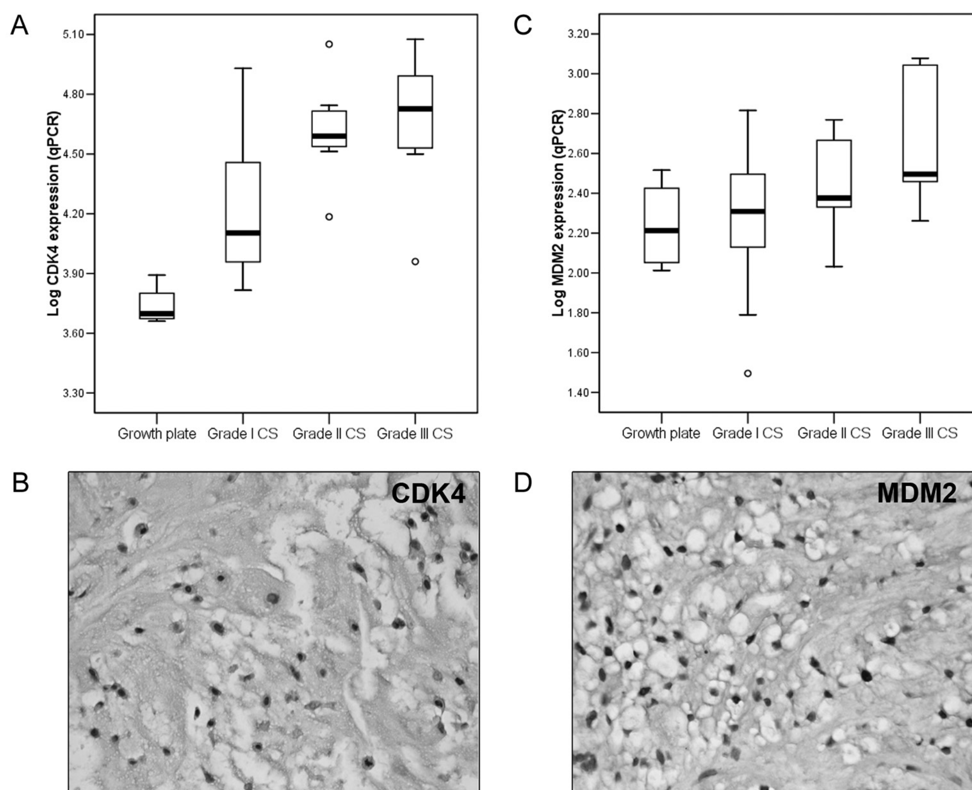

Figure 4.2 CDK4 (A) and MDM2 (C) mRNA expression levels relative to the growth plate are shown. Nuclear protein expression of CDK4 (B) and MDM2 (D) was determined (magnification 40x).

	CDK4		MDM2		cMYC	
Enchondroma	6/12	50%	0/20	0%	0/20	0%
Chondrosarcoma grade I	4/20	20%	1/19	5%	3/25	12%
Chondrosarcoma grade II	11/21	52%	7/25	28%	2/28	7%
Chondrosarcoma grade III	6/9	67%	6/13	46%	3/17	18%

Table 4.2 Immunohistochemical staining of 90 FFPE samples of enchondroma and chondrosarcoma patients

increasing histological grade in chondrosarcomas (Pearson's R=0.368, p=0.009 and figure 2E, R=0.356, p=0.007, respectively). Of the 29 high-grade chondrosarcomas that were previously studied for CDKN2A/p16 protein expression[15] (Table 3.3) and that were selected for further study, cyclin D1 was expressed in 62% (17/27). Moreover, 8 of 28 tumours were negative for p21, a CDK4/Cyclin D1 inhibitor activated by p53. Eight of 24 high grade

		pRb pathway				***p53 pathway***	
	L-number	**p16**	**CDK4**	**Cyclin D1**	**p21**	**MDM2**	**p53**
1	147	+	-	-	-	n/a	-
2	164	+	+	+	+	-	-
3	171	-	+	-	+	-	-
4	172	-	+	+	+	n/a	-
5	181	-	+	-	-	-	-
6	182	+	+	-	+	-	-
7	184	-	+	+	+	+	+
8	187	-	+	+	-	-	-
9	190	-	+	-	-	n/a	-
10	250	+	-	+	+	-	+
11	253	-	-	n/a	-	+	n/a
12	260	+	-	+	+	-	+
13	265	+	-	+	+	+	+
14	266	-	-	+	-	-	+
15	278	+	+	-	+	+	-
16	286	-	-	-	+	n/a	-
17	304	+	-	+	+	+	-
18	333	-	-	+	+	-	+
19	536	+	-	+	-	-	-
20	629	+	+	+	+	-	+
21	654	-	-	+	+	-	+
22	795	-	+	+	+	+	+
23	802	+	+	-	+	-	-
24	813	+	+	n/a	n/a	-	n/a
25	822	+	-	+	+	-	+
26	861	-	+	-	-	n/a	-
27	903	+	-	+	+	-	-
28	908	+	+	+	+	+	+
29	1066	-	+	-	+	+	-
	Total	Negative	Positive	Positive	Negative	Positive	Positive
		14/29 (48%)	16/29 (55%)	17/27 (62%)	8/28 (28%)	8/24 (33%)	11/27 (41%)
	Summary		***28/29 (96%)***			***21/29 (72%)***	

Table 4.3 Twenty-nine high-grade chondrosarcomas and associated alterations in the key players of the pRb and p53 pathways. + and - indicate positive and negative results, respectively. Immunohistochemical results for p16 were published previously[15] N/a: data not available.

chondrosarcomas (33%) were positive for MDM2 and in 11/27 p53 was overexpressed. These results emphasise that aberrations in the pRb pathway occur in the majority (28/29, 96%) of high-grade chondrosarcomas. In fact, central cartilaginous tumours harbouring aberrations in the pRb pathway had shorter disease-free survival (Log Rank test p=0.018), although this was not independent of histological grade.
While a correlation between mRNA expression of CDK4 and MDM2 with 12q13 amplification was evident, only in 2 of 4 tumours with an amplification (L795 and L1066) CDK4 and MDM2 protein expression was found (data not shown). Nuclear c-MYC protein expression was found in 0/20 enchondromas and in only 8/70 (11%) chondrosarcomas (Table 4.2). The difference in c-MYC mRNA expression between enchondromatosis and solitary tumours could also not be confirmed at the protein level (X^2 p=0.983).

Discussion

The aim of our study was to investigate whether the pRb and p53 pathways harbour potential targets for therapy of inoperable or metastatic chondrosarcomas. We present the first *in vitro* evidence for an important role of *CDKN2A/p16* and *CDK4* in chondrosarcoma cell survival and proliferation.
Unfortunately, there is nothing with curative intent to offer patients with inoperable or metastatic high-grade chondrosarcoma. 12q13 and 8q24 amplifications and 9p21 deletions are reported and suggest an important role for cell cycle regulators. Therefore, we investigated whether the pRb and p53 pathways carry a specific target that could be used for future targeted therapy of high grade central chondrosarcoma, similar to the attempts currently being made for other types of cancers. We demonstrate alterations of the pRb pathway in the vast majority of high-grade central chondrosarcomas.
Increased CDK4 expression, both at the mRNA and protein level, was found in 16 of 29 high-grade central chondrosarcomas and correlated with increasing histological grade and, consequently, poor prognosis. Increased CDK4 expression was also shown previously in a pRb negative chondrosarcoma cell line by Asp et al.[16]. Reducing *CDK4* expression in chondrosarcoma cell lines resulted in decreased survival and cell proliferation, confirming the important role of *CDK4* in chondrosarcoma progression.
Loss of p16 was previously shown in chondrosarcoma specimens and cell lines by Asp et al.[7, 16] and by us[15]. We now show the functional implications of the p16 loss in central chondrosarcoma by overexpressing p16 in three p16 negative chondrosarcoma cell lines. We found decreased cell growth upon p16 overexpression, which is probably caused by senescence, since increased apoptosis was not observed (data not shown).
Other players in the pRb pathway are also affected in central chondrosarcoma, as was also reported previously[7,14-17,35]. We show that the pRb pathway is

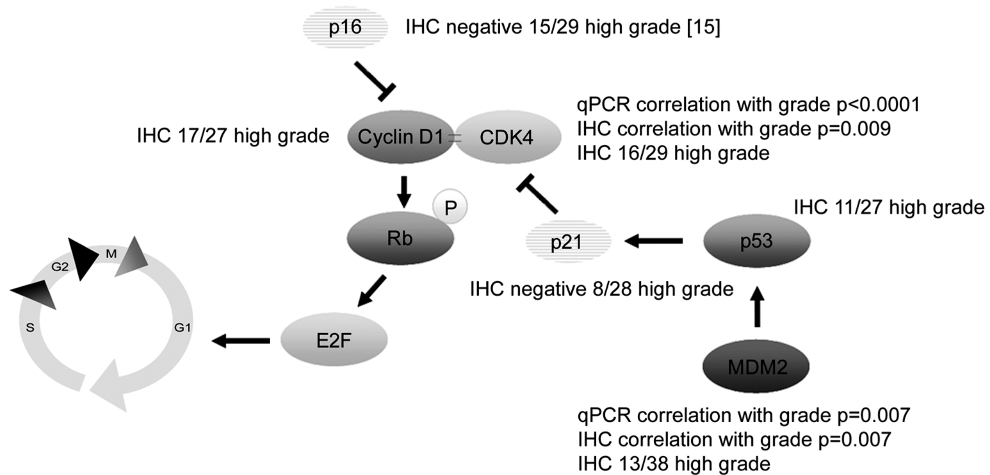

Figure 4.3 Changes in the pRb and p53 pathway promoting cell cycle passage in high-grade central chondrosarcoma, as found in the present and previous studies. Correlations are positive, unless stated otherwise.

affected in 96% of the high-grade central chondrosarcomas, either by a decrease in the amount of CDKN2A/p16 (48%), an increase in the amount of CDK4 (55%), or expression of cyclin D1 (62%) (Table 4.3, figure 4.3). In addition, we show that *MDM2* overexpression is correlated with increased histological grade. Thirteen of 38 (34%) high-grade tumours showed staining for MDM2, indicating that p53 degradation through MDM2 is also associated with tumour progression in a subset of central chondrosarcomas. Thus, alterations in p53 and pRb pathways are non-redundant in high grade central chondrosarcoma. Crosstalk between the pRb and p53 pathway occurs via the p53 response gene, $p21^{WAF1}$. The $p21^{WAF1}$ protein can inhibit the CDK4-cyclin D1 complex upon overexpression (Figure 4.3). Surprisingly, p21 expression was also shown to be associated with increasing grade in chondrosarcoma[36].

The reported amplification of the *c-MYC* locus (8q24)[21] could not be verified in our previous arrayCGH experiments[6]. cDNA microarray demonstrated differences in mRNA expression of the oncogene *c-MYC* in enchondromatosis-related versus solitary tumours[23], which we confirmed in the present study using qPCR. C-MYC overexpression is observed in a large number of malignancies[22]. Based on the low c-MYC protein expression levels we detected in chondrosarcoma, the importance of oncogene *c-MYC* in central chondrosarcoma development or progression is questionable.

Chondrosarcomas are highly insensitive to classical chemotherapeutics that interfere with the cell cycle, like methotrexate and 5-fluorouracil, and to radiation therapy. Surgery is currently the only therapeutic option. We now

show that central chondrosarcomas gain defects in the pRb pathway upon progression in grade, and that in the majority of high-grade chondrosarcomas either *CDKN2A/p16* expression is inhibited, *CDK4* is activated, or both. Our *in vitro* experiments with shRNA against *CDK4* and overexpression of CDKN2A/p16 gene suggests that a number of therapeutic strategies may become possible including the use of CDK4 inhibitors. Functionally intact pRb signalling is a prerequisite for the effectivity of CDK4 inhibition. Despite LOH of 13q14 has been found in a subset of chondrosarcoma[5,13,14], pRb mutations were not found[5]. Moreover, we show deliberate pRb expression in three chondrosarcoma cell lines.
In MCF7 breast cancer cells, *CDK* inhibitors were effective in treating tumours that overexpress the CDK4-cyclin D1 complex or that have lost INK4a function[37]. Heat shock protein 90 (HSP90) inhibitors could also be potential proliferation blockers in chondrosarcoma, since the stabilisation of CDK4, among other proteins, is regulated by HSP90 (reviewed in Whitesell et al.[38]). Our results indicate that studying the effect of compounds targeting *CDK4*, as soon as they are made available for (pre-)clinical use, would be the next step to investigate alternative, targeted treatment for high-grade central chondrosarcomas.

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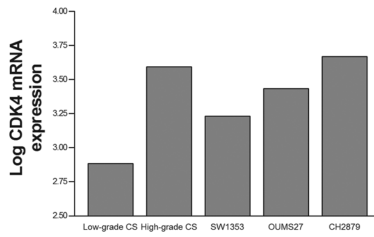
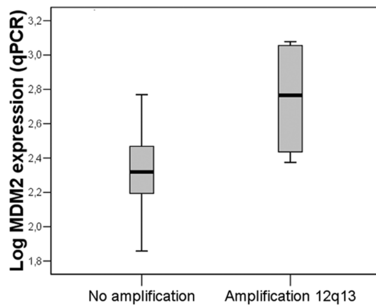
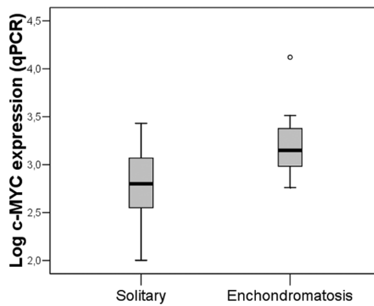


Supplementary figure 4.1 Central chondrosarcoma cell lines SW1353, OUMS27, and CH2879 show high levels of CDK4 mRNA in qPCR, comparable to the levels in high grade chondrosarcoma.



Supplementary figure 4.2 Central chondrosarcomas, showing gain at 12q13 at arrayCGH, express significantly higher mRNA levels of MDM2.



Supplementary figure 4.3 c-MYC mRNA levels are significantly increased in enchondromatosis patients.

shRNA oligonucleotide	Forward sequence (5'-3')
1	gatccgGACATATCTGGACAAGGCttcaagagaGCCTTGTCCAGATATGTCCTT ttttggaaa
2	gatccgCCCACACAAGCGAATCTCTttcaagagaAGAGATTCGCTTGTGTGGGTTttttggaaa
3	gatccgTCTTTGCCTTTATCTCTGAttcaagagaTCAGAGATAAAGGCAAAGATT ttttggaaa

Supplementary table 4.1. CDK4 shRNA oligonucleotide sequences.

Protein	Origin	Clone	Dilution	Species	Antigen retrieval	Positive control
IHC						
CDK4	Biosource	DCS-31	1:40	Mouse	Citrate	Skin
Cyclin D1	NeoMarkers	SP4	1:200	Rabbit	Tris-EDTA	Tonsil
MDM2	Zymed	IF2	1:250	Mouse	Citrate	Atypical lipomatous tumour
cMYC	Epitomics	Y69	1:100	Rabbit	EDTA	Burkitt lymphoma
p21	Calbiochem	Ab-1	1:400	Mouse	Citrate	Sigmoid
p53	NeoMarkers	DO-7	1:1000	Mouse	Citrate	Colon caricinoma
Immunoblotting						
CDKN2A/p16	NeoMarkers	16P04	1:800	Mouse		
CDK4	Santa Cruz Biotechnology	C-22	1:200	Rabbit		
	Abcam	DCS-35	1:250	Mouse		
MDM2	*Mol Cell Biol* 1993;13:4107-14	4B2	1:3	Mouse		
	+ Santa Cruz Biotechnology	SMP14	1:500	Mouse		
Phospho-pRb (ser 807/811)	Cell Signalling Technology	CS# 9308S	1:1000	Rabbit		
Total pRb	Pharmingen BD	G3-245	1:500	Mouse		
α Tubulin	Sigma Aldrich	DM1A	1:2000	Mouse		

Supplementary table 4.2. Procedures and details of the primary antibodies used for immuohistochemistry (IHC) and immunoblotting.

Gene	Primer sequence (5'-3')
CDK4 sense	TTCGTCGAAAGCCTCTCTTCTGTG
CDK4 antisense	TCCACGGGGCAGGGATACATC
MDM2 sense	AGATTCCAGCTTCGGAACAA
MDM2 antisense	TTTTTGTGCACCAACAGACTT
c-MYC sense	ACCACCAGCAGCGACTCTGA
c-MYC antisense	TCCAGCAGAAGGTGATCCAGACT

Supplementary table 4.3. Primer sequences used for qPCR.

5. Kinome profiling of chondrosarcoma reveals Src-pathway activity and dasatinib as option for treatment

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Abstract
Chondrosarcomas are notorious for their resistance to conventional chemo- and radiotherapy, indicating there are no curative treatment possibilities for patients with inoperable or metastatic disease. We therefore explored the existence of molecular targets for systemic treatment of chondrosarcoma using kinome profiling. Peptide array was performed for 4 chondrosarcoma cell lines and 9 primary chondrosarcoma cultures with GIST882, mesenchymal stem cells, and colorectal cancer cell lines as controls. Activity of kinases was verified using immunoblot and active Src- and PDGFR signalling were further explored using imatinib and dasatinib on chondrosarcoma *in vitro*. The AKT1/GSK3B pathway was clearly active in chondrosarcoma. In addition, the PDGFR pathway and the Src kinase family were active. PDGFR and Src kinases can be inhibited by imatinib and dasatinib respectively. While imatinib did not show any effect on chondrosarcoma cell cultures, dasatinib showed a decrease in cell viability at nanomolar concentrations in 7 out of 9 chondrosarcoma cultures. However, inhibition of phosphorylated Src (Y419) was found both in responsive and non-responsive cells. In conclusion, using kinome profiling we found the Src pathway to be active in chondrosarcoma. Moreover, we showed *in vitro*, that the inhibitor of the Src pathway, dasatinib, may provide a potential therapeutic benefit for chondrosarcoma patients which are not eligible for surgery.

Introduction

Curative treatment of chondrosarcoma of bone is restricted to surgery, since this tumour is reported to be extremely chemo- and radiotherapy resistant against conventional therapeutic modalities[1-3]. Therefore, there is not much to offer with curative intent to patients with metastatic disease or with tumours at inoperable sites. Whereas low-grade chondrosarcomas (grade I) are treated by marginal or intraleasional excision, followed by margin improvement by application of fenol or cryosurgery[3,4], high-grade chondrosarcoma is treated by, often mutilating, large en-bloc resection or amputation. The metastatic rate of chondrosarcoma is directly related to histological grade[5], currently being the only predictor of outcome, while histological grade is highly subjected to interobserver variability[6,7]. Grade I chondrosarcomas almost never metastasize, while metastases occur in 10% of grade II chondrosarcomas and in 71% of grade III chondrosarcomas[8]. The 10 year survival rate for patients with high grade chondrosarcoma is poor: 64% for grade II and only 29% for grade III tumours.

Few recurrent genetic alterations were found in chondrosarcoma, pointing towards a loss of cell cycle control of chondrosarcoma, such as gain of CDK4[9,10] and loss of p16[11,12]. Decreased cell viability was demonstrated after restoration of p16 expression or the knock-down of CDK4 by shRNA in chondrosarcoma cells *in vitro*[10]. So far there is little evidence for a role for kinase inhibitors in chondrosarcoma treatment. In addition to CDK4[10], AKT[13] and Flk-1/KDR, PDGFRB[14] and FGFR1[15] were shown to be activated and suggested as possible targets in the treatment of chondrosarcoma.

In the present study we explored new treatment options for chondrosarcoma using kinome profiling. Kinases are enzymes that phosphorylate tyrosine/ serine or threonine residues on other proteins. They play a major role in signalling cascades that determine cell cycle entry, cell survival and differentiation fate, which are often deregulated in tumours. Kinases are excellent targets for anti-cancer therapy since they work as a molecular switch; their regulation is reversible, rapid (merely in seconds) and does not require new protein synthesis (reviewed in[16]).

Kinome profiling allows the detection of kinase activity in cell lysates by detecting the level of substrate phosphorylation. This produces a comprehensive description of cellular signal transduction in a particular sample, which can be assigned to specific pathways, as has been shown by Diks *et al.*[17]. Kinome profiling identified the AKT1/GSK3B pathway, PDGFRB and the Src pathway, as potential targets for chondrosarcoma treatment. We showed that inhibition of the Src pathway by dasatinib indeed resulted in decreased cell viability in 7 out of 9 chondrosarcoma cell cultures *in vitro*.

Material and methods

Reagents

Imatinib mesylate (Glivec/Gleevec, STI571) was obtained from Novartis (Basel, Switzerland) and dasatinib (Sprycel, BMS- 354825) from Bristol-Myers Squibb (New York, USA). Both drugs were dissolved in DMSO.

Cell culture

Chondrosarcoma cell lines, chondrosarcoma primary cultures (Table 5.1) and a gastro intestinal stromal tumour cell line, GIST882 [18] were cultured in RPMI 1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK), supplemented with 10% heat-inactivated foetal calf serum (Gibco). GIST882, a gastrointestinal stromal tumour cell line carrying a homozygous exon 13 missense mutation (K642E) in KIT[18] and known to be imatinib sensitive, was used, treated and untreated, as a proof of principle of the Pepchip® technique. Two cell cultures of normal bone marrow derived mesenchymal stem cells (MSCs) (L2361 and L2370) were used as non-neoplastic counterpart control. In addition, an independent set of 5 colorectal carcinoma cell lines were tested (HT29, RKO, LS180, SW480, SW837) to estimate specificity.
Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. The cartilaginous phenotype of the chondrosarcoma cultures was confirmed by RT-PCR, showing mRNA expression of collagens I, 2B, 3, and 10, Aggrecan and SOX9[19].

	Sample	Type	Grade	Gender	Age	Passage
1	OUMS27[39]	Cell line	III	m	*na*	19
2	CH2879[40]	Cell line	III	f	35	58
3	SW1353*	Cell line	II	f	72	53
4	C3842[41]	Cell line	II	m	38	16
5	L784	Primary culture	II	m	40	11
6	L821	Primary culture	I	f	53	12
7	L835	Primary culture	III	m	55	15
8	L869	Primary culture	II	m	52	27
9	L1081	Primary culture	II	f	47	11
10	L1250	Primary culture	III	f	38	10
11	L2252	Primary culture	I	f	29	7
12	L2279	Primary culture	I	f	43	7
13	L2388	Primary culture	II	m	42	4

Table 5.1 Chondrosarcoma cultures. *na*: not available. *SW1353 was obtained from American Type Culture Collection (Manassas, VA, USA).

Kinome array analysis

A kinase substrate peptide array was used containing 1024 different kinase substrates spotted in triplicate with 16 negative, and 16 positive controls (Pepchip Kinomics, Pepscan Presto, Lelystad, the Netherlands). Cells were harvested during their exponential growth phase. Cells were washed 3 times with cold PBS. Cell lysis buffer (Cell Signalling Technology, Cambridge, MA, USA) supplemented with 1mM PMSF was used. Lysates were processed through a QIAshredder (Qiagen, Germantown, MD, USA) for 10 minutes and a filter (Millipore, Billerica, MA, USA) for 5 minutes both at 4°C to retain optimal kinase activity. For concentration of the lysates and discarding of the lysis buffer, a 10kD spin column (Millipore) was used for 30 minutes at 4°C. Concentration of the protein lysate was measured using the *DC* Protein Assay (Biorad, Hercules, CA, USA). For kinase array analysis 50µg of the protein was diluted in kinase buffer (Cell Signalling Technology) in the presence of complete mini EDTA free (Roche, Basel, Switzerland) and 1 mM PMSF. To 62,5µl of the lysate 12,5µl activation mix (TrisHCl pH7.5 20mM, MgCl2 10mM, Glycerol 5%, Brij-35 0.01%, BSA 0,05mg/ml, ATP 5µM and [γ-^{33}P]ATP 20µCi) was added. A 24x60 mm cover slip was used. The slides were incubated in a humid chamber for 2 hours at 37°C. Subsequently, slides were rinsed in PBS/Triton X-100 1% twice, then washed twice in NaCl 2.0M/Triton X-100 1% for 15 minutes, followed by washing in distilled water. All buffers were used at 37°C and washing was performed in a rotation stove at 37°C. Subsequently the slides were dried in a 50 ml tube in a centrifuge at 2000 rpm. The measurement of the ^{33}P signal was performed using a Biomolex reader for real time digital imaging of radioisotopes (Biomolex, Oslo, Norway). At least 1×10^6 hits were collected.

Data analysis

Biomolex Readback V3.6 and Biosplit software (Biomolex) were used to create a list with intensities using a grid. For further data mining R-packages Affyio and Limma were used (http://www.bioconductor.org). Quality of the triplicates and distribution of the data was assessed and quantile normalisation (Affyio) was performed. Phosphorylated substrates in chondrosarcoma cultures were compared with those in MSCs using Limma which provides functions to summarise results using a linear model to perform hypothesis tests and adjust the p-values for multiple testing[20]. In addition, the data set of 13 chondrosarcoma cell cultures was compared with an independent set of 5 colorectal carcinoma cell lines using Limma. Subsequently, phosphorylation signals of all chondrosarcoma cultures were averaged and the top 100 (the common denominators) were imported for core analysis in Ingenuity Pathway Analysis (http://www.ingenuity.com). IPA is a literature based program that calculates the probability of involvement of identifiers, in this case combinations of kinases, in 74 known canonical pathways.

Immunoblotting

10 mg of each sample, stored in kinase buffer at -80°C, was run on SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) using electrophoresis. Membranes were pre-incubated with 5% skinned milk in phosphate buffered saline-Tween 0.05%. After incubation with first and secondary antibodies, the membranes were developed with ECL™ Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and visualised by exposure to X-ray films (Hyperfilm ECL, Amersham Biosciences, Buckinghamsire, UK).

Rabbit monoclonal antibodies against phospho-c-Raf (Ser338) (56A6), phospho-MEK1/2 (Ser217/221) (41G9), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), phospho-p90RSK (Ser380) (9D9), phosphorylated AKT (Ser473) (D9E), phosphorylated AKT (Thr308) (C31E5), pan AKT (C67E7), GSK3B (27C10) and rabbit antibodies against phosphorylated c-Raf (Ser259) and phosphorylated GSK3B (Ser9) were obtained from Cell signalling Technology. Jurkat cells, treated with LY294002 or Calyculin A were used as a negative and postive control for AKT phosphorylation, respectively. Polyclonal antibody to phosphorylated Src (Y419) was obtained from R&D Systems (Minneapolis, MN USA). Monoclonal antibody to total Src (clone GD11) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Pro-caspase-3 (37 kDa) and beta-tubulin antibodies were from Cell Signalling Technology and Sigma Aldrich (St. Louise, MO, USA), respectively.

In vitro proliferation assays

Response of chondrosarcoma primary cultures to escalating doses of imatinib (range 1.0 – 100 µM) was measured by cell count using a Bürker chamber. GIST882 was used as positive control. Response of chondrosarcoma cell lines and primary cultures to escalating doses dasatinib (range 5.0 nM – 1.0 µM) was measured either by tritium incorporation assay (OUMS27, CH2879, SW1353, L784, L869) or WST-1 colorimetric assay (Roche Diagnostics GmbH, Penzberg, Germany) (L1081, L1250, L2252, L2388), which measures mitochondrial activity as described previously[21]. C3842, L821 and L835 did not reach an adequate proliferation rate to allow inhibition experiment. Results were compared to a poor responding (ALL CR) and a well responding acute lymphoblastic leukemia (ALL CM) cell line and as well as to GIST882, known to respond to dasatinib at ~10 nM[22]. In brief, 20,000 cells were seeded in a 96 wells plate and were allowed to incubate with the drugs for 72 hours. ^{3}H was added and the following day cells were harvested using the FilterMate system (Perkin Elmer, Waltham, MA, USA). ^{3}H-thymidine incorporation was measured using a TopCount scintillation counter (Perkin Elmer). All experiments were performed in duplicate and in the presence of a maximum of 0.1% DMSO. In vitro experiments were performed at least three times. Graphs show data from one representative experiment. Error

bars indicate the standard error of the mean.

Results

Identification of active kinases in chondrosarcoma cultures.

Through kinome profiling of 13 chondrosarcoma cultures we created a list of phosphorylated targets and their corresponding active kinases. The top 100 of chondrosarcoma targets was analysed using IPA, ranking the importance of the corresponding kinases in chondrosarcomas. The specificity of this list of substrates for chondrosarcoma was verified by comparing the intensity of the signals with those for normal MSCs, using Limma (Supplementary table 5.1). Based on the average spot intensity and how frequent their targets were present in the top 100 of substrates, 21 kinases were identified by IPA analysis (Table 5.2). The AKT pathway (AKT1 and GSK3B) was found to be the most active pathway in chondrosarcoma, followed by Titin and RPS6 kinase. Also the Src pathway (FYN and LCK) and the Ras/Raf/MEK/ERK pathway stimulated by PDGFRB were active in chondrosarcoma. In addition, Aurorakinase B and CDC2 were found.

Verification of kinome profiling

Kinome profiling of untreated GIST882 revealed an active Ras/Raf/MEK/ERK pathway, which is activated by KIT (Supplementary table 5.2). GIST882 carries an activating KIT mutation[18]. Subtracting the average intensities of untreated GIST882 from the 1.0 µM imatinib treated GIST882 revealed that indeed the Ras/Raf/MEK/ERK pathway was targeted by imatinib, which was confirmed by analysis using IPA (data not shown). Inhibition of the Ras/Raf/MEK/ERK pathway in GIST882 by imatinib was verified by immunoblot, using the same lysates as hybridised on the kinase array. Staining for cRaf, MEK1/2, ERK1/2 and 90RSK was decreased after 1.0µM imatinib treatment (Figure 5.1A), confirming that the Ras/Raf/MEK/ERK pathway was targeted by imatinib.

In addition, comparison of the substrate phosphorylation patterns of all substrates of 13 chondrosarcoma cell cultures and the two MSC cultures (Supplementary table 5.2) and the 5 colorectal carcinoma cell lines (Supplementary table 5.3) was performed, revealing 167 and 175 differently phosphorylated spots, respectively (adjusted $p<0.01$).

Phosphorylation of AKT in chondrosarcoma cell cultures could be verified by immunoblot at both the serine 473 and the threonine 308 position in all cultures (Figure 5.2). Whereas total GSK3B was present in nearly all samples, phosphorylation levels of GSK3B at serine 9 were indeed very low in the chondrosarcoma cell cultures confirming active GSK3B. cRaf, which links the AKT pathway with the MEK/ERK pathway, was also detected by immunoblot in all primary cultures.

	Intensity	Kinase	Nr hits	Description	Drugs
1	681.09	AKT1	9	V-akt murine thymoma viral oncogene homolog 1	Enzastaurin
2	587.329	GSK3B	4	Glycogen synthase kinase 3 beta	Enzastaurin
3	501.354	TTN	1	Titin	
4	446.35	RPS6KA5	5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	
5	410.859	FYN	1	FYN oncogene related to SRC, FGR, YES	Dasatinib
6	410.859	LCK	1	Lymphocyte-specific protein tyrosine kinase	Dasatinib
7	376.366	CDC2	4	Cell division cycle 2, G1 to S and G2 to M	Flavopiridol
8	360.768	AURKB	2	Aurora kinase B	AZD-1152
9	343.572	PAK2	1	P21 (CDKN1A)-activated kinase 2	
10	330.902	PIP5K3	2	Phosphatidylinositol-3-phosphate/ phosphatidylinositol 5-kinase, type III	
11	324.603	CSNK2A1	6	Casein kinase 2, alpha 1 polypeptide	
12	288.843	CAMK1D	4	Calcium/calmodulin-dependent protein kinase ID	
13	272.598	EPHB2	1	EPH receptor B2	
14	269.754	RHOA	1	Ras homolog gene family, member A	
15	268.982	MAP2K1	2	Mitogen-activated protein kinase kinase 1	PD 0325901
16	267.397	AKAP1	1	A kinase (PRKA) anchor protein 1	
17	262.279	PRKACA	1	Protein kinase, cAMP-dependent, catalytic, alpha	
18	244.71	CSNK1A1	1	Casein kinase 1, alpha 1	
19	241.637	CAMK2G	3	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	
20	239.691	PDGFRB	1	Platelet-derived growth factor receptor, beta polypeptide	Dasatinib, Sunitinib, Imatinib, Sorafenib, Becaplermin
21	202.938	SDK1	1	Sidekick homolog 1, cell adhesion molecule (chicken)	

Table 5.2 Results from the top 100 phosphorylated substrates (not shown), their corresponding kinases and targeted drugs in chondrosarcoma cultures. Nr hits: how many times the kinases were present in the top 100 spots. For each kinase only the highest average expression is shown here

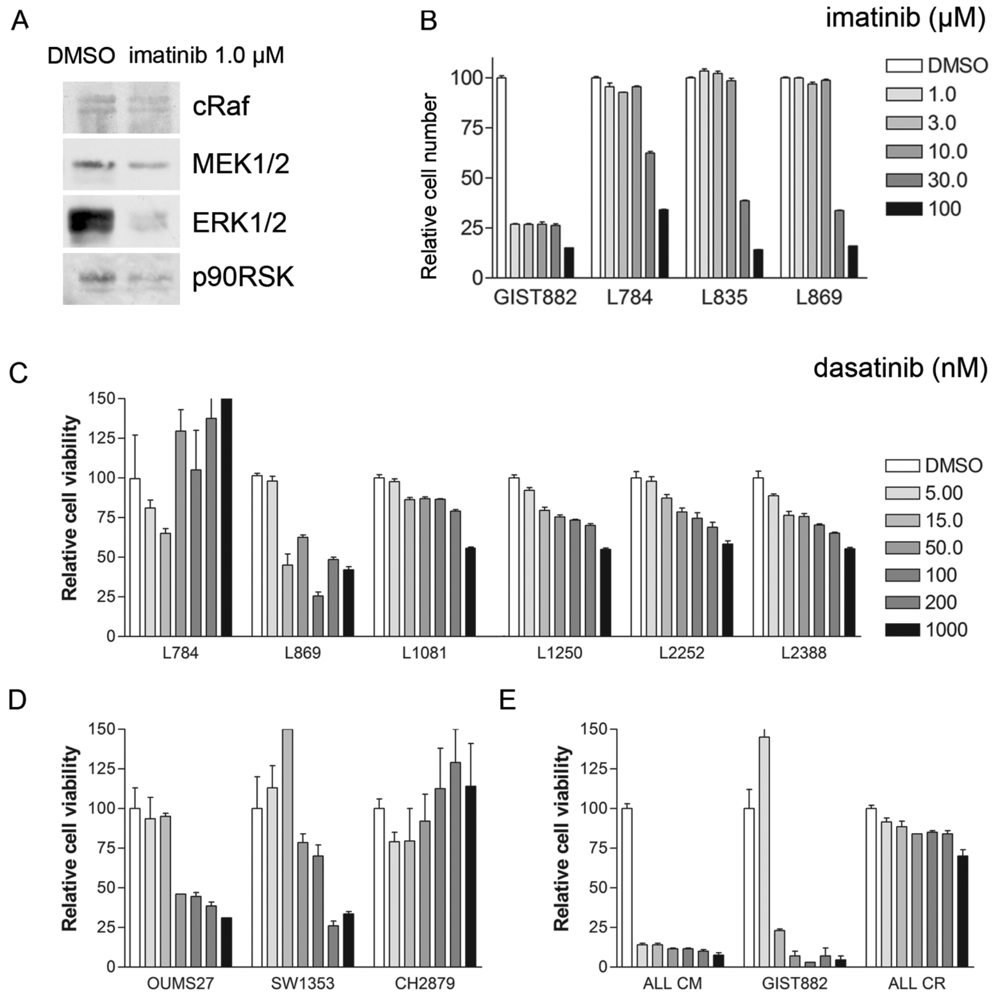

Figure 5.1: Chondrosarcoma cultures are susceptible to dasatinib but not to imatinib. (A) Treatment of GIST882 with 1.0 μM imatinib leads to a decrease in levels of cRaf, phosphorylated MEK 1/2, ERK 1/2 and p90RSK verifying interference with the Ras/Raf/MEK/ERK pathway by imatinib. (B) Imatinib treatment decreases cell numbers in the imatinib responsive GIST882 cell line. However four chondrosarcoma cultures did not respond to imatinib treatment, unless toxic concentrations of 30 and 100 μM were used. (C) Dasatinib causes decreased cell viability in 5 of 6 chondrosarcoma primary cell cultures. A decrease in cell viability of 20% at 15 nM and 40-50% at 1000 nM was observed in L1081, L1250, L2252 and L2388. Primary culture L869 responded with a 50% decrease in cell growth at 15 nM treatment. (D) Cell lines OUMS27 and SW1353 showed a decrease in viability at 50 nM dasatinib, whereas both primary culture L783 and cell line CH2879 showed a response only at low concentrations (5 and 15 nM), while at higher concentrations no effect was found. (E) In postitive controls ALL CM and GIST882 a decrease in cell viability of more than 80% was observed, while a limited effect was found in dasatinib resistant leukemia cell line CR.

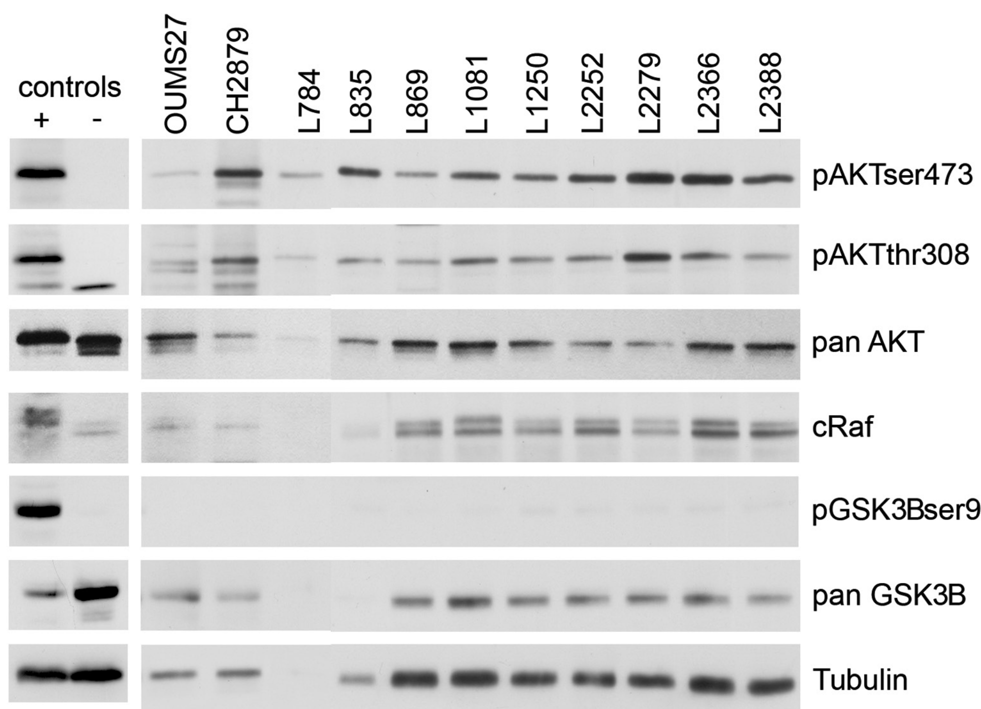

Figure 5.2 Chondrosarcoma cultures are characterized by AKT phosphorylation. Findings of kinome profiling by Pepchip® analysis were verified by immunoblot. All cultures showed phosphorylation of AKT at serine 473 and threonine 308, although levels of pan AKT were variable. While pan GSK3B was detected in nearly all samples, phosphorylation at serine 9 was absent confirming active GSK3B. Phosphorylated Raf was detected in nearly all samples. Jurkat treated with LY294002 (-) or Calyculin A (+) served as a negative and positive control of AKT phosphorylation, respectively.

Chondrosarcomas do not respond to imatinib treatment in vitro

In table 5.2 the currently used kinase inhibitors specific for the active kinases in chondrosarcoma are shown. Dasatinib and imatinib, targeting the Src kinase family and KIT/PDGFR pathway respectively, were available to us. Sensitivity of chondrosarcoma cells to both drugs was tested. Whereas GIST882 showed a profound decrease of cell number relative to the DMSO control at lower dosages of imatinib, the chondrosarcoma primary cultures did not show any effect, only at high concentrations of imatinib, probably due to non specific toxicity, rather than on target effects (Figure 5.1B).

Chondrosarcomas are responsive to dasatinib treatment in vitro

Seven out of 9 chondrosarcoma cell cultures responded to dasatinib treatment with a decrease in cell growth. Primary cultures L1081, L1250, L2252, L2388 showed 20% decreased cell viability at 15 nM and 40-50% decreased viability at 1000 nM of dasatinib treatment, as measured by

either ^{3}H incorporation or WST-1 assay (Figure 5.1C).The strongest effect was observed in primary culture L869, with a 50% reduction in cell viability compared to the DMSO control at 15 nM dasatinib (Figure 5.1C). Inhibition of cell growth of the chondrosarcoma cell lines SW1353, OUMS27 occurred at 50 nM (Figure 5.1D). In contrast, both primary culture L784 (Figure 5.1C) and cell line CH2879 (Figure 5.1D) did not show any effect. Positive controls ALL CM and GIST882 and negative control ALL CR are shown in figure 5.1E.

Dasatinib treatment of chondrosarcoma decreased Src-phosphorylation but does not result in caspase-3 mediated apoptosis

To investigate the effect of dasatinib on Src signalling, GIST882, a good responsive chondrosarcoma cell culture (L869) and a non-responsive chondrosarcoma cell line (CH2879) were treated with increasing doses of dasatinib for 6 hours. Whereas levels of total Src do not decrease upon dasatinib treatment, a decrease in phosphorylated Src (pSrc) (Y419) was found in GIST882 (Figure 5.3A). At a dosis of 6.25 nM dasatinib the pSrc staining has disappeared. Also in L869, the responsive cell culture, levels of phosphorylated Src decrease gradually, although both bands of the staining are present (Figure 5.3B). Strikingly, in CH2879 the level of phosphorylation of Src decreased upon dasatinib treatment as well (Figure 5.3C), while no effect on cell viability was found. Staining for pro-caspase 3 (37 kDa) showed a gradual decrease in GIST882 upon dasatinib treatment starting at 12.5 nM, suggesting pro-caspase-3 cleaving and active caspase-3 mediated apoptosis (Figure 5.3A)[23]. Caspase-3 staining seems to increase slightly at 50 nM and then decreases further until 200 nM allthough tubulin was slightly variable. Pro-apoptotic action of dasatinib has been described previously[22]. However, caspase-3 mediated apoptosis could not be demonstrated in both chondrosarcoma cell cultures (Figure 5.3B and 5.3C).

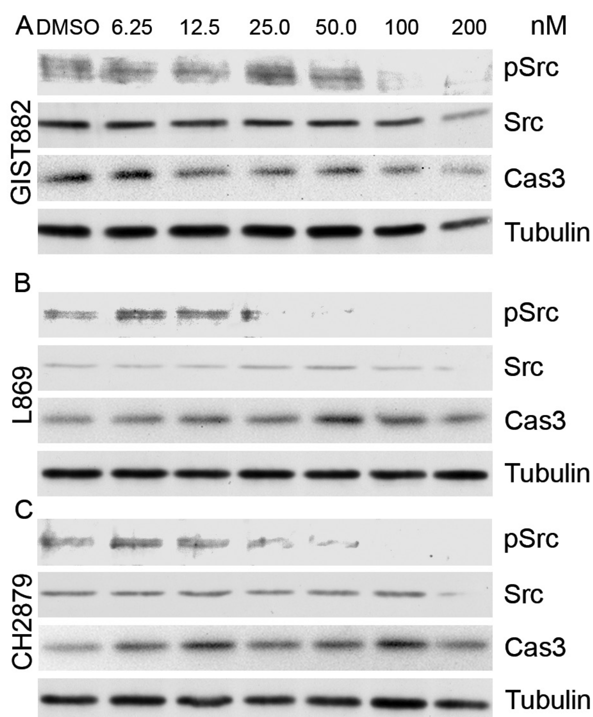

Figure 5.3 Decrease in cell viability by dasatinib seems to be unrelated to inhibition of phosphorylated Src and to caspase 3 mediated apoptosis in chondrosarcoma. (A) while levels of total Src do not decrease upon dasatinib treatment in GIST882, a decrease in phosphorylated Src (pSrc) (Y419) is shown upon dasatinib treatment. At a dosis of 6.25 nM dasatinib the upper band of the pSrc staining has disappeared (arrow). Staining for pro-caspase 3 shows a gradual decrease in GIST882 upon dasatinib treatment. (B) in L869, the dasatinib sensitive cell culture, levels of pSrc decrease gradually, although both bands of the staining are present. A decrease of pro-caspase 3 could not be demonstrated in L869.
(C) also in CH2879, the non-responsive cell culture, the level of phosphorylation of Src decreases upon dasatinib treatment. Again, no decrease in pro-caspase-3 could be observed.

Discussion

Chondrosarcomas are highly resistant to conventional chemo- and radiotherapy, and as a consequence there is no curative treatment option for patients with inoperable or metastatic disease. Kinome profiling was used to search for drugable kinases in chondrosarcoma.

A major caveat in large scale phosphorylation studies is the promiscuity of kinases in the absence of *in vivo* regulation signals, which may lead to false-positive results. Ideally, by comparing kinase profiles with and without a certain stimulus, for instance a kinase inhibitor, kinases being activated or deactivated upon the stimulus can be detected, as was previously reported[17]. However, since there are no compounds available that are known to have any effect in chondrosarcoma, we averaged 13 chondrosarcoma cultures to get an impression of the most active kinases and the pathways they are involved in.

Using GIST882 we demonstrated that the Pepchip® kinome profiling platform correctly identified the pathways that are known to play an important role in GIST and that their activity is decreased by imatinib treatment. Using mesenchymal stem cells and an independent set of colorectal carcinoma cell

lines we also demonstrated tumour specificity and tumour type specificity, respectively.
Analysing kinome profiles of 13 chondrosarcoma cell cultures identified several drugable targets. Validation of the results by immunoblot as well as the inhibition of chondrosarcoma cell viability *in vitro* by dasatinib demonstrate that this is an elegant approach to identify targets for treatment of tumours for which so far no systemic treatment options are available.
Analysing the top 100 activated substrates of the 13 chondrosarcoma cell cultures revealed 21 active kinases, of which AKT and GSK3B were shown to be the most active. The phosphorylation of AKT was confirmed by immunoblot. This pathway can be blocked by Enzastaurin, a PKCB-selective inhibitor, that has been shown to suppress angiogenesis and induces apoptosis in colorectal cancer and glioblastoma xenografts, by targeting AKT and GSK3B[24]. In contrast to AKT, GSK3 is constitutively active and becomes functionally inactivated after phosphorylation. GSK3 has a central function in physiological (i.e. transcription, apoptosis and cell cycle progression) and pathological (i.e. diabetes mellitus, Alzheimer and carcinogenesis) processes (reviewed in[25]). In chondrosarcoma cell cultures we demonstrated the absence of phosphorylation at serine 9, indicative for active GSK3B. An important role for the AKT kinase in chondrosarcoma survival was previously suggested by Jang et al.[13].

Kinome profiling of chondrosarcoma also revealed an active Src pathway. Src plays a role in the regulation of embryonic development and cell growth[26]. Mutations in Src are involved in the malignant progression of colorectal cancer[27]. We identified activity of Fyn and Lck in chondrosarcoma, which are, together with Yes, Fgr, Hck, Blk, Lyn and Frk, members of the Src family. The Src pathway can be targeted by dasatinib. Dasatinib is well-known for its efficacy in the treatment of chronic myelogenous leukemia and Philadelphia chromosome–positive ALL[28], in which dasatinib inhibits the Abl-kinases[29]. Recently, dasatinib has also been shown to be effective in the treatment of cells derived from solid tumours, i.e. prostate cancer[30] and head and neck squamous cell carcinoma[31]. In previous research, also expression of Abl kinase was shown in chondrosarcoma by immunohistochemistry with interestingly, a negative correlation with histological grade[32]. We show decreased cell viability after dasatinib treatment in the majority (7/9) of chondrosarcoma cell cultures, although a maximum of 60% of inhibition of cell growth was reached, while the effect in GIST882 and the leukemia cell lines were more profound. This difference may be explained by secondary events which stimulate cell growth in chondrosarcoma, ie. the loss of cell cycle inhibition, which was previously shown to occur in 96% of the tumours[10]. Likewise, GISTs have been shown to become refractory to initial successful response to imatinib due to loss of cell cycle control[33].

Strikingly, dose dependent inhibition of Src kinase phosphorylation by dasatinib, as measured by autophosphorylation at Y419, was found both in a responsive (L869) and in a non-responsive (CH2879) cell culture. This suggests that growth inhibition induced by dasatinib might be independent of Src kinase phosphorylation. Thus, dasatinib might exert its function via other pathways in chondrosarcoma, ie. by inhibition of Abl kinases, fibroblast growth factor receptor kinases or PDGF receptor kinases[34] or AKT[35]. Activity of the latter two is demonstrated in the present study.
While dasatinib was suggested to induce caspase-3 mediated apoptosis in the control GIST882 cell line, this was not observed in chondrosarcoma cell cultures. This suggests that dasatinib inhibits chondrosarcoma cell growth through other mechanisms, for example by inducing G1 arrest. However, immunoblotting for pSrc and caspase-3 was performed on one responsive and one non-responsive cell line only and extrapolating these results to all chondrosarcomas should be done with caution.
More experiments are needed to further explore the mechanism underlying growth inhibition and whether the effects of dasatinib on chondrosarcoma growth can be increased by combination with another cytostatic compound to reach higher growth inhibition rates.

Despite the finding of PDGFRB activity using the Pepchip® and the fact that PDGFRA protein expression [36] and activity of the alpha and beta receptor were reported previously in the absence of gain-of-function mutations[14], we were not able to decrease cell viability of chondrosarcoma cell cultures by imatinib treatment. In contrast Klenke *et al.* showed SU6668, which inhibits tyrosine kinases PDGFRB, Flk-1/KDR and FGFR1, to repress chondrosarcoma growth via antiangiogenesis *in vivo*[15]. One must take into account that we studied the effect of dasatinib and imatinib in chondrosarcoma *in vitro*, and that an additional effect *in vivo* through the inhibition of angiogenesis may be possible, as has been described for dasatinib[37].

Also Flavopiridol and AZD-1152, inhibitors of CDC2 and Aurora kinase activity respectively, were suggested for chondrosarcoma treatment, by our Pepchip® approach (Table 5.2). Flavopiridol is a pan cyclin-dependent kinase inhibitor, not only targeting CDC2, also known as cyclin dependent kinase 1, but also cyclin dependent kinase 2 and 4. Previously we reported the amplification of 12q13[9], the locus of CDK4, increased expression of CDK4 and a decrease in cell viability using shRNA to knock down CDK4 expression *in vitro*[10]. We suggested the use of CDK4 inhibitors in the treatment of chondrosarcoma, which is now being emphasised accordingly in the present study. Also RPS6kinase was found to be active in chondrosarcoma, which was found to predict the response to mTOR inhibitors in sarcoma[38]. RPS6 kinase is responsible for the phosphorylation of ribosomal protein S6, which we previously found to be deleted and downregulated in a subset of

chondrosarcomas[9]. This suggests that the tumour cells may try to overcome this deletion by phosphorylation. No array CGH data were available for the cell cultures described here.

In the present study we report the kinome profiling of 13 chondrosarcoma cell cultures and by averaging the profiles we identified activity of the Src pathway. Accordingly, Src inhibitor dasatinib decreased cell viability in seven out of nine chondrosarcoma cell cultures. Our experiments suggest that dasatinib is a potential treatment option in chondrosarcoma treatment. Future studies *in vivo* should be performed to confirm these data and to investigate the combination with conventional chemotherapy and possible additional effects through the inhibition of angiogenesis.

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Supplementary table 5.1 Top 100 phosphorylated substrates and their corresponding kinases in chondrosarcoma cultures as used for IPA analysis.

Average intensity in CS cultures	Substrate	Protein	Psite	Corresponding kinase	Fold Change MSC (log2)	Adj p-value MSC
1043.42	RKKKVSSTKRH	Cytohesin-1	S394	PKC	1.83	0.001
746.95	RKGYRSQRGHS	Vitronectin	S381	PKC	1.00	0.056
709.03	RARSTSLNERP	Tuberin	S939	AKT1	1.64	0.008
559.42	SPPRSSLRRSS	Transcription elongation factor A-like1	S37	PKC; GSK3	0.18	0.684
515.29	LRRSLSRSMSQ	Telethonin	S157	Titin	0.77	0.082
510.00	MQPDNSSDSDY	CD5	T434	PKA	-0.35	0.671
476.27	GGRGGSRARNL	Heterogeneous nuclear ribonucleoprotein K	S302	PKCdelta	1.03	0.028
455.97	LKPGSSHRKTK	Bruton's tyrosine kinase	S180	PKCbeta	1.55	0.001
444.65	RRRMASMQRTG	E1A binding protein p300	S1834	AKT; p70S6 kinase; pp90Rsk	0.53	0.195
440.26	HLRSESQRQRR	Guanine nucleotide binding protein, alpha Z polypeptide	S27	PKC	0.88	0.199
424.12	RPRNYSVGSRP	6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 2	S483	AKT	1.32	0.003
419.61	KKKIATRKPRF	Metabotropic glutamate receptor 1	T695	PKC	1.75	0.001
391.21	DNSSDSDYDLH	CD5	T453	Lck; Fyn	-2.09	0.001
377.39	LRQLRSPRRAQ	Ras associated protein Rab4	S204	CDC2	0.63	0.091
376.28	SSQRVSSYRRT	Desmin	S12	Aurora kinase B	0.56	0.255
369.05	ARIGGSRRERS	EP4 receptor	S354	PKC	0.29	0.543
367.99	EPKRRSARLSA	HMG14	S7	RPS6 kinase alpha 3; PKA; PKC	-0.01	0.996
349.08	SRKGYSRKGFD	Peptidylglycine alpha amidating monooxygenase	S930	PKC	0.21	0.678
347.92	RRRLSSLRAST	Ribosomal protein S6	S236	PAK2	0.02	0.985
346.84	RSNPPSRKGSG	Connexin 32	S233	PKC; PKA	0.54	0.190
346.69	AAKRASRIYNT	Regulator of G protein signalling 10	S176	PKA	0.50	0.170
338.52	LRRRLSDSSFI	Synapsin II	S10	PKA	0.33	0.543
337.91	RGRGSSVGGGS	ASK1	S83	AKT1	1.00	0.085
326.09	RTRRISQTSQV	Ryanodine receptor 2	S2808	PKA	2.40	0.000
324.60	RKRKPSTSDDS	DNA topoisomerase II aplha	S1469	CK2	1.92	0.001
319.79	RPRRRSSAVSL	Cyclic GMP inhibed phosphodiesterase B	S295	AKT1	1.79	0.001
315.24	TEDQYSLVEDD	Phosphatidylinositol 3 kinase, regulatory subunit, alpha	S608	Phospho-inositide-3-kinase (catalytic subunit gamma)	-1.03	0.092
307.96	APKAPSKKEKK	DAB2	S24	PKC	1.40	0.003
307.63	PPRRSSIRNAH	p47-phox	S304	PKCzeta; PKCalpha; PKCbeta-II; PKCdelta	-1.16	0.010
303.16	IKRQLSMTLRG	PCTAIRE protein kinase 1	S12	PKA	2.26	0.000
300.32	STPAPSRTASF	ATP citrate lyase	S450	GSK3	0.55	0.228
298.00	RERKSSAPSHS	GAB2	S159	AKT1	1.55	0.009
293.33	NRSFLSLKHTP	Notch2	*nd*		3.03	0.000
290.18	RKRRWSAPESR	Ataxin 1	S776	ATK1	2.46	0.000
288.71	SDSRKSMRQST	TCFL1	S132	PKC	-0.08	0.888
280.59	LRRRLSDSNFM	Synapsin I	S9	CaMKI; PKA	-0.15	0.737
278.56	LARRPSYRKIL	Activating transcription factor 1	S63	CaMKI; PKA; S6K	1.73	0.009
277.57	TRVPPSRRGPD	Alpha 2A adrenergic receptor	S232	PKC	1.89	0.000
277.25	APERASSVYTR	CCR5	S336	GRK	0.33	0.614

276.73	KQQPGSPRRIS	Retinoblastoma like 1	S975	*nd*	1.20	0.060
276.59	TKALQSPKRPR	TFII-I	S668	EphB2	1.72	0.003
276.04	AVRLRSSVPGV	Vimentin	S72	Rho kinase	1.17	0.029
272.05	PTPPLSPSRRS	c-Myc	T58	CK2; GSK	-0.01	0.996
270.99	VSRSTSFRGGM	Keratin 18	S52	CAMK; RPS6 kinase alpha 3; PKCepsilon	-0.21	0.678
269.23	KPKDASQRRRS	c-Src	S12	PKC	-0.80	0.082
267.23	GLRRSSKFALK	Beta-2-adrenergic receptor	S262	PKC	1.39	0.007
266.07	SKRRNSEFEIF	Tryptophan hydroxylase	S58	PRKA1	1.77	0.000
265.80	WRRKSSDRKGG	HLA-A	S337	Protein kinase (cAMP dependent, regulatory type I alpha)	1.13	0.008
264.80	LTRIPSAKKYK	PEA15	S104	PKC	0.75	0.062
264.72	GEKRASSPFRR	Nucleolar phosphoprotein p130	S623	PKA	0.33	0.367
263.62	LSRRPSYRKIL	cAMP response element-binding protein 1	S133	PKA; MAPK; CaMK; RSK2	-0.22	0.617
257.08	GPRRRSRKPEA	Centromeric protein a	S7	Aurora kinase B	1.27	0.009
251.37	RRRAASMDSSS	AFX 1	S196	AKT1	1.57	0.002
250.33	MRRQRSAPDLK	Kinesin family member 1C	S1092	CK2	0.76	0.126
249.76	KIRRLSAAKQQ	LKB1	S424	p90RSK; MSK1; S6K1; PKA; LKB1	0.69	0.083
246.15	RRKAATMRERR	Myogenic differentiation antigen 1	T115	PKC	0.59	0.216
244.00	PSRSYSERDFE	Solute carrier family anion exchange, member 3	S67	PKCepsilon	-0.82	0.209
243.41	SVRMLSGSKEK	Opioid receptor mu	S268	CAMKII	1.57	0.008
241.37	GQLRGSATRAL	Casein kinase 1, epsilon	S323	CK1epsilon	1.58	0.008
237.13	RQRSTSTPNVH	RAF1	S259	PKA; AKT1	-0.35	0.553
235.65	VAARATLRRSN	Calcium sensing receptor	T888	PKC	3.77	0.000
235.20	YRERMSSNRPN	Meprin,beta subunit	S687	PKC	1.97	0.003
235.05	KKGARSRRLFS	Guanine nucleotide binding protein, alpha 15 subunit	S336	PKCalpha	2.42	0.000
234.35	LLSELSRRRIR	eIF2 alpha	S52		1.64	0.001
229.53	GKNRPSSGSLI	Quinoid dihydropteridine reductase	S223	CAMKII	1.72	0.001
227.56	VSEDNSEDEIS	Acetyl-CoA carboxylase alpha	S29;S66	CK2	0.38	0.671
227.44	LARRPTKGIHE	Guanine nucleotide binding protein, alpha13	T203	PKA	1.22	0.059
226.89	RDRSSSAPNVH	B-Raf	S364	AKT; SGK	1.58	0.001
226.56	TWRRGSTAGGA	Calpain, large polypeptide L1	T80	*nd*	0.68	0.122
225.54	RKGHEYTNIKY	SHP2	Y542	PDGFRbeta	1.51	0.020
224.28	ASVPPSPSLSR	Glycogen synthase 1	S645	GSK3beta	1.18	0.008
223.11	LSGRGSNYGSL	NPRA	S538	*nd*	0.13	0.847
220.60	IVPGKSPTRKK	RAP1 GTPase activating protein 1	S484	CDC2	0.97	0.092
220.52	RKRKNSRVTFS	NIPP1	S199	PKA	1.62	0.001
220.49	RAREGSFESRY	Phospholipase C, gamma1	S1248	PKCmu	-0.46	0.446
219.07	RSRSRSPRPRG	ErbB3	S1123	Phospholipid kinase; Phospho-inositide kinase	-0.05	0.950
216.15	SRKRLSQDAYR	p47-phox	S320	PKCalpha; PKCbeta-II; PKCdelta	1.88	0.003
212.91	VPSYDSFDSED	C ets 1 protein	S285	CaMK2	-1.33	0.029
212.35	LLREASARDRQ	Vanilloid receptor 1	S801	PKCepsilon	1.42	0.003
212.11	ALVRGTPVRGA	Sam68	T317	CDC2	1.96	0.000

210.65	RLRPRTRKVKS	Neutrophil cytosolic factor 4 40kDa	T154	PKC	0.03	0.984
207.46	KFRTPSFLKKN	Adducin 3	S693	PKCalpha	-0.51	0.185
207.21	VLAQPSTSRKR	CHK2	S19	PKA; PKG	-0.47	0.281
206.28	LLRSGSSPNLN	E1A binding protein p300	S89	PKCdelta	1.11	0.009
204.18	APDEGSDLFYD	Cell division cycle 34	S203	CK2	-2.00	0.001
203.93	IAVRKSRDKAK	CCAAT/Enhancer binding protein, beta	T235	ERK2	1.59	0.001
202.48	GARRSSWRVIS	14-3-3-Eta	S59;S60	SDK1	-0.55	0.296
201.66	RPDPSSFSRPR	Opioid receptor	S344	PKC	0.02	0.985
200.16	EAKRKSPKKKE	Coilin	S184	CDK2-cyclin E	-0.09	0.911
199.29	RDRHLSFSGSG	CD44	S291	PKC	1.00	0.059
199.21	PSSRASSRASS	Connexin 43	S367	*nd*	1.81	0.001
197.56	QLRRPSDRELS	NFKB3	S263	PKA	0.33	0.451
196.98	NSRRPSRAMWL	Vitronectin	S397	PKA	0.97	0.013
196.61	KKKKPSRLKGD	APC	S2054	PKA	-0.57	0.138
196.07	VHRDLSRDRPL	Kell blood group protein	S63	PKC	2.02	0.000
193.15	ALRRESQGSLN	Regulator of G protein signalling 14	S260	PKA	2.14	0.001
192.06	HSLPLSPASTR	NFKB1	S907	GSK3beta	0.59	0.216
191.92	GFTRKSVRKAQ	Protein phosphatase 2, regulatory subunit B56, alpha	S28	PKR	-0.49	0.302
187.50	GLYSRSGSLSG	PKN	S374	PKN	-0.03	0.984
186.47	LQRSSSFKDFA	SH3 protein expressed in lyphocytes	S27	*nd*	0.00	0.996

Supplementary table 5.2 Limma analysis of substrate phosphorylation of 13 chondrosarcoma cell cultures versus 2 MSCs. Significant differences shown (p<0.01)

Fold change (log2)	adj.P.Val	Substrate	Protein	Corresponding kinase	Psite
-3.20	2.11E-06	IDRTESLNRSI	Lymphocyte specific protein	MAPkinase	S204
-3.42	2.82E-06	AGDLESPLSEE	PPAR	MAPK	S21
3.77	3.03E-06	VAARATLRRSN	Calcium sensing receptor	PKC	T888
-3.96	3.64E-06	FESIESYDSAD	C ets 1 protein	CaMK2	S257
-3.56	9.58E-06	SQRRESFLYRS	"cAMP-specific 3',5'-cyclic phosphodiesterase 4B"	PKA	S133
-2.48	2.87E-05	TLVIAYLMMRQ	Dual specificity phosphatase 3	ZAP70_	Y138
-2.61	3.01E-05	SptCtrl	Used for production/QC	*nd*	
2.46	3.22E-05	RKRRWSAPESR	Ataxin 1	ATK1	S776
2.93	3.46E-05	QKIHISKKWGF	Ribosomal protein L10	RPL10	S168
2.31	3.46E-05	YKFPSSPLRIP	Retinoblastoma 1	CDK4	S795
3.03	4.01E-05	NRSFLSLKHTP	Notch2	*nd*	
2.12	5.65E-05	EVPRRSGLSAG	Methyl CpG binding domain protein 3	Aurora_kinase_A	S24
2.40	8.03E-05	RTRRISQTSQV	Ryanodine receptor 2	PKA	S2808
2.03	8.84E-05	PAARASKKILL	Beta-adrenergic receptor kinase1	PKC	S29
-2.28	9.04E-05	ETNNDYETADG	CD32	Lyn;Blk;Fyn;SYK	Y279
-2.37	9.95E-05	LEVSDSESDEA	SPIB transcription factor	CK2	S146
2.31	9.95E-05	RARLLSDAANV	Crystallin beta A2	*nd*	S31
-2.57	9.95E-05	IDAFSDYANFK	"Protein tyrosine phosphatase, receptor type, alpha"	*nd*	Y798
-2.89	1.08E-04	NegCtrl	Mixed peptides without STY	*nd*	
2.35	1.08E-04	TKWYRSPRLLL	MAPK4	MAPK4	S196
-2.27	1.14E-04	RSRSGSIVELI	Huntingtin	AKT	S421
-1.91	2.00E-04	NADDSYEPPPV	B-cell linker protein	SLP-76	Y96
1.97	3.15E-04	SAIRQSPSPPP	"cAMP-specific 3',5'-cyclic phosphodiesterase 4A"	*nd*	S447
2.26	3.17E-04	IKRQLSMTLRG	PCTAIRE protein kinase 1	PKA	S12
2.34	3.66E-04	RTRTDSYSAGQ	mTOR	AKT	S2448
-2.75	4.00E-04	SLYASSPGGVY	Vimentin	CDC2	S56
-2.77	4.04E-04	TTGGESADELE	Occludin	CK2beta	S408
-2.31	4.27E-04	IESDIYAEIPD	Focal adhesion kinase 2	FAK2	Y402
-2.22	4.27E-04	PDEILYVNMDE	AXL receptor tyrosine kinase	auto	Y821
-2.00	4.30E-04	DDRHDSGLDSM	I-Kappa-B-alpha	*nd*	S32
1.77	5.34E-04	SKRRNSEFEIF	Tryptophan hydroxylase	PRKA1	S58
2.42	5.50E-04	KKGARSRRLFS	"Guanine nucleotide binding protein, alpha 15 subunit"	PKCalpha	S336
2.02	5.50E-04	VHRDLSRDRPL	Kell blood group protein	PKC	S63
2.26	5.74E-04	GSRRSSAIGIE	RAP1 GTPase activating protein 1	PKA	S499
-2.01	5.91E-04	PGADLSQYKMD	CtIP	ATM	S664
2.14	6.09E-04	IFRRPSLPAIS	Cyclic GMP inhibited phosphodiesterase B	PKA	S318
-1.80	6.09E-04	SSLDVYDGRFL	Acetylcholinesterase	*nd*	S164
1.96	6.09E-04	ALVRGTPVRGA	Sam68	CDC2	T317
-2.42	6.09E-04	AEGSAYEEVPT	"Phospholipase C, gamma1"	FGFR1	Y472
1.57	6.19E-04	KKRKRSRWNQD	Splicing factor 1	PKG-1	S20
-1.74	6.50E-04	DVLKFYDSNTV	PAK2	Src	Y130
1.85	6.50E-04	GERKKTLAGTP	Polo like kinase	*nd*	T210
-1.85	6.58E-04	PTAAGTPNKET	CALDESMON 1	CDC2_Kinase	S759
1.86	6.58E-04	IKRLRSQVQVS	Hepatocyte nuclear factor 4-alpha	AMPK	S304
-1.78	8.34E-04	VLDDEYVSSFG	Protein tyrosine kinase TXK	Fyn	Y420
-2.14	8.34E-04	KDEGSYTLEEP	Syndecan 3	Tyrosine_specfic_kinase	Y361

-1.75	1.08E-03	EEPSIYESVRV	DAPP1	Lck;PI3-kinase	Y139
1.83	1.08E-03	KVEPASPPYYS	"Peroxisome proliferator activated receptor, gamma"	ERK2_JNK1	S112
1.64	1.18E-03	PELARYLNRNY	Hrs	EGFR	Y329
1.78	1.33E-03	FSEITSPSKRS	ORC1	CDK2	S273
-2.07	1.33E-03	SptCtrl	Used for production/QC	*nd*	
1.89	1.33E-03	TRVPPSRRGPD	Alpha 2A adrenergic receptor	PKC	S232
-1.61	1.43E-03	KLFSSSVSEGF	Kell blood group protein	CK2	S383
-1.95	1.56E-03	NVVPLYDLLLE	"Estrogen receptor, alpha"	c-Src;Lck	SY537
1.92	1.60E-03	RKRKPSTSDDS	DNA topoisomerase II alpha	CK2	S1469
2.02	1.61E-03	RPPTLSPIPHI	Retinoblastoma 1	Cyclin_D1;Cdk4	S780
2.02	1.65E-03	GKRHRYRVLSS	Antisense ERCC1	PKA	Y80
1.43	1.65E-03	PosCtrl	Mixed kinase substrate peptides	*nd*	
-1.69	1.65E-03	DKQVEYLDLDL	GAB1	Insulin_receptor;EGFR;HGFR	Y627
-2.00	1.65E-03	APDEGSDLFYD	Cell division cycle 34	CK2	S203
1.81	1.70E-03	PSSRASSRASS	Connexin 43	*nd*	S367
1.61	1.92E-03	RGKKKSGALVL	RhoA	PKA	S188
1.75	1.94E-03	KKKIATRKPRF	Metabotropic glutamate receptor 1	PKC	T695
-1.42	1.94E-03	EPLPPSYVAAS	Erythropoietin receptor	JAK2	Y504
-1.92	1.94E-03	ILDREYYSVQQ	Ron	Ron	Y1238
2.37	2.03E-03	SVSVETQGDDW	Hematopoietic cell specific LYN substrate 1	CK2alpha_1	T16
1.87	2.03E-03	QLGPPSPVKMP	Pituitary tumour-transforming protein 1	CDC2	S165
-1.51	2.03E-03	RIRTQSFSLQE	Nitric oxide synthase 3	AKT1	S1176
1.62	2.03E-03	RKRKNSRVTFS	NIPP1	PKA	S199
1.78	2.03E-03	GGARASPATQP	Sam68	ERK	S58
1.41	2.06E-03	QSKRSTMVGTP	PAK3	"Pyruvate_dehydrogenase_kinase,_ isoenzyme_1"	T423
-1.55	2.14E-03	FMAKVYSDPQP	FGF receptor 1	FGFR1	Y280
1.79	2.25E-03	RPRRRSSAVSL	Cyclic GMP inhibited phosphodiesterase B	AKT1	S295
1.68	2.31E-03	PGLRRSPIKKV	B-Myb	CDK2	S577
-1.52	2.52E-03	VDPMLTPEERH	Amyloid precursor like protein 2	CDC2-kinase	T736
-1.73	2.52E-03	TDDEMTGYVAT	MAPK14	MAP2K3	T180
-2.35	2.52E-03	NegCtrl	Mixed peptides without STY	*nd*	
-1.90	2.52E-03	ELDQGSLATSF	IKK beta	NIK;MAP3K7;MEKK1;_IKK_alpha	S177
2.14	2.52E-03	ALRRESQGSLN	Regulator of G protein signalling 14	PKA	S260
1.88	2.52E-03	RRRFSSLHFMV	Caspase 9	ERK1;ERK2;MEK1;MAP2K2	T125
1.56	2.52E-03	FSEHRTQVSLK	Cell cycle checkpoint kinase II	CHK2	T432
1.58	2.53E-03	RDRSSSAPNVH	B-Raf	AKT;SGK	S364
-2.55	2.57E-03	NDDDVYRSLEE	VAV2	EGFR	Y142
-1.47	2.59E-03	ASGYISSLEYP	"Complement component 1, subcomponent r"	CK2	S206
-1.91	2.66E-03	LKRSLSEMEIG	Serum response factor	Potassium_voltage_ gated_channel_ subfamily_A_member_2;	S103
1.42	2.66E-03	APLLSTPKRER	Kinesin like 4	Cyclin_B2	T463
1.64	2.66E-03	LLSELSRRRIR	eIF2 alpha	PKR;GCN2;Eukaryotic_translation_ initiation_factor_	S52
-1.84	2.87E-03	DESVDYVPMLD	"PDGF receptor, beta"	SHP2	Y751
1.59	2.93E-03	IAVRKSRDKAK	"CCAAT/Enhancer binding protein, beta"	ERK2	T235
-1.77	3.19E-03	AFEEGSQSTTI	ATM	Auto	S1981
-2.01	3.25E-03	KEVSKYSDIQR	"PDGF receptor, alpha"	PDGFRalpha	Y754
1.78	3.25E-03	YETFKSIMKKS	NFKB3	PKCzeta	S298
2.26	3.29E-03	VPKRKSLVGTP	p21 activated protein kinase 6	Nd	S560
1.49	3.29E-03	KNKPRSPVVEL	Beta-adrenergic receptor kinase1	MAPkinase	S670
1.57	3.29E-03	SHLRNSPEDKR	Eukaryotic translation initiation factor 4E binding protein 1	Dual-specificity_ tyrosine_phosphorylation-regulated_kinase_2	S101
-1.63	3.29E-03	VLDDQYVSSVG	Bone marrow kinase BMX	c-Src	Y566

-1.92	3.36E-03	GQESEYGNITY	SHP1	c-Abl;Lck	Y536
1.55	3.39E-03	LKPGSSHRKTK	Bruton's tyrosine kinase	PKCbeta	S180
1.82	3.50E-03	MRRSVSEAALA	Lipase hormone sensitive	nd	S554
1.83	3.50E-03	RKKKVSSTKRH	Cytohesin-1	PKC	S394
-1.78	3.52E-03	PGIDLSQVYEL	ABL	AKT	S465 466
-1.60	3.52E-03	SptCtrl	Used for production/QC	*nd*	
1.54	3.52E-03	PosCtrl	Mixed kinase substrate peptides	*nd*	
-1.49	3.52E-03	ISEGTTLKDLI	"TGF beta receptor, type I"	"TGF_beta_receptor,_type_II"	T176
1.26	3.77E-03	PosCtrl	Mixed kinase substrate peptides	*nd*	
1.97	3.85E-03	LTTGVYVKMPP	CD152	"JAK2,_Protein_tyrosine_kinase_TXK"	Y201
-2.09	3.92E-03	DNSSDSDYDLH	CD5	Lck;Fyn	T453
1.39	4.03E-03	AKIQASFRGHM	Neurogranin	PKCalpha	S36
1.39	4.11E-03	GQVIMSIRTKL	Ribosomal protein L10	Ribosomal_protein_L10	S137
-1.76	4.11E-03	PEPYASPPQPG	Steroidogenic factor 1	ERK2	S203
-1.50	4.17E-03	RLLDSSQIVII	E2F transcription factor 1	ATM;ATR	S31
-1.85	4.17E-03	KEDPIYDEPEG	Docking protein 1	Insulin_receptor	Y362
-1.72	4.17E-03	FEGFSYVNPQF	Protein kinase C alpha	SYK	Y658
1.45	4.17E-03	IRSSMSGLHLV	Acetyl-CoA carboxylase alpha	AMPK	S80
1.72	4.19E-03	GKNRPSSGSLI	Quinoid dihydropteridine reductase	CAMKII	S223
1.90	4.25E-03	RPRSATWPLPR	FKHR	AKT1	T24
-2.04	4.28E-03	FDDPSYVNVQN	Shc	TRK-T3;Insulin_receptor;MAP_kinase;Fyn;Lck	Y427
-1.36	4.42E-03	KDDKLTPKIGF	Ezrin	CDK5	T234
1.47	4.42E-03	PGTRLSLARMP	Glial fibrillary acidic protein	CaMKII;PKC;Rho_kinase	S38
-1.77	4.56E-03	TTVELYSLAER	"Protein kinase C, theta"	Lck	Y90
1.51	4.59E-03	LDSAQSPGPSW	CDX2	MAPkinase	S60
-1.63	4.59E-03	FDAHIYEGRVI	Protein kinase C delta	PDGF;Lyn_kinase	Y64
-1.68	4.68E-03	EPVQLTPDDED	"Protein kinase C, zeta"	PKCzeta	T560
-1.37	4.68E-03	ISRQHSYDNIV	"Glutamate receptor ionotropic, N-methyl D-aspartate subunit 2A"	CaMKII	S1291
1.84	4.68E-03	FRRQLSEPANS	ETS variant gene 1	Ribosomal_protein_S6_kinase_alpha_5;	S191
-2.44	4.83E-03	IRESESTAGSF	Lck	PKC	S158
1.62	5.02E-03	NSIAKTYVGTN	MAP2K5	*nd*	T315
-2.00	5.02E-03	IGADSSEEKFL	Statherin	*nd*	S22
1.57	5.42E-03	RRRAASMDSSS	AFX 1	AKT1	S196
1.42	5.50E-03	ALDFRTPRNAK	Opioid receptor mu 1	Beta-adrenergic_receptor_kinase_2	T182
-1.25	5.56E-03	AERPLTQEELL	TCFL1	CAMKII	T168
1.32	5.56E-03	RPRNYSVGSRP	"6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2"	AKT	S483
1.37	5.56E-03	KKKKGSLDSDN	"Solute carrier family 4, sodium bicarbonate cotransporter, member 4"	PKA	S982
-1.44	5.70E-03	TAEAPSVLELG	Crystallin beta A4	*nd*	S35
1.30	5.86E-03	YQQRNSPGVPT	Golgin 95	CDC2;PP2A	S25
-1.25	6.09E-03	FKTEGPDSD	p53	CK2alpha_1;p38_MAP_kinase;PKR;CDK2	S392
-1.41	6.16E-03	FMMTPYVVTRY	JNK1	MAP2K4	Y223
1.40	6.16E-03	APKAPSKKEKK	DAB2	PKC	S24
1.62	6.45E-03	SQKYMSFTDKS	PAK1	PAK1	S144
-1.24	6.61E-03	NegCtrl	Mixed peptides without STY	*nd*	
1.52	6.76E-03	QVKALYDFLPR	Protein tyrosine kinase TXK	Protein_tyrosine_kinase_TXK	Y91
-1.35	6.76E-03	FPDQAYANSQP	SIT	*nd*	Y188
-1.62	6.76E-03	LMDNAYFAEAD	Growth hormone receptor	*nd*	Y534
1.17	6.76E-03	EVERTYLKTKS	"Glutamate receptor ionotropic, N-methyl D-aspartate subunit 2A"	Src	Y1105
1.92	6.76E-03	MRKKISNAQLQ	Cullin 5	PKA	S730

1.74	6.76E-03	KDFSSSKRMNT	Syntaxin binding protein 1	PKC	S313
1.36	6.76E-03	IHMVYSKRSGK	Small nuclear ribonucleoprotein 70 kD	*nd*	S137
1.97	6.76E-03	YRERMSSNRPN	"Meprin,beta subunit"	PKC	S687
-1.36	6.76E-03	IIGEKSFRRSV	"Protein kinase C, mu"	PKCmu	S738
-2.36	7.28E-03	AEEEFSDSEEE	Histone deacetylase 1	CK2	S421
1.42	7.42E-03	LLREASARDRQ	Vanilloid receptor 1	PKCepsilon	S801
1.34	7.50E-03	RTAPYTPNLPH	SMAD4	ERK2	T277
1.41	7.64E-03	GTDLEYLKKVR	O-linked N-acetylglucosamine transferase	Tyrosine_kinase	T989
-1.37	7.64E-03	HKGHLSEGLVT	MAPK6	MAPK6	Y189
1.88	7.64E-03	SRKRLSQDAYR	p47-phox	PKCalpha;PKCbetaII;PKCdelta	S320
1.53	7.64E-03	KSEPISPPRDR	Myocyte specific enhancer factor 2A	CDK5	S408
-1.47	7.93E-03	ENTLPTPMVLQ	Mnk2	*nd*	T379
1.38	8.24E-03	SVPTPSPLGPL	"CK2, alpha 1"	CK2alpha_1	Y182
1.31	8.24E-03	PTRKISASEFD	Phosphodiesterase 5A	cGMP_dependent_protein_kinase	S102
1.25	8.43E-03	PQPPKSPGPHS	RAD9	*nd*	S336
-1.29	8.63E-03	VTKLLTDVQLM	Heat-shock transcription factor 1	CK2	T142
1.72	8.80E-03	TKALQSPKRPR	TFII-I	EphB2	S668
-2.10	8.87E-03	PIGEDEESESD	Vesicular monoamine transporter 2	CK1;CK2	S511
1.43	9.18E-03	TKRNSSPPPSP	ATP2B1	PKA	S1178
-1.33	9.43E-03	SptCtrl	Used for production/QC	*nd*	
-1.37	9.45E-03	TATEGQYQQQP	Lyn	Csk_homologous_kinase	Y508
-1.09	9.65E-03	GSAAPYLKTKF	STAT3	JAK1;c-Src	Y705

Limma comparison of substrate phosphorylation of chondrosarcoma cultures with mesenchymal stem cell cultures was performed, quantifying (fold change) the differential expression (log scores). Note that positive log score means higher substrate phosphorylation in chondrosarcoma than in MSC. Psite: phosphorylation site on the spotted substrate. *Nd.* Not determined.

Supplementary table 5.3. Limma analysis of substrate phosphorylation of 13 chondrosarcoma cell cultures versus 5 colorectal carcinoma cell cultures. Significant differences shown (p<0.01)

Fold change (log2)	adj.P.Val	Substrate	Protein	Corresponding kinase	Psite
-2.91	5.04E-09	SQRRESFLYRS	"cAMP-specific 3',5'-cyclic phosphodiesterase 4B"	PKA	S133
-2.68	8.45E-09	IDAFSDYANFK	"Protein tyrosine phosphatase, receptor type, alpha"	*nd*	Y798
-2.26	1.00E-08	MEDYDYVHLQG	p130CAS	FAK	Y666
-3.12	2.81E-08	IESDIYAEIPD	Focal adhesion kinase 2	FAK2	Y402
2.70	2.81E-08	LKPGSSHRKTK	Bruton's tyrosine kinase	PKCbeta	S180
-2.46	4.96E-08	SILPFTPPVVK	SMAD2	ERK	T8
-3.34	4.96E-08	LGQRIYQYIQS	DYRK1B	DYRK1B	Y271
2.10	8.04E-08	SKRRNSEFEIF	Tryptophan hydroxylase	PRKA1	S58
-2.35	1.24E-07	EDENLYEGLNL	CD79A	*nd*	Y188
1.95	1.97E-07	KKRKRSRWNQD	Splicing factor 1	PKG-1	S20
-2.34	2.20E-07	LEVSDSESDEA	SPIB transcription factor	CK2	S146
2.17	9.93E-07	GSKNKSPSKAA	High mobility group AT-hook 2	Cdc2_kinase	S59
-1.98	1.24E-06	TAPEYYAPEVH	Titin	*nd*	Y24917
1.79	1.24E-06	PSEVPTPKRPR	High mobility group AT-hook 1	CDC2_kinase	T52
2.99	1.24E-06	RKKKVSSTKRH	Cytohesin-1	PKC	S394
2.77	1.36E-06	RKRKPSTSDDS	DNA topoisomerase II alpha	CK2	S1469
2.51	1.38E-06	RERKSSAPSHS	GAB2	AKT1	S159
1.69	1.56E-06	LTRIPSAKKYK	PEA15	PKC	S104
1.95	1.76E-06	NSIAKTYVGTN	MAP2K5	*nd*	T315
-2.27	1.76E-06	LALSLTADQMV	"Estrogen receptor, alpha"	MAPK14	T311
-2.39	1.87E-06	IKRQLSMTLRG	PCTAIRE protein kinase 1	PKA	S12
2.31	2.27E-06	EAKRKSPKKKE	Coilin	CDK2-cyclin_E	S184
1.87	2.28E-06	APKAPSKKEKK	DAB2	PKC	S24
-1.59	4.00E-06	SSRRQSVLVKS	"Glutamate receptor, ionotropic kainate 2"	PKA	S715
-1.60	4.72E-06	ERGQEYLILEK	Tec tyrosine kinase	Tec_tyrosine_kinase	Y206
1.95	4.74E-06	RSRSRSPRPRG	ErbB3	Phospholipid_kinase_ Phosphoinositide_kinase	S1123
1.73	6.10E-06	NNTSSSPQPKK	p53	CDK2	S315
-1.71	1.35E-05	RGQEVYVKKTM	TEK receptor tyrosine kinase	*nd*	Y992
2.22	1.78E-05	ARIGGSRRERS	EP4 receptor	PKC	S354
1.88	1.79E-05	SRKGYSRKGFD	Peptidylglycine alpha amidating monooxygenase	PKC	S930
1.85	2.45E-05	QHRSSSSAPHH	Calcium channel voltage dependent beta 2 subunit	DNA-dependent_protein_ kinase_catalytic_subunit	S478
1.62	2.45E-05	KKPKKKSALLL	Ras related protein 1A	PKA	S180
1.59	2.76E-05	YQRRASDDGKL	RAF1	PKA	S43
-1.80	2.92E-05	PVAPLSPARLQ	ELK3	MAPK14;EphB2	S363
1.51	3.57E-05	RARLLSDAANV	Crystallin beta A2	*nd*	S31
-2.29	3.75E-05	PosCtrl	Mixed kinase substrate peptides	*nd*	
1.75	4.14E-05	KAARKSAPSTG	"H3 histone, family 3A"	*nd*	S10
1.70	4.19E-05	KFSKFSLYKQL	PLD1	PKCalpha	S561
1.36	4.19E-05	RKKRISVKKKQ	Cytohesin 2	PKC	S392
-1.76	5.12E-05	QERRGSNVALM	Protein tyrosine phosphatase nonreceptor 7	PKA	S83
1.67	5.12E-05	ESRSGSNRRER	Wiskott-Aldrich syndrome protein interacting protein	PKCtheta	S488
2.15	5.12E-05	LQRSSSFKDFA	SH3 protein expressed in lymphocytes	*nd*	S27
1.67	5.12E-05	PSRKGSGFGHR	Connexin 32	PKC	S229
-1.40	5.75E-05	TAEPDYGALYE	"Phospholipase C, gamma1"	Lyn	Y771
1.55	6.23E-05	RRRMASMQRTG	E1A binding protein p300	AKT;p70S6_ kinase;pp90Rsk	S1834
1.84	6.59E-05	RRRLSSLRAST	Ribosomal protein S6	PAK2	S236

-2.04	7.19E-05	VGKIFSNVRIN	Caveolin 1	CK2	S88
1.75	9.28E-05	RKRHNSISEAK	“Phospholipase C, beta 3”	PKG	S1105
1.56	1.01E-04	SYSHHSGLEYA	Caveolin 2	c-Src	Y19
-1.41	1.02E-04	FEGFSYVNPQF	Protein kinase C alpha	SYK	Y658
1.70	1.04E-04	RGRLRSADSEN	MEKK3	PKA	S337
-2.04	1.24E-04	PSEEGYQDYEP	Synuclein alpha	SYK	Y136
1.36	1.31E-04	GTDLEYLKKVR	O-linked N-acetylglucosamine transferase	Tyrosine_kinase	T989
1.47	1.51E-04	AMRNFSRSDHL	Early growth response protein 1	CK2	S301
-1.54	1.90E-04	IDPFTYEDPNE	EphB3	EphB3	Y614
1.68	2.01E-04	RLRTHSIESSG	MAP2K4	AKT1	S80
-1.30	2.15E-04	ILDREYYSVQQ	Ron	Ron	Y1238
-1.29	2.16E-04	ASGLSSQSDIL	BRCA1	ATM	S1387
1.25	2.30E-04	SRRPKSSLPPV	Myosin light chain kinase	PAK2	S1208
1.71	2.45E-04	PPRRSSIRNAH	p47-phox	PKCzeta;PKCalpha;PKCbetaII;PKCdelta	S304
-1.36	2.58E-04	PLVQRGSANGL	Beta-adrenergic receptor kinase1	PKA	S685
-1.38	2.58E-04	NegCtrl	Mixed peptides without STY	*nd*	
1.20	2.69E-04	SptCtrl	Used for production/QC	*nd*	
1.18	3.65E-04	KGGKYSVKDKE	L1 cell adhesion molecule	p90rsk	S1152
1.65	4.25E-04	RDRSSSAPNVH	B-Raf	AKT;SGK	S364
1.30	4.66E-04	KRRQTSMTDFY	Cyclin-dependent kinase inhibitor 1A	AKT1	S146
1.45	4.66E-04	MAKRNTVIGTP	Serine/Threonine protein kinase4	Serine/threonine_protein_kinase_3	T180
1.37	4.71E-04	QQREKTRWLNS	Adducin 1	Rho-kinase	T445
-3.41	5.25E-04	MNEVTYSTLNF	Carcinoembryonic antigen-related cell adhesion molecule 1	MAP3K10	S508
-1.19	6.51E-04	STEPQYQPGEN	c-Src	Csk;Fgr_PTPase	Y530
1.33	6.51E-04	RDKYKTLRQIR	Moesin	Rho-Kinase	T564
-1.26	6.51E-04	TSLAQYDSNSK	Bone marrow kinase BMX	BTK;ITK;Tec	Y216
-1.35	6.51E-04	TEENIYQVPTS	DAB1	c-Src	Y220
1.36	6.51E-04	TDGNRSSHSRL	BH3 interacting domain death agonist	CK1;CK2	S65
-1.15	7.57E-04	LSNPAYRLLLA	Discoidin domain receptor	Discoidin_domain_receptor	Y513
-1.37	7.57E-04	RTAASSLALVS	Uridine nucleotide receptor	*nd*	S334
-1.43	7.58E-04	DDEDAYGNYDN	3-Phosphoinositide dependent protein kinase 1	c-Src	Y373
1.22	8.18E-04	GERKKTLAGTP	Polo like kinase	*nd*	T210
1.25	8.77E-04	VRRRQSVELHS	Aquaporin 2	PKA	S256
1.32	9.04E-04	GVERSSPSKAP	BRCA1	CDK2	S1497
1.54	9.04E-04	NQNSSSDSEAE	T-cell transcription factor 4	CK2	S60
1.39	9.04E-04	ALVRGTPVRGA	Sam68	CDC2	T317
-1.18	9.04E-04	GFSRKSHTFLP	CNPase	PKC	S9
1.25	9.04E-04	IVPGKSPTRKK	RAP1 GTPase activating protein 1	CDC2	S484
-1.95	9.68E-04	NDDDVYRSLEE	VAV2	EGFR	Y142
0.92	9.92E-04	PNSSKTYGIKW	“Calpain, large polypeptide L1”	*nd*	S360
1.84	1.05E-03	VLAQPSTSRKR	CHK2	PKA;PKG	S19
1.17	1.28E-03	RRRAVSMDNSN	Forkhead box protein O3A	AKT1	S253
-1.34	1.29E-03	RESSVYDISEH	NR2B	CaMKII	S383
1.11	1.46E-03	TTTAPSLSGKG	Beta-catenin	CK1	S45
-1.13	1.46E-03	SNDKVYENVTG	FAK	GRB2	Y925
-1.16	1.46E-03	IKDDEYNPAQG	Fgr	Fgr	Y412
1.08	1.46E-03	SptCtrl	Used for production/QC	*nd*	
-1.25	1.46E-03	PGMKIYIDPFT	EphB1	EphB1	Y594
-0.92	1.58E-03	NGRPDYIIVTQ	Aryl hydrocarbon receptor	auto	Y378
2.99	1.59E-03	LARRPTKGIHE	“Guanine nucleotide binding protein, alpha13”	PKA	T203
-0.97	1.59E-03	GPRLVSNHSLH	Filamin A	CAMKII	S2523

2.28	1.61E-03	EDEESYDTESE	I-Kappa-B-alpha	CK2	S288
-1.11	1.84E-03	AEKPFYVNVEF	BCR	BCR	Y177
1.50	1.84E-03	RKGYRSQRGHS	Vitronectin	PKC	S381
-0.98	1.84E-03	FMMTPYVVTRY	JNK1	MAP2K4	Y223
-0.94	1.84E-03	LSRRPSYRKIL	cAMP response element-binding protein 1	PKA;MAPK;CaMK;RSK2	S133
-0.90	1.84E-03	NSRRPSRAMWL	Vitronectin	PKA	S397
1.13	1.84E-03	TKALQSPKRPR	TFII-I	EphB2	S668
-1.04	2.02E-03	MPDNLYTFVLK	LNK	ZAP70	Y273
1.36	2.05E-03	KPKDASQRRRS	c-Src	PKC	S12
-1.29	2.05E-03	AKKEESEESDD	Ribosomal phosphoprotein large P1	*nd*	S101
1.14	2.13E-03	EKPRLSFADRA	"Protein kinase C, theta"	*nd*	S676
-1.17	2.16E-03	LTIDRYLAIVH	CCR2	JAK2	Y139
-1.31	2.31E-03	AGDLESPLSEE	PPAR	MAPK	S21
-1.00	2.43E-03	QQLQLSPLKGL	Ribonucleotide reductase M2 subunit	CDC2	S20
1.05	2.47E-03	EDTTSTFAGTP	Serum/glucocorticoid regulated kinase 2	3-Phosphoinositide_dependent_protein_kinase_1	T253
-0.85	2.63E-03	ESRASTFAGTP	Protein kinase C delta	Lck	T507
-1.33	2.65E-03	RKGHEYTNIKY	SHP2	PDGFRbeta	Y542
-1.01	2.82E-03	SASPYTPEHAA	p73	Cyclin_A;Cyclin_B;CDC2;CDK2;Cyclin_E	T86
-0.97	2.86E-03	AEPHTYEEPGR	EphA8	EphA8	Y616
0.79	2.93E-03	LHRDKTPLHQK	B-Myb	CDK2	T494
1.40	3.02E-03	GSRRGSFDATG	Desmoplakin	PKA	S2849
-1.08	3.20E-03	GTLRTSISVER	Bradykinin receptor 2	PKC	S373
-1.21	3.49E-03	EDSTYYKASKG	FAK	c-Src	Y577
1.04	3.49E-03	DDRHDSGLDSM	I-Kappa-B-alpha	*nd*	S32
-1.19	3.63E-03	FFPFHSPSRLF	"Crystallin, alpha B"	*nd*	S19
-1.15	3.66E-03	VYESPYSDPEE	ZAP70	ZAP70	Y319
-0.87	3.66E-03	TDEDIYLLGKA	Sialyltransferase 1	*nd*	Y391
0.93	3.66E-03	PELARYLNRNY	Hrs	EGFR	Y329
1.07	3.66E-03	KSEPISPPRDR	Myocyte specific enhancer factor 2A	CDK5	S408
-1.20	3.66E-03	GWMVHYTSKDT	"Protein kinase C, mu"	c-Src;ABL	Y432
-0.84	3.71E-03	LARRPSYRKIL	Activating transcription factor 1	CaMKI;PKA;S6K	S63
1.30	3.76E-03	GPRRRSRKPEA	Centromeric protein a	Aurora_kinase_B	S7
-1.03	3.76E-03	KVVALYDYMPM	Bruton's tyrosine kinase	BTK;ABL	Y223
-1.24	3.80E-03	LGSQSYEDMRG	CD19	Lyn_kinase;Bruton's_tyrosine_kinase	Y531
-1.15	3.96E-03	EGKHLYTLDGG	RACK1	Src	Y228
1.06	4.35E-03	WRRKSSDRKGG	HLA-A	"Protein_kinase,_cAMP_dependent,_regulatory,_type_I,_alpha"	S337
-1.03	4.39E-03	LEDNDYGRAVD	AKT1	c-Src	Y326
-0.94	4.39E-03	DVHMVSDSDGD	FAS associated factor 1	CK2beta	S289
-0.79	4.69E-03	HVEDLYVEGLP	TFII-I	Bruton's_tyrosine_kinase	Y398
-0.92	4.80E-03	GLQMGSNRGAS	Transgelin	PKC	S181
-0.76	4.80E-03	YTRVQSMALPP	Forkhead box protein L2	PAK1	S263
1.00	4.80E-03	EEGEMYEDDEE	"Tubulin, beta-4"	MAPkinase	Y437
1.44	5.01E-03	IRESESTAGSF	Lck	PKC	S158
-0.94	5.18E-03	DVLKFYDSNTV	PAK2	Src	Y130
-1.26	5.20E-03	EELAEYAEIRV	SIGLEC4A	Fyn	Y620
0.83	5.42E-03	SptCtrl	Used for production/QC	na	
-1.66	5.47E-03	TTEAIYEEIDA	TOM1 like 1	Fyn	Y460
1.11	5.53E-03	PDVPRTPVGKF	CDC25C	Polo_like_kinase	S198
-1.34	5.60E-03	KTRDQYLMWLT	"Phosphatidylinositol 3 kinase, regulatory subunit, alpha"	Insulin_receptor	Y580
-1.67	5.70E-03	MGQAGSTISNS	Connexin 43	CK1delta	S325

-0.86	5.80E-03	EADGVYAASGG	FES tyrosine kinase	FES_tyrosine_kinase	Y713
-1.13	5.82E-03	KTKFASDDEHD	Autoantigen La	*nd*	S366
-0.97	5.82E-03	SANAIYSLAAR	CBL	EGFR	Y774
-1.36	6.04E-03	EKKAYSFAGTV	Ribosomal S6 kinase 1	"Pyruvate_ dehydrogenase_kinase,_ isoenzyme_1"	S227
-1.10	6.09E-03	QESEDYSQPST	Oncoprotein Mdm2	c-abl_kinase	Y394
1.05	6.33E-03	RDRHLSFSGSG	CD44	PKC	S291
0.82	6.99E-03	ALTEDSTQTSD	Formyl peptide receptor 1	Beta-adrenergic_ receptor_kinase1	S328
0.94	7.18E-03	TQRQNSAQLGM	CDC 25A	Cell_cycle_checkpoint_ kinase	S123
1.18	7.34E-03	KERRRTESINS	HAND1	PKA;PKCalpha	T107
1.36	7.40E-03	DFEGFSYVNPQ	Protein kinase C alpha	PKCalpha	S657
0.88	7.44E-03	GFFSSSESGAP	Clathrin light polypeptide B	CK2	S11
-1.08	7.55E-03	GFTEESGDDEY	TCFL1	CK2	S41
1.04	7.55E-03	SQKYMSFTDKS	PAK1	PAK1	S144
-1.00	7.55E-03	GDDEASATVSK	Rhodopsin	RK	S334
-0.93	7.55E-03	GSDSDSEVDKK	PC4	CK2alpha_1	S19
1.02	7.77E-03	HYLDETEQWEK	Complement component 3	CK	T1031
-1.07	7.81E-03	VSSAASVYAGA	Keratin 18	p34cdc2	S33
0.95	7.99E-03	LRRSLSRSMSQ	Telethonin	Titin	S157
1.00	8.29E-03	SptCtrl	Used for production/QC	na	
1.27	8.34E-03	DAPPLSPFPHI	Retinoblastoma like 1	CDK4	S964
0.77	8.37E-03	IIGEKSFRRSV	"Protein kinase C, mu"	PKCmu	S738
0.99	8.42E-03	VSRSTSFRGGM	Keratin 18	CAMK;Ribosomal_ protein_S6_kinase_ alpha3;PKCepsilon	S52
0.84	8.43E-03	KKKKPSRLKGD	APC	PKA	S2054
0.93	8.43E-03	GFTRKSVRKAQ	"Protein phosphatase 2, regulatory subunit B56, alpha"	PKR	S28
-1.02	8.61E-03	QQQEVYGMMPR	Spectrin	Csk	Y1176
1.05	9.02E-03	FMRRTSLGTEQ	EP4 receptor	PKA	S222
-1.42	9.89E-03	EGDEIYEDLMR	VAV1	Lck	Y174
0.78	9.95E-03	KIRRLSAAKQQ	LKB1	p90RSK	S424

Limma comparison of substrate phosphorylation of chondrosarcoma cultures with colorectal carcinoma cell lines was performed, quantifying (fold change) the differential expression (log scores). Note that positive log score means higher substrate phosphorylation in chondrosarcoma than in colorectal carcinoma. Psite: phosphorylation site on the spotted substrate. *Nd.* Not determined.

Supplementary table 5.4. Top 25 activated kinases in untreated GIST882

	Average intensity	Substrate	Corresponding kinase
1	2313.85	IKRQLSMTLRG	PKA
2	1126.63	RKGHEYTNIKY	PDGFRbeta
3	893.56	GLRRSSKFALK	PKC
4	756.77	LSRRPSYRKIL	PKA;MAPK;CaMK;RSK2
5	698.83	RTRRISQTSQV	PKA
6	640.91	NRSFLSLKHTP	*Nd*
7	608.82	RRRFSSLHFMV	ERK1;ERK2;MEK1;MAP2K2
8	581.82	LARRPSYRKIL	CaMKI;PKA;S6K
9	541.54	KKWKQSVRLIS	Death associated protein kinase 1
10	520.65	IHMVYSKRSGK	*Nd*
11	499.88	GFSRKSHTFLP	PKC
12	485.98	LGQRIYQYIQS	DYRK1B
13	473.90	SSRRQSVLVKS	PKA
14	455.12	MRKKISNAQLQ	PKA
15	437.41	RKRRWSAPESR	ATK1
16	430.21	RKAKRSLAPRF	PKR
17	420.23	LNRIQTQIRVV	PKC
18	409.27	GARRSSWRVIS	SDK1
19	403.69	RPPTLSPIPHI	Cyclin D1;Cdk4
20	394.64	KKGARSRRLFS	PKCalpha
21	386.26	YKFPSSPLRIP	CDK4
22	379.85	TKWYRSPRLLL	MAPK4
23	372.94	RKRKNSRVTFS	PKA
24	369.24	GGRGGSRARNL	PKCdelta
25	365.05	LSGRGSNYGSL	*Nd*

Nd: Not determined

6. COX-2 expression in chondrosarcoma: a role for celecoxib treatment?

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Submitted

Abstract

Chondrosarcomas are resistant to conventional chemo- and radiotherapy. A subset of chondrosarcomas arises secondarily in the benign tumour syndromes Enchondromatosis (EC) and Multiple Osteochondromas (MO) and prevention of tumour development would greatly improve prognosis. We therefore investigated the effect of selective COX-2 inhibition on chondrosarcoma growth.

COX-2 expression was studied in central- and peripheral cartilaginous tumours. The effect of COX-2 inhibition was assessed in four chondrosarcoma cell lines using celecoxib and NS-398 treatment. COX-2 activity (prostaglandin E_2 (PGE_2) ELISA) and cell viability were measured. The (prophylactic) effect of celecoxib on chondrosarcoma growth *in vivo* was studied during 8 weeks using a xenograft model of cell line CH2879 in immunoincompetent nude mice.

High COX-2 protein expression was mainly found in solitary peripheral chondrosarcoma and in enchondromatosis-related central chondrosarcoma, which was confirmed by qPCR. After 72 hours of celecoxib treatment, a significant decrease in cell viability was observed in three chondrosarcoma cell lines. *In vivo*, celecoxib initially slowed tumour growth in chondrosarcoma xenografts, however after prolonged treatment relapsed tumour growth was observed. Tumour volume was negatively associated with celecoxib serum levels, and seemed smaller in the high-dose prophylactic treatment group.

We confirmed expression of COX-2 in 65% of chondrosarcomas, and COX-2 inhibition by celecoxib diminished cell viability *in vitro*. However, the *in vivo* data suggest no role for celecoxib in the treatment of adult high grade chondrosarcoma. The role of high-dose prophylactic celecoxib in preventing development of benign and malignant cartilage tumours in EC and MO patients deserves further investigation.

Introduction

Chondrosarcoma of bone is a malignant cartilage-forming tumour, which is highly insensitive to classical chemotherapeutics and radiation therapy. Chondrosarcomas are histologically divided into three grades, which is currently the only objective predictor of metastasis. Grade I tumours rarely metastasize with a 10 year survival rate of 83%, while 10 year survival for grade III tumours decreases to 29% due to metastatic disease[1]. Marginal or intralaesional excision of tumours can result in local recurrence with increased histological grade[2]. Currently, surgical removal of the tumour is the only option for curative treatment. There is no treatment with curative intent for patients with metastatic disease or inoperable tumours.

The majority (80-85%) of conventional chondrosarcomas arise in the medullar cavity of bone and are designated as primary central chondrosarcomas[3]. For less than 1% of central chondrosarcomas, there is clinical evidence of a pre-existing (benign) enchondroma[3,4]. Enchondromas occur mostly as solitary leasions, although they may occur as multiple leasions in the context of enchondromatosis (Ollier disease), which is a rare non-hereditary syndrome[5]. The risk of malignant progression is increased up to 30-35% in enchondromatosis patients[6] as compared to solitary enchondroma (<1%).

At the surface of bone, peripheral chondrosarcomas arise secondary to a pre-existing osteochondroma. Multiple osteochondromas (MO) is an autosomal dominant hereditary disorder which occurs in children and young adolescents. Malignant progression of hereditary osteochondroma is slightly more frequent than in solitary leasions (1-5% vs. 1%). Preventing new tumour formation and malignant progression in enchondromatosis and multiple osteochondroma patients would greatly benefit their prognosis.

In colorectal cancer, a protective effect of prostaglandin synthesis inhibitors (also known as non-steroidal anti-inflammatory drugs (NSAIDs)) has been suggested against development and growth of the tumours. Celecoxib and rofecoxib, both selective COX-2 inhibitors, were shown to reduce the number and size of colorectal polyps in the adjuvant treatment of Familial Adenomatous Polyposis (FAP) patients[7, 8]. Also aspirin was found to have a chemopreventive effect on adenoma recurrence in patients in which a non-FAP related adenoma had been removed[9]. NSAIDs block attachment sites for arachidonic acid on the COX enzyme, thereby inhibiting prostaglandin production[10]. Whereas COX-1 is constitutively expressed under physiologic conditions, COX-2 is induced by cytokines and free radicals, making it a suitable target for (anti-cancer) therapy.

Endo et al. reported high COX-2 expression in a substantial amount of chondrosarcomas (16/72) by immunohistochemistry, which was associated with high histological grade and poor prognosis[11]. However, Sutton found no correlation of COX-2 protein expression and histological grade in 24 chondrosarcomas (6/9 grade I tumours; 4/6 grade II and 1/6 grade III), whereas 8 enchondromas were negative[12].

We investigated whether COX-2 inhibition could play a role in either the treatment of high grade chondrosarcomas or the prevention of malignant progression of tumours associated with Enchondromatosis or Multiple Osteochondromas. Therefore we determined COX-2 mRNA and protein expression in patient material. We investigated the effects of COX-2 inhibition on COX-2 protein expression, PGE_2 levels, and cell viability in 4 high grade chondrosarcoma cell lines *in vitro*. In addition, a chondrosarcoma xenograft model of immunoincompetent nude mice was used to study the effects of COX-2 inhibition *in vivo*.

Material and methods

COX-2 expression in patient material of cartilaginous tumours

Conventional central and peripheral cartilaginous tumours were selected based on accepted clinicopathological and radiological criteria[3]. Juxtacortical-, mesenchymal-, dedifferentiated-, and clear-cell chondrosarcomas were excluded. In total, formalin-fixed paraffin-embedded specimens from 66 patients (Table 6.1) and fresh-frozen material of 34 patients was studied. Histological grading was performed according to Evans[1]. All specimens 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.

COX-2 immunohistochemistry (Table 6.2) of tumour tissue was independently semi-quantitatively scored for cytoplasmic staining, as described previously[13], without knowledge of the clinicopathological data. Scores were given for intensity (1 = weak, 2 = moderate, 3 = strong) and percentage of positive cells (1 = 0-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75%-100%). To avoid tumours

	Central						**Peripheral**						**OVERALL**	
	Solitary		EC		TOTAL		Solitary		MO		TOTAL			
	pos	%	pos	%	pos	%	pos	%	pos	%	pos	%	pos	%
Benign	3/9	33	0/6	0	3/15	20	2/8	25	1/9	11	3/17	18	6/32	19
Malignant	1/6	17	6/6	100	7/12	58	12/13	92	3/9	33	15/22	68	22/34	65
Grade I CS	0/4	0	1/1	100	1/5	20	6/6	100	2/5	40	8/11	73	9/16	56
Grade II CS	1/2	50	3/3	100	4/5	80	6/7	86	1/4	25	7/11	64	11/16	69
Grade III CS	-	-	0/2	0	0/2	0	-	-	-	-	-	-	0/2	0
ALL (Benign +Malignant)	4/15	27	6/12	50	10/27	37	14/21	67	4/18	22	18/39	46	28/66	42

Table 6.1 COX-2 protein expression in cartilaginous tumours

Antigen	Clone	Applic	Manufac.	Origin	Against	Positive control	Pre-incubation	Secondary	Antibody conc.	AR
COX-2	PG-46	IHC (human)	Nuclilab	Rabbit	Human	Colorectal carcinoma	60' blocking solution*	Anti rabbit HRP	1 100	Citrate
COX-2	PG-46	IHC (mouse)	Nuclilab	Rabbit	Human	Colorectal carcinoma	60' blocking solution*	Anti rabbit envision*	1 100	Citrate
Ki-67	MIB-1	IHC (mouse)	Dako	Mouse	Human	Any tumour	None	Anti mouse IgG1/HRP	1 100	Citrate
Cleaved caspase-3 (Asp175)		IHC (mouse)	Cell signalling	Rabbit	Human	Burkitt lymphoma	None	Anti rabbit envision	1 100	Citrate
CD31 (Pecam-1)		IHC (mouse)	Santa Cruz	Rabbit	Mouse	Any	30' NGS 5%	Anti rabbit envision	1 500	None
COX-2		IB	Cayman chemicals	Mouse	Human	Colorectal carcinoma	60' 5% non-fat dry milk in PBS/0.05%Tween	Anti mouse HRP	0,25 ng/ml	
Tubulin		IB		Mouse	Human	Any	60' 5% non-fat dry milk in PBS/0.05%Tween	Anti mouse HRP	1 1000	

Table 6.2 Antibodies and protocols used for immunohistochemistry and immunoblotting. *for human tissue anti rabbit HRP was used IHC: Immunohistochemistry, IB: Western blot, NGS: normal goat serum, *blocking solution: 0,01M Tris, 0,1M MgCl2, 5% Tween 20, 1% BSA and 5% normal goat serum. AR; antigen retrieval.

with single positive cells being regarded as positive a cut-off level of total sum ≥3 was applied.

RNA was isolated from fresh frozen tumour tissue of 34 central tumours as described previously[14]. Growth plate samples (n=4) were used as normal counterpart controls. Messenger RNA expression of *COX-2* was studied using quantitative RT-PCR (forward primer *GAATCATTCACCA-GGCAAATTG,* reverse primer *TCTGTACTGCG-GGTGGAACA*), as previously described[15]. For normalisation GENORM was used[16].

Inhibition of COX-2 in chondrosarcoma in vitro

Chondrosarcoma cell lines derived from chondrosarcoma grade II (SW1353, American Type Culture Collection (ATCC), Manassas, VA), grade III (CH2879[17] and OUMS27[18]) and a recurrent chondrosarcoma grade II in enchondromatosis (C3842[19]) were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Cell line HT29 (ATCC), expressing high COX-2 levels, was used as a control. Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. The cartilaginous phenotype of the chondrosarcoma cell lines

was confirmed by RT-PCR, showing mRNA expression of *collagens I*, *2B*, *3,* and *10*; *Aggrecan;* and *SOX9*[20]. Protein extraction and immunoblotting were performed as described previously[21]. A WST-1 colorimetric assay (Roche Diagnostics GmbH, Penzberg, Germany) was used as described previously[21] to measure metabolic activity representing the amount of viable cells in response to celecoxib (Pfizer, NY, USA) and NS-398 (Cayman chemicals, Ann Arbor, MI, USA). $5.0*10^3$ chondrosarcoma cells (CH2879, OUMS27 and C3842) and $1,5*10^3$ HT29 and SW1353 cells were seeded. Celecoxib and NS-398 were diluted in DMSO. After 24 hours, increasing concentrations of the drugs were added (5, 10 and 25 µM) or 0,1% DMSO. C3842 was not included in the NS-398 assay.
For measuring PGE_2 levels, cells of OUMS27, CH2879, C3842, and HT29 were seeded in a 24 wells plate at a density of $5.5*10^4$, and SW1353 at a density of $2.0*10^4$. Twenty-four hours after seeding, cells were treated with celecoxib in increasing dosages (5, 10 and 25 µM) or with 0.1% DMSO. After 72 hours, PGE_2 concentration in the medium was determined by a PGE_2-specific enzyme-linked immunoassay (Cayman chemicals, Ann Arbor, MI, USA) according to the manufacturer's protocol. mRNA was extracted and CYP19A1 mRNA levels were determined to assess aromatase activity[20]. Five percent serum supplementation was used for all experiments.

In vivo chondrosarcoma model
Sixty Swiss male nude mice (Crl:NU(Ico)-Foxn1nu (IFFA-CREDO, Lyon, France)) were randomly assigned to one of five groups (Figure 6.1). Low doses contained 500ppm celecoxib, high doses 1000ppm. The selective COX-2 inhibitor celecoxib (CS-58635) was incorporated into a modified nude mouse diet (Altromin Gesellschaft fur Tierernahrung mbH, Lage, Germany) and irradiated to accomplish sterility. Mice were able to feed and drink ad libitum. Amount of food consumed was monitored individually. Prophylaxis was started 7 days prior to inoculation, as steady-state levels of celecoxib are reached in five days in humans (Pfizer). At week 1 all mice were injected with $2*10^6$ CH2879 cells, subcutaneously on the back. Since chondrosarcomas have a relatively slow growth rate, we waited 21 days for the tumours to reach a size large enough for external analysis, before treatment with celecoxib was started. Treatment was prolonged for 30 days, after which the mice were sacrificed. The mice were weighted once a week. Calipers were used to measure the tumour volume *in vivo* using the following formula: $(length*whith^2)/2$. Blood samples, obtained by aortal punction at time of sacrifice, were allowed to coagulate and were centrifuged for 10 minutes at 13000 rpm; supernatants were collected and stored at -20°C until HPLC analysis. HPLC was performed by Case Bel (Borgerhout, Belgium) as described previously[22].
The excised tumours were fixed in 0,1% formalin for 24 hours and embedded in paraffin. To study possible toxicity of celecoxib, tissue of the heart, lungs,

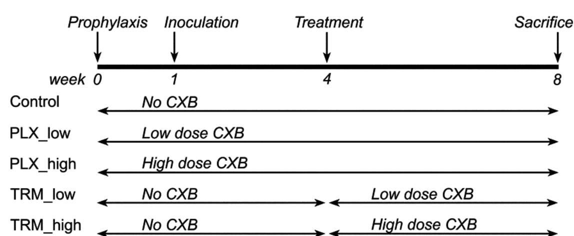


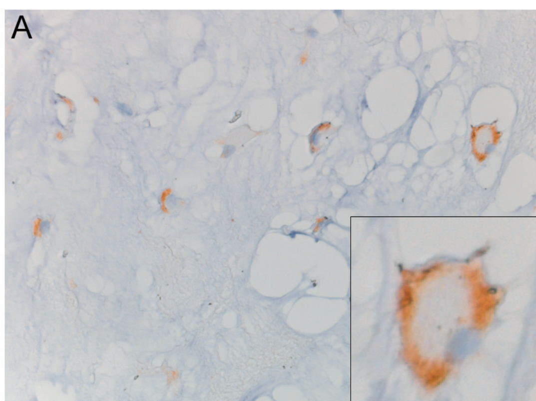
Figure 6.1 Sixty immunoincompetent nude mice were randomly assigned to either prophylactic low dose celecoxib (PLX_low), prophylactic high dose celecoxib (PLX_high), treatment low dose celecoxib (TRM_low), treatment high dose celecoxib (PLX_high) or control group (Control).

liver, and kidney were taken at autopsy for histological analysis. Xenografted tumours were analysed by H&E, Toluidine blue and immuhistochemistry for COX-2, Ki-67, cleaved caspase-3 and CD31 (Table 6.2). COX-2 staining was scored as negative, very weak or positive. Ki-67 and cleaved caspase-3 staining were digitally scored using confocal microscopy (Nuance 2.6.0 Cambridge research and Instrumentation Inc. Woburn, MA, USA). A minimum of 2000 cells were counted and the percentage of positive cells was automatically calculated (ImageJ 1.37V, Wayne Rasband, National Institutes of Health, USA)), to assess microvessel density, CD31-positive vessels were counted in 10 high-power fields per tumour. An independent experiment was performed using grade II chondrosarcoma xenograft model, with high celecoxib prophylaxis (n=12) and treatment (n=12) group.

Statistics
Comparison between groups was performed using Pearson Chi-Square (immunohistochemistry) and Students's t-test (RT-PCR). P values <0.05 were considered significant.

Results

Higher COX-2 expression in chondrosarcoma and in enchondromatosis.
In total, in 65% of the chondrosarcomas tumour cells demonstrated cytoplasmic COX-2 protein expression (Figure 6.2A). Malignant tumours were more often positive for COX-2 than benign tumours (58% vs. 20%, p=0.040 for central and 68% vs. 18%, p=0.002 for peripheral, Pearson Chi-Square). In the group of central chondrosarcomas positivity was mainly seen in enchondromatosis-related chondrosarcomas (6/6), whereas peripheral solitary tumours were more often positive (12/13) than MO related chondrosarcomas (3/9). Results are summarised in table 6.1.
Also at mRNA level enchondromatosis-related tumours tend to show higher COX-2 expression, as compared to solitary tumours (p=0.056 Student's t-test) (Figure 6.2B). However, no difference in COX-2 mRNA expression between benign (n=7) and malignant tumours (n=27) was found (Student's t-test p=0.58) (not shown).



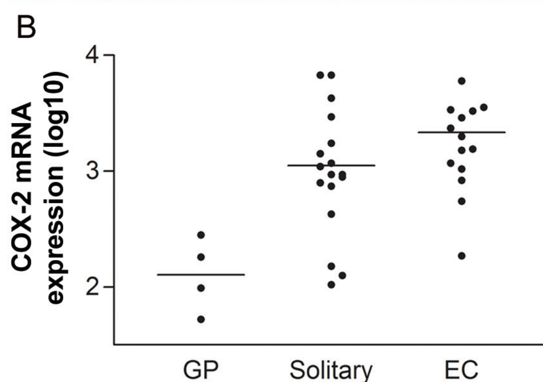


Figure 6.2 (A) Cytoplasmic COX-2 expression in central chondrosarcoma grade I. (B) COX-2 mRNA was higher expressed in central cartilaginous tumours compared to growth plate (GP) samples and tumours related to enchondromatosis (EC) showed higher expression of COX-2 than solitary tumours.

COX-2 inhibitors decrease proliferation of chondrosarcomas in vitro

All four chondrosarcoma cell lines demonstrated COX-2 protein expression in variable levels (Figure 6.3A). After 72 hours of 25 µM celecoxib treatment, a decrease in cell viability was observed, comparable to HT29, in the three cell lines that were derived from solitary tumours (Figure 6.3B). For OUMS27 a decrease in cell viability was observed already at 10 µM celecoxib. C3842 did not respond to celecoxib (Figure 6.3B), not even when treatment was prolonged for 48 hours (data not shown). Effects of NS-398 were more subtle (Figure 6.3C).

Response to celecoxib is independent of COX-2 activity

By PGE_2 ELISA we showed that in CH2879, OUMS27 and C3842 the COX enzymes are active and that a dose of 5,0 µM celecoxib was enough to significantly decrease PGE_2 levels (Figure 6.3D). Remarkably, we were not able to detect COX activity in HT29 and SW1353, which both responded well to celecoxib treatment. Relative CYP19A1 mRNA levels were decreased twofold upon celecoxib treatment in SW1353, whereas in CH2879 and C3842 CYP19A1 levels were increased twofold (data not shown).

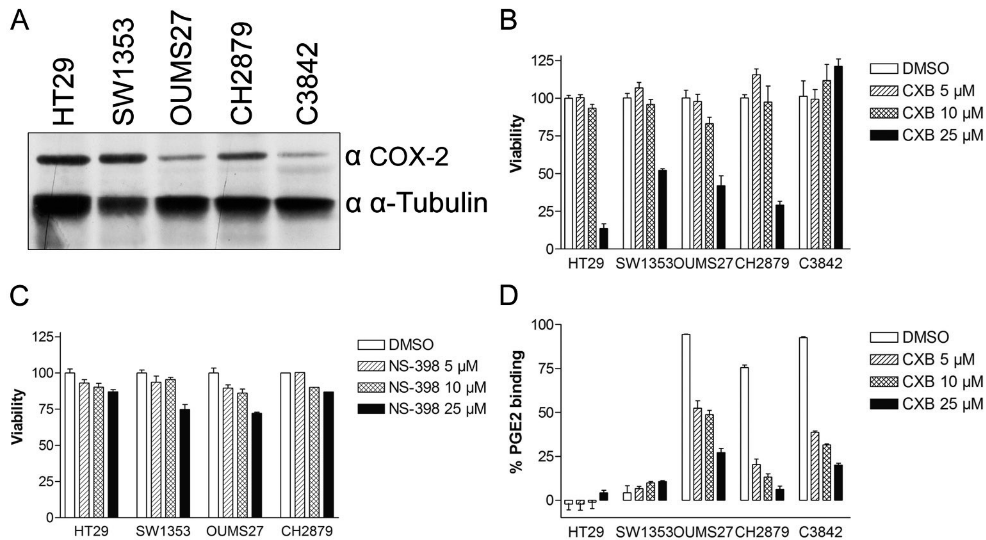


Figure 6.3 (A) COX-2 protein expression in cell lines (B) decreased viability (SW1353, OUMS27 and CH2879) upon 25 µM celecoxib treatment. (C) NS-398 has a moderate effect (d) COX-2 activity is absent in SW1353 and decreased upon 5 µM celecoxib treatment in OUMS27, CH2879 and C3842.

Celecoxib initially slows tumour growth in chondrosarcoma xenografts

During the *in vivo* study, all mice had comparable body weights and amounts of food administered (6.4A). Six mice died during the experiments, both in the celecoxib and the control groups. Serum levels of celecoxib corresponded to the dose of celecoxib administered, although levels were variable (Figure 6.4B). Tumour size was negatively correlated to celecoxib serum levels (r=-0.39) (Figure 6.4C). At 4 weeks, the tumour volume seemed smaller in the groups receiving celecoxib prophylaxis, which was significant for the high dose -, as compared to the control group (Student's t-test p=0.053 (low dose) and p=0.028 (high dose) respectively) (Figure 6.4D). Histological evaluation of the heart, lungs, liver, spleen, and kidneys of the mice did not reveal any signs of toxicity.

Relapse of tumour growth in xenografts after prolonged treatment

At week 6 a relapse was observed, most clearly in the low celecoxib prophylaxis-, and treatment groups. At the end of the experiment (week 8), only the high prophylaxis group showed smaller tumour volumes than the control group (Figure 6.4E). However due to large variation in tumour volume within the groups, statistical calculations are not meaningful. The growth curves of the tumours showed that at week 6 the tumours started to grow even faster than the control group (Figure 6.4F). This was exactly the time point at which the growth curves of the mice flattened (Figure

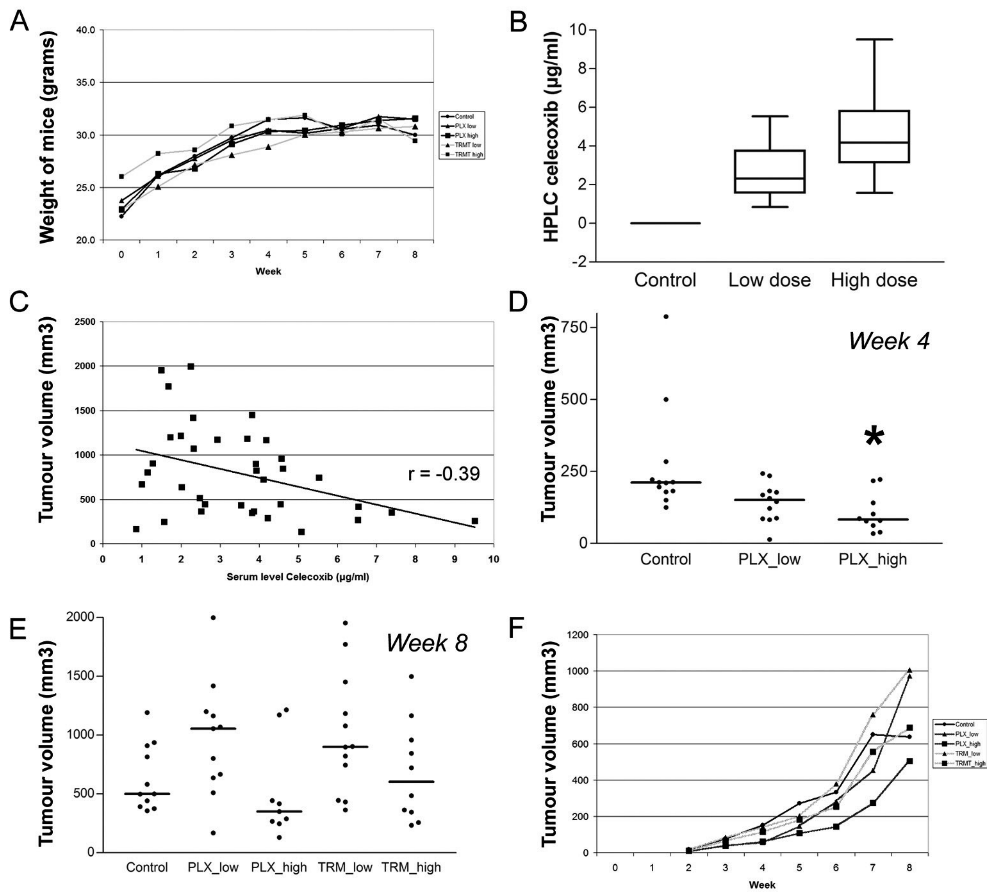


Figure 6.4 (A) Mice body weight increases till week 6 (B) Celecoxib blood levels correspond to celecoxib dose (C) tumour volume is negatively correlated with celecoxib serum levels (r=-0.39). (D) Lower tumour volume in prophylaxis group at week 4, (E) tumours exposed to low dose celecoxib are larger than controls at week 8, although variation within the groups is considerable. (F) Overall growth curve of the tumours showing relapse at week 6 for those exposed to low dose celecoxib. (*: significantly different from controls).

6.4A), suggesting the end of puberty. The independent experiments, in which high celecoxib doses were used, showed similar results for the grade II chondrosarcoma xenograft model (data not shown).

Evaluation of tumour tissue

Tumours were highly cellular with limited cytonuclear atypia and a limited amount of extracellular matrix (Figure 6.5A). Toluidine-blue staining confirmed the deposition of proteoglycans (Figure 6.5B). Whereas in all celecoxib-treated groups COX-2 staining was absent or very weak (Figure 6.5C), strong COX-2 expression was observed in 50% of the control tumours

(Figure 6.5D). Proliferation was seen in all groups (Figure 6.5E), with higher Ki-67 expression in the treated tumours at week 8 (Student's t-test low dose p=0.040 and high dose p=0.018) (Asterisks, figure 6.5F). Cleaved caspase-3 expression was low (mean 0.746%, range 0.2-1.7%), as was microvessel density (mean 18.9 per 10 hpf, range 4.0-40.0 per hpf) and no differences between groups were found (Figures 6.5G-H).

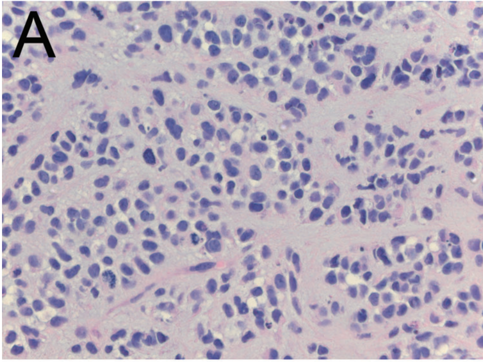
Discussion

Since there is nothing to offer with curative intent to patients with metastatic or inoperable chondrosarcoma, there is a desperate need for new therapeutic options. Furthermore, the prevention of development of new tumours and especially of malignant transformation of benign precursor leasions in patients with enchondromatosis and multiple osteochondromas would greatly improve the prognosis of these children and young adults. In this study, we investigated the potential of selective COX-2 inhibition for the treatment of chondrosarcoma.

We demonstrated expression of COX-2 in a subset of enchondromas (20%), osteochondromas (18%), and central- (58%) and peripheral (68%) chondrosarcomas, confirming published results[11,12]. Interestingly, COX-2 protein and mRNA expression was mainly found in enchondromatosis-related tumours, whereas correlation with histological grade could not be confirmed.

Chondrosarcoma cell viability decreased after administration of high (super-physiologic) levels of celecoxib and NS-398, while a physiologic dosage of celecoxib was able to abolish most COX-2 activity in three cell lines. Despite the lack of COX-2 activity in SW1353, a decrease in cell viability was found in response to celecoxib, which suggests a COX-2 independent mechanism, which was previously suggested for colorectal cancer (reviewed by Grosch et al.[23]). In breast cancer COX-2 inhibitors were found to suppress aromatase activity in both a PGE_2 dependent and independent manner[24], and Cleton-Jansen et al. showed that chondrosarcoma growth could be inhibited by aromatase inhibitors *in vitro*[20]. The decrease in CYP19A1 activity in SW1353 during celecoxib treatment suggests that this growth inhibitory effect is exerted via the inhibition of aromatase.

Since three of four chondrosarcoma cell lines responded to celecoxib *in vitro*, we also tested celecoxib in chondrosarcoma xenografts. Celecoxib treatment was initially effective in slowing the growth rate of chondrosarcoma. Moreover, tumour size was inversely correlated with celecoxib serum levels, measured at the end of the experiment. However, a relapse was observed in week 6, which was especially prominent in the mice receiving low dose celecoxib. In addition, the treated tumours showed an increased proliferation rate. Interestingly, at week 6 the growth curve of the mice flattened, suggesting the end of puberty, which suggests hormonal influences on tumour growth. Here, this effect cannot be attributed to estrogens, since CH2879 is estrogen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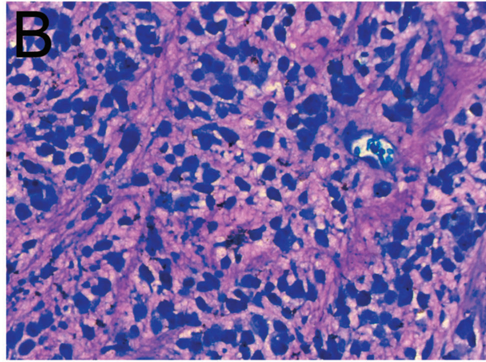
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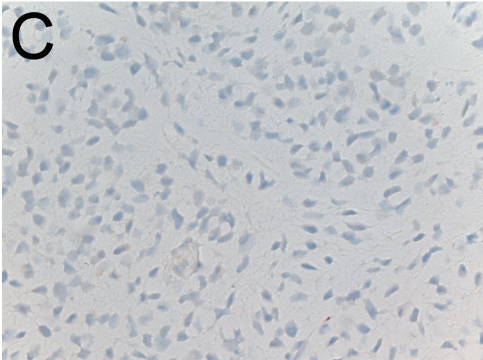
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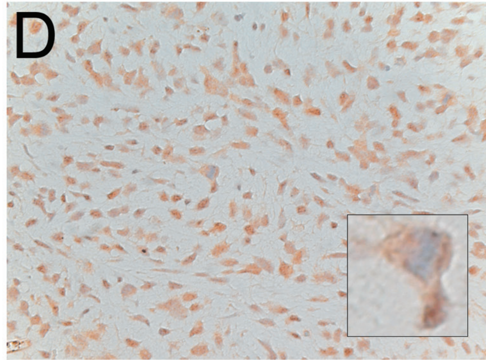
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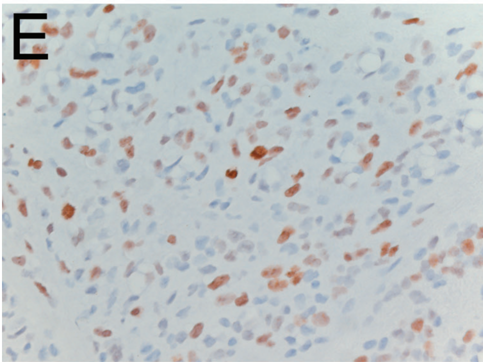
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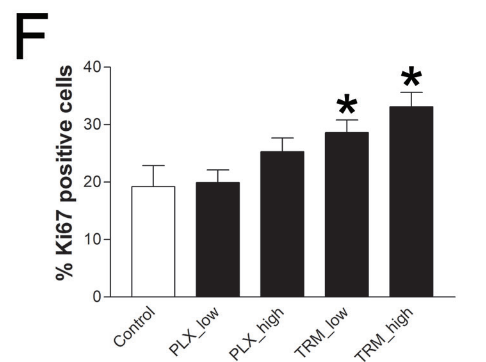
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% Ki67 positive cells
40
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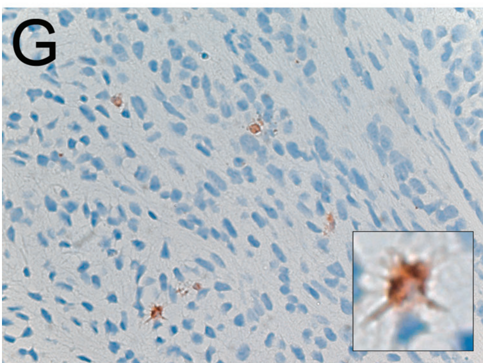
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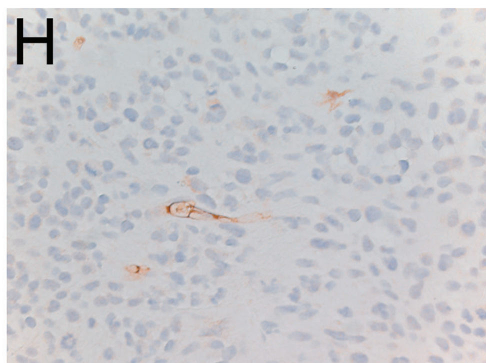
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receptor negative[17].
One of the mechanisms of tumour inhibition of celecoxib is thought to be the inhibition of angiogenesis. The relapse at week 6 might reflect a time point where celecoxib can no longer inhibit angiogenesis allowing vessel ingrowth. At sacrifice no differences in microvessel density were found, suggesting that either differences in microvessel density are completely overcome or a different mechanism was responsible for the initial decreased tumour growth. Unfortunately, we were not able to study tumour characteristics or blood parameters during the experiment.
In analogy to the prevention of new adenoma formation in familial adenomatous polyposis, prevention of development and malignant transformation of enchondromas and osteochondromas in patients with enchondromatosis and multiple osteochondromas might be beneficial. Although we showed COX-2 expression to be higher in enchondromatosis related tumours compared to solitary tumours, the enchondromatosis derived cell line C3842 did not respond to celecoxib treatment *in vitro*. In vivo, a growth-inhibiting effect was shown in the first 4 weeks of the study, in the prepubertal mice, which was abolished at the moment the mice reached adulthood. This might suggest that celecoxib is more effective in prepubertal patients. Moreover, there was a trend for high-dose prophylactic celecoxib treatment to have smaller tumours at the end of the study. However, the high dose prophylaxis group showed a higher proliferation rate as compared to the control. Thus, our results are not conclusive on whether the paediatric population of multiple osteochondromas and enchondromatosis patients might benefit from celecoxib treatment and further studies should be performed. In addition, it should be noted that our model is suboptimal to study the effect of celecoxib on prevention of malignant transformation, since we used high grade chondrosarcoma xenografts. Unfortunately, there is no suitable *in vivo* model for enchondromatosis or multiple osteochondromas, and xenografts from low-grade chondrosarcomas are difficult to obtain.
During long-term clinical trials, COX-2 inhibitors were shown to have cardiovascular side effects. Celecoxib trials were discontinuated earlier[25] and rofecoxib was withdrawn by the FDA[26]. However, children and adolescents are at low risk of cardiovascular disease, which renders the use of celecoxib relatively safe. Accordingly, celecoxib is prescribed to juvenile rheumatoid arthritis patients from the age of 2 years (reviewed in Frampton et al.[27]). Next to its potential anti-tumour effect, celecoxibs analgetic effect will be beneficial for multiple osteochondromas and enchondromatosis patients.

left page **Figure 6.5** (A) H&E staining of the CH2879 xenograft. (B) Toluidine blue staining confirmed proteoglycan content. (C) Absent or very weak COX-2 staining in treated tumours (D) strong COX-2 staining in 50% of the controls.(E,F) Higher proliferation rate (Ki-67) in the celecoxib treated tumours . (G) Caspase 3-mediated apoptosis is limited. (H) Microvessels detected by CD31.

In conclusion, we confirmed expression of COX-2 in 65% of chondrosarcomas, and COX-2 inhibition diminished cell viability *in vitro*, although this was independent of COX-2 activity. However, using a grade II and III chondrosarcoma xenograft model, we observed a switch from tumour inhibitory to tumour promoting effects when mice reached adulthood, suggesting that even though tumour volume was negatively associated with celecoxib serum levels, there is no role for celecoxib in the treatment of adult high-grade chondrosarcoma. The role of high-dose prophylactic celecoxib in preventing development of benign and malignant cartilage tumours in prepubertal EC and MO patients deserves further investigation.

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7. Discussion and future prospectives

Chapter 7

Contents

7.1 Histological distinction between benign and low-grade malignant cartilaginous tumours

7.2 Prognostic markers superior to histological grading

7.3 New therapeutic strategies for metastatic and irresectable chondrosarcoma

7.4 Concluding remarks

7.1 Histological distinction between benign and low-grade malignant cartilaginous tumours

In the clinical management of asymptomatic enchondroma a wait-and-see approach is justified, whereas grade I chondrosarcomas are treated by curettage followed by the application of a local adjuvant such as cryosurgery, or phenolisation[1-3]. The difficult histological distinction between benign- and malignant central cartilaginous tumours of bone was assessed in **chapter 2** of this thesis. A panel of 18 specialised bone tumour pathologists affiliated to Eurobonet, an European Commission granted network of excellence to study the biology and pathology of bone tumours, was asked to evaluate a set of 16 cases. A low-weighted kappa value (κ=0.78) confirmed the variability between the observers, which was most striking in the distinction between enchondroma and low-grade central chondrosarcoma (κ=0.54). A similar study was conducted in the United States of America, showing similar results with regard to interobserver variability[4].

In **chapter 2** a second separate set of 20 enchondromas and 37 chondrosarcomas of which full clinical, radiological and pathologic data were available, were analysed for individual morphologic criteria. Diagnostic power of these criteria was evaluated using 10 years follow-up data, including radiological assessment as golden standard. The two most significant findings to discriminate grade I chondrosarcoma from enchondroma were entrapment of host lamellar bone and mucoid/myxoid matrix degeneration. The application of a combination of 5 parameters allowed optimal differentiation between enchondromas and grade I central chondrosarcomas: the finding of host bone entrapment, high cellularity, open nuclear chromatin, mucoid/myxoid matrix degeneration and patient age of above 45 years. In addition, clinical symptoms were shown to be helpful in the differentiation between benign and malignant cartilaginous tumours, since spontaneous pain was reported in 35% of the patients with an enchondroma and in 62% of patients with a grade I chondrosarcoma.

Incisional surgical and the closed trocar (Jamshidi) biopsy are the most frequently used techniques for obtaining a representative tissue sample for histologic evaluation[5]. Evaluation of some of the above mentioned histological parameters, especially entrapment of host lamellar bone, may however be difficult on small needle biopsies. Based on the findings presented in **chapter 2**, it is advisable to evaluate at least the mucoid/myxoid matrix degeneration and, if possible, entrapment of host lamellar bone when grading cartilaginous tumours in daily practice. These two findings were the most discriminating and are considered more objective than high cellularity or open chromatin.

In conclusion, objective markers that discriminate on a molecular level are still needed to guide clinical decision making. In **chapter 3** it was shown that the expression of WNT strongly differed between enchondromas

and grade I chondrosarcomas. While only 17% of enchondromas showed nuclear accumulation of β-catenin, this was present in 48% of grade I chondrosarcomas. Although this certainly is an interesting finding from a biological point of view, the differences are not large enough to promote the use of β-catenin immunohistochemistry as a diagnostic marker to distinguish enchondroma and grade I chondrosarcoma. Until molecular markers are found that discriminate between benign and malignant cartilaginous tumours, clinical decision making will be based on histological assessment by the pathologist using mucoid/myxoid matrix degeneration and entrapment of host lamellar bone as important criteria and is facilitated by a multidisciplinary approach, including also radiologists and surgeons.

7.2 Prognostic markers superior to subjective histological grading

In the majority of bone tumour referral centres, grade I chondrosarcomas are treated by intraleasional surgery followed by some kind of local adjuvant treatment. Grade II chondrosarcomas however, are surgically resected with a wide margin, by en-bloc resection, or sometimes even amputation. In this respect the differentiation between grade I and grade II is even more important than the differentiation between benign and grade I chondrosarcoma, which was discussed in the previous paragraph.

In **chapter 2** it was shown that not only the distinction between benign and low-grade malignant cartilaginous tumours is difficult. Also at the other end of the spectrum, the bone tumour specialised pathologists differed considerably in grading the malignant tumours. A weighted kappa value of $\kappa=0.80$ was found for the variance amongst the observers in discerning grade I from grade II chondrosarcomas, which is slightly better than the κ-value calculated for the discrimination between enchondroma and grade I chondrosarcoma. Statistically, $\kappa=1$ indicates complete agreement between all observers, while $\kappa=0$ indicates there is no agreement among the raters (other than what would be expected by chance). In clinical practice staging of a cartilaginous tumour by a pathologist guides the operative modality used. Under-grading followed by a less aggressive treatment might lead to a higher percentage of recurrences and worse prognoses for the patients and is therefore not acceptable. On the contrary, over-grading followed by a therapy that is too aggressive, might lead to unnecessary morbidity. Again, this stresses the urgent need for molecular markers that are able to predict outcome of cartilaginous tumours.

A search for biomarkers alternative to histological grading is described in **chapter 3**, in which growth signalling was studied in a large series of central chondrosarcomas. In order to find new prognostic markers, biomarkers should be correlated to long term follow up. The expression of TGFB was found to be gradually upregulated with grade, as was previously described for peripheral chondrosarcomas[6] (Figure 7.1). Unfortunately, a correlation with outcome was not found in this study. From a biological point of view the increased expression of TGFB is interesting. TGFB is important in many signalling pathways promoting cell proliferation, cell survival, tissue vascularisation and extracellular matrix remodelling[7]. Acquisition of a malignant phenotype due to increase in TGFB expression was also suggested in peripheral chondrosarcomas[6] (Figure 7.1).

In **chapter 4** cyclin dependent kinase 4 (CDK4) and MDM2 were found to be positively correlated with histological grade, although correlation with clinical outcome was not found in this study, despite the availability of long term follow up data. Both *CDK4* and *MDM2* are located at 12q13,

a genetic locus that is often found to be amplified in high grade central chondrosarcomas[8-10]. Like was discussed for TGFB, the expression of both CDK4 and MDM2 could also be described as a crescending spectrum depending on histological grade.

In the considerable number of reports in literature in which prognostic markers for chondrosarcoma are investigated, conclusions are drawn from comparing data from chondrosarcoma groups that were separated based on parameters that are shown to be highly observer dependent (**chapter 2**). In order to find new prognostic markers, it is recommended to only use tumours of which a long follow up including distant staging is available in order to find prognostic markers that are independent from and superior to histological grading. For example outcome measurements as metastasis free survival or disease free survival after wide margin surgery could be used as such objective markers.

7.3 New therapeutic strategies in chondrosarcoma treatment

In **chapter 3** it was hypothesised that pathways in the human growth plate that are responsible for normal growth regulation of bone are involved in the development of enchondromas and central chondrosarcomas. Central cartilaginous tumours were shown to have normal *EXT1* and *EXT2* expression, which is in contrast to peripheral tumours with low *EXT1* and *EXT2* expression due to genetic loss in sporadic tumours[11] or inactivating mutations in hereditary tumours[12-14]. In central cartilaginous tumours, active Indian hedgehog signalling was found. Indian hedgehog is important for the regulation of normal longitudinal bone growth. Cyclopamine, a hedgehog pathway inhibitor, was hypothesised to inhibit central chondrosarcoma cell growth. Cyclopamine is currently used in the treatment of children with medulloblastoma[15]. A pre-clinical evaluation of the use of cyclopamine in central chondrosarcoma treatment was performed *in vitro* in six chondrosarcoma cell cultures in **chapter 3**. Despite the finding of hedgehog activity in chondrosarcomas *in vivo* and *in vitro*, cyclopamine had a growth inhibitory effect in only one of six chondrosarcoma cell cultures *in vitro*. In this cell culture the transcription of *GLI1* decreased upon cyclopamine treatment, while the other cell cultures did not respond with respect to *GLI1* expression levels. Unexpectedly, growth was even stimulated by cyclopamine in one of the cultures, although *GLI1* levels were not increased. Therefore selecting the eligible subjects for cyclopamine treatment will be difficult. In addition, a drawback of the clinical use of cyclopamine is the inhibition of Sonic hedgehog, which is important for brain development[16]. Also the interference of cyclopamine with the signalling in the human growth plate could lead to growth retardation in paediatric patients. The use of cyclopamine could be more save in adult patients. However in medulloblastoma, which is the most common malignant brain tumour in children carrying an activating *PTCH* mutation, the use of cyclopamine is being investigated, although not clinically implemented due to these side effects[17].

Ninety-six % of high-grade central chondrosarcomas were shown to carry one or more alterations in the expression of proteins important for cell cycle regulation, especially in the pRB pathway, studied in **chapter 4**. The importance of loss of *p16* for chondrosarcoma cell proliferation was shown by overexpressing *p16* by lentiviral transduction *in vitro,* which caused inhibition of cell growth in three chondrosarcoma cell lines. By using shRNA against *CDK4*, also *CDK4* expression was shown to be important for cell growth. Although these cell cycle proteins are expressed in every cell in the human body, there are certain treatment strategies directed against these proteins. Flavoperidol is the most well known, shown to induce G1 arrest with inhibition of cyclin-dependent kinase (CDK) 2 and CDK4 in human breast carcinoma cells[18]. However, in a phase II consortium study conducted in 2000 in metastatic renal cancer no significant effect was

observed and major side effects such as asthenia and severe diarrhoea were reported in the majority of patients[19]. In addition, Flavoperidol is described as a sensitising agent to doxorubicin in small cell lung cancer cells that were pRb-negative, both *in vitro* and *in vivo*[20].
Another indirect strategy is suggested, by using Heat Shock Protein (HSP) inhibitors that inhibit the stabilisation of CDK4 by HSP90[21]. In addition, the kinomics study presented in **chapter 5** also revealed flavoperidol as a potential target for therapeutic treatment of chondrosarcoma. Unfortunately, Flavoperidol was not available to conduct an *in vitro* study on the effect on chondrosarcoma growth. In the future, the use of Flavoperidol or HSP inhibitors in chondrosarcoma treatment should be studied more in detail in a preclinical setting *in vitro* and *in vivo* using animal models.

In addition to the hypothesis driven approaches concerning EXT and cell signalling pathways, new treatment options for chondrosarcoma were studied using kinome profiling. Kinases play a major role in signalling cascades that determine cell cycle entry, cell survival and differentiation fate, which are often deregulated in tumours. Kinases are excellent targets for anti-cancer therapy since they work as a molecular switch; their regulation is reversible, rapid and does not require new protein synthesis[22]. Antineoplastic activity of imatinib mesylate, which inhibits KIT, PDGFR, and BCR-ABL kinases, was shown to be successful in the treatment of gastrointestinal stromal tumours (GISTs)[23] and chronic myeloid leukemia[24]. GISTs harbour an activating mutation of the KIT receptor kinase or PDGFRα receptor kinase, and are refractory to conventional chemotherapy.
In **chapter 5**, 13 chondrosarcoma cell cultures (both cell lines as well as short term cultures of clinical samples) were assessed for the activity of kinases. The common denominators in this respect between these 13 cell cultures were activity of the AKT1/GSK3β pathway, PDGFRβ and the Src pathway.

AKT and GSK3 have a central function in physiological processes as transcription, apoptosis and cell cycle progression, and also in pathological processes as diabetes mellitus, Alzheimer and carcinogenesis[25]. AKT was previously shown to be important in chondrosarcoma survival[26].
PDGFRA protein expression[27] and activity of the alpha and beta receptor were reported previously in the absence of gain-of-function mutations[28], and its expression was shown to be correlated with adverse prognosis in chondrosarcoma[27].
Like AKT, Src is a major regulator of cellular processes, as well. For instance, Src plays a role in the regulation of embryonic development and cell growth[29]. In addition, mutations in Src are involved in the malignant progression of colorectal cancer[30].

The AKT1/GSK3β pathway can be inhibited by Enzastaurin hydrochloride, also known as LY317615. Enzastaurin is an acyclic bisindolylmaleimide that was initially developed as an adenosine triphosphate-competitive, selective inhibitor of PKCß[31]. Enzastaurin was shown to suppress signalling through the AKT pathway, induce apoptosis, and suppress growth of human colorectal cancer and glioblastoma xenografts[32]. In a phase II trial, Enzastaurin was well-tolerated and associated with prolonged progression free survival in patients with relapsed or refractory diffuse large B-cell lymphoma, the most common type of non-Hodgkin's lymphoma[33]. Therefore, the effect of Enzastaurin on chondrosarcoma cell growth *in vitro* is currently under investigation at our department.

Chapter 5 was focussed on the inhibition of the Src pathway by dasatinib, which indeed resulted in decreased cell viability in seven out of nine chondrosarcoma cell cultures *in vitro*. Interestingly, these inhibiting effects were not moderated by the Src pathway alone, since decrease of active Src was found both in the non-responsive and in the responsive cell cultures. This suggests that other pathways might be targeted by dasatinib, such as AKT[34], in chondrosarcomas.

More experiments are needed to further explore the mechanism of growth inhibition by dasatinib. Since an intermediate response of 50% of cell growth inhibition was found in four of the seven responding chondrosarcoma cultures, the effect of dasatinib might be increased by combination with another cytostatic compound and higher growth inhibition rates might be established. A combinatory therapy with a mTOR inhibitor is a good candidate for this, since also RPS6kinase was found to be active in chondrosarcoma. RPS6kinase was found to predict the response to mTOR inhibitors in sarcoma[35]. RPS6 kinase is responsible for the phosphorylation of ribosomal protein S6, which we previously found to be deleted and downregulated in a subset of chondrosarcomas[9].

The *in vitro* results described in **chapter 5** should be further studied in a preclinical model *in vivo.* The presence of blood vessels or cartilaginous matrix might have extra effects on growth inhibition on chondrosarcoma cells by dasatinib. Also, i*n vitro* data are not always reproducible *in vivo*, as was shown for celecoxib in **chapter 6**. Currently, a phase II clinical trial using dasatinib in advanced sarcomas opened for amongst others chondrosarcomas for which patients will be recruited until 2013 (www. clinicaltrials.gov Identifier NCT00464620).

Although PDFGRβ was shown to be active as a kinase in chondrosarcoma cell cultures by kinome analysis, and despite the finding of receptor activity correlated with adverse outcome[27], the inhibition of PDGFRβ by imatinib

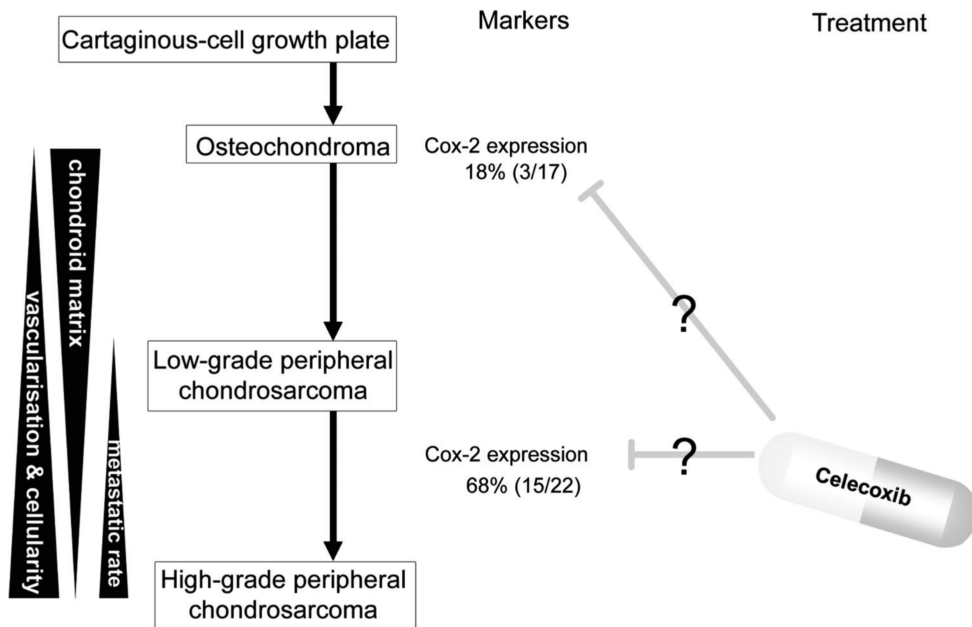

Figure 7.1 Implications for potential therapeutic strategies in peripheral chondrosarcoma. A multistep model of the progression of osteochondroma towards secondary peripheral chondrosarcoma as introduced in **chapter 1** is shown. Expression of COX-2 and celecoxib as a new potential target for therapeutic strategy in peripheral chondrosarcomas is described in **chapter 6** of this thesis. Celecoxib was not tested *in vitro* (question mark).

mesylate did not provoke any effect on the cell growth of chondrosarcoma cell cultures. Imatinib resistance has been shown to occur in GIST due to loss of cell cycle control as well[36]. Although in GISTs these are secondary events, loss of cell cycle control, which was shown to occur in 96% of chondrosarcomas (**chapter 4**), might cause imatinib resistance in chondrosracoma cells. Alternative to secondary events, it was shown that after switching off mutated receptors in GIST by imatinib, heterologous wild-type receptors maintain signalling activation[37]. Such a mechanism could be present in chondrosarcomas, explaining their resistance. Also, it would be interesting to investigate whether this pathway can be attacked at another level, for example by the use of monoclonal antibodies against the PDGF receptor.

Another kinase that was shown to be particularly active in chondrosarcomas is Aurorakinase B. Aurorakinase B functions in the attachment of the mitotic spindle to the centromere. While Aurorakinase A localises to centrosomes, aurorakinase B localises to microtubules near kinetochores, specifically to the specialised microtubules called K-fibers[38]. Overexpression of these

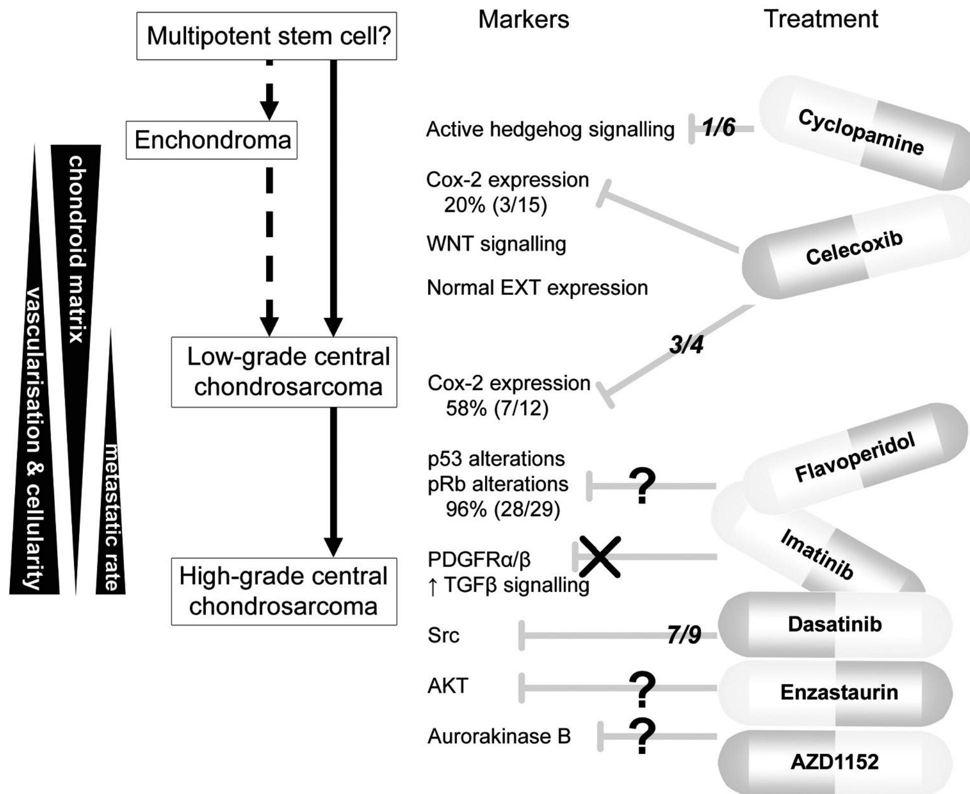

Figure 7.2 Implications for potential therapeutic strategies in central chondrosarcoma
A multistep model of the progression of central chondrosarcoma as introduced in **chapter 1** is shown. Markers and their corresponding treatment options as investigated in this thesis are shown. *In vitro* inhibitory effects of IHH inhibitor cyclopamine, selective COX-2 inhibitor Celecoxib and Src inhibitor Dasatinib in chondrosarcoma were described in **chapter 3**, **chapter 6** and **chapter 5**, respectively. Imatinib mesylate was shown not to be a potential drug in the treatment of chondrosarcoma (**chapter 5**). A possible role for Flavoperidol was suggested (**chapter 4 and 5**), as was suggested for Enzastaurin and AZD1152, based on the finding of AKT and Aurorakinase B using kinomics (both **chapter 5**).

kinases causes unequal distribution of genetic information, leading to aneuploidy characteristic of high-grade central chondrosarcomas (figure 7.2). Inhibition of Aurorakinase B by the pyrazoloquinazolines class of drug AZD-1152 is a recent topic of preclinical cancer studies. In 2007, AZD-1152 was shown to induce growth arrest, apoptosis, and sensitisation for vincristine and daunorubicin in acute leukemia cells, both *in vitro* and *in vivo*[39]. Also the growth of human colorectal and lung tumour xenografts in immunodeficient mice was inhibited by AZD-1152[40]. In addition to sensitising tumour cells to chemotherapeutical agents, AZD1152 can also sensitise to ionising radiation. Interestingly, it was shown that p53-deficiency

of tumour cells exacerbated this effect[41]. So far, inhibition of Aurorakinase B in central chondrosarcomas seems to be a potential therapeutic strategy which deserves more investigation in a pre-clinical setting. The sensitising effect to ionising radiation should be addressed, as well.

The last hypothesis driven approach to find new targets for the therapeutic treatment of chondrosarcoma was based on the presence of COX-2 expression in chondrosarcomas. In **chapter 6** the effect of the specific COX-2 inhibitor celecoxib on chondrosarcoma cell growth was investigated. In addition, **Chapter 6** illustrates that success of an agent *in vitro* does not guarantee its success *in vivo***.** Growth of chondrosarcoma cell cultures was strongly inhibited by celecoxib *in vitro* and a similar effect was found during the first 6 weeks of treatment of xenotransplanted nude mice. A promising role of high-dose prophylactic celecoxib in preventing development of chondrosarcoma was found which deserves further investigation, especially in the context of enchondromatosis and multiple osteochondromas in which the importance of prevention of malignant progression is evident to improve prognosis.

However, in a later stage of the celecoxib treatment, a disconcordant effect was found in the groups treated with low dosage celecoxib, showing catch up growth of the tumours. Future studies should also gain more insight in the mechanisms causing this growth advantage of low dose celecoxib in chondrosarcomas by collecting serum parameters, such as sex steroid hormones, of the animals during the study.

An *in vivo* model in which the tumours can be monitored real time, for example using chondrosarcoma cell lines transduced with luciferase constructs would be of great use in future studies and is currently under development. Also studying the effect of celecoxib on a low-grade chondrosarcoma *in vivo* model would be interesting, since this study was performed using a grade II and grade III chondrosarcoma cell line. In addition an *in vivo* model of peripheral chondrosarcoma, which were also shown to express COX-2, should be studied to further elucidate the role of celecoxib in the treatment of benign and malignant cartilage tumours in MO patients, although such a model is still to be developed (figure 7.1).

7.4 Concluding remarks

The results presented in this thesis have created insight into the major difficulty in the classification and histological grading of cartilaginous tumours. A new algorithm, based on 5 patient-related, and tumour-related characteristics, is proposed to separate benign from malignant cartilaginous tumours, with host bone entrapment and mucoid/myxoid changes being the most important findings in support of malignant potential.

Grading of malignant cartilaginous tumours using a three-tiered grading scheme was shown to be highly variable between different observers, even among those being specialised in bone tumour pathology. The distinction between grade I and grade II guides clinical management to either intraleasional surgery or wide-margin surgery. Despite many attempts, including the ones in this thesis, no molecular markers superior to histological grading were discovered so far. More studies to reveal new markers need to be performed in the near future; for example using protein identification by proteomics, which could lead to the discovery of yet unknown or unexpected biomarkers that i.e. are important in the formation of metastasis in chondrosarcoma. However, it is most important to interpret the results of these studies based on long term follow up, not on subjective histological grading. Thereby, the importance of long term storage including standardisation, automated enrichment of annotations from hospital information systems and disease registries, insight in overlapping collections of different forms of tissue banking and cooperation in national and international networks of patient material is stressed[42].

This thesis presents the identification of new targets for therapeutic treatment of chondrosarcoma, using a relatively new array technique to identify active kinases in chondrosarcoma cell cultures. Src inhibitor dasatinib was investigated more in detail, and in the majority of chondrosarcoma cell cultures cell growth was inhibited. In the near future, the growth inhibition of chondrosarcoma by dasatinib and the underlying mechanism should be tested in a chondrosarcoma model *in vivo*.
In addition to dasatinib, CDK inhibitor Flavoperidol, Heat Shock Protein-inhibitor, Aurorakinase inhibitor AZD1152 and AKT inhibitor Enzastaurin were identified as candidates for therapeutic intervention in chondrosarcomas and their actions and mechanisms should be studied pre-clinically *in vitro* and if promising subsequently *in vivo* (Figure 7.2). Moreover, the use of selective COX-2 inhibition was evaluated preclinically. More experiments are needed to investigate its precise mechanism of growth inhibition since *in vitro* and *in vivo* results were inconclusive.

The results described in this thesis have led to a better understanding of molecular events that are important in the growth of conventional chondrosarcoma. Based on these and previous findings targets for therapeutical treatment of chondrosarcoma were investigated *in vitro* and *in vivo*. In addition, new targets were identified using large scale kinomics, which are expected to lead to the implementation of new therapy modalities for patients that have inoperable or metastatic chondrosarcoma.

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Nieuwe strategieën voor de behandeling van chondrosarcomen

Nederlandse samenvatting

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Nieuwe strategieën voor de behandeling van chondrosarcomen

Chondrosarcomen: entiteit, gradering, behandeling en prognose
Chondrosarcomen zijn kwaadaardige, kraakbeenvormende tumoren die in of op het bot groeien. In de groep conventionele chondrosarcomen, veruit de grootste groep (85%), wordt onderscheid gemaakt tussen centrale chondrosarcomen, die ontstaan in de mergholte van de lange pijpbeenderen, en de perifere chondrosarcomen, die ontstaan aan het oppervlak van het bot. Naast de conventionele chondrosarcomen, beschreven in dit proefschrift, omvat de groep chondrosarcomen ook zeldzame chondrosarcoom soorten, zoals het clear cell chondrosarcoom, het mesenchymale chondrosarcoom en het gededifferentieerde chondrosarcoom.

Conventionele chondrosarcomen komen voor bij ongeveer 1 op 50.000 personen, bij mannen en vrouwen even vaak. De leeftijd waarop de diagnose chondrosarcoom gesteld wordt, is tussen de 30 en 60 jaar. Chondrosarcomen worden met name aangetroffen in de lange pijpbeenderen (dus vooral in benen en armen) die groeien volgens enchondrale ossificatie, dat wil zeggen, met behulp van een groeischijf. In de groeischijf bevinden zich kraakbeencellen die blijven delen, totdat een individu onder invloed van hormonen aan het einde van de puberteit komt en de groeischijven dichtgaan, oftewel verbenen. De meeste chondrosarcomen worden gevonden in de heupbotten, de bovenbenen, de armen en de ribben.

Van zowel centrale als perifere chondrosarcomen worden ook benigne (goedaardige) voorloperstadia gevonden. Centraal groeiende benigne kraakbeentumoren worden enchondromen genoemd, perifeer groeiende benigne kraakbeentumoren osteochondromen. De kans dat een enchondroom kwaadaardig wordt, en dus verandert naar een chondrosarcoom, wordt geschat op minder dan 1%. In 40% van de centrale chondrosarcomen worden echter resten van een goedaardige voorloper teruggevonden. Ook osteochondromen worden in minder dan 1% kwaadaardig. Perifere chondrosarcomen ontstaan per definitie uit een osteochondroom. Om deze reden worden perifere chondrosarcomen ook wel secundaire chondrosarcomen genoemd.

Conventionele kraakbeentumoren worden meestal als solitaire (enkele) afwijkingen gevonden. Echter, bij zowel perifere als centrale kraakbeentumoren is er een syndroom bekend waarbij meerdere goedaardige kraakbeentumoren voorkomen. Multipele Osteochondromen (MO) betreft een erfelijke ziekte waarbij meerdere osteochondromen ontstaan in combinatie met deformatie van de botten van de armen. MO openbaart zich al op kinderleeftijd en vaak blijven de patiënten klein van gestalte. MO-patiënten hebben een licht verhoogde kans dat de osteochondromen kwaadaardig worden, geschat op 1-3%. Het voorkomen van meerdere enchondromen is zeer zeldzaam en wordt enchondromatosis (EC) genoemd, beter bekend als de ziekte van

Ollier. In tegenstelling tot MO is EC geen erfelijke ziekte. De enchondromen groeien vooral in de handen en voeten, wat tot ernstige disfunctie leidt. EC wordt op zowel kinderleeftijd als bij volwassenen manifest. De kans dat een enchondroom kwaadaardig wordt, is sterk verhoogd bij EC-patiënten, namelijk tot circa 33%.

Chondrosarcomen maken deel uit van een klinisch-pathologisch gedefinieerd spectrum, dat verdeeld wordt in vier groepen: benigne tumoren, graad I chondrosarcomen, graad II chondrosarcomen en graad III chondrosarcomen. De onderverdeling van dit spectrum gebeurt internationaal nog immer aan de hand van de in 1960 door Evans opgestelde criteria. Aan de hand van deze criteria worden behandelplannen opgesteld en de prognose voorspeld. Graad I en II chondrosarcomen geven zelden metastases (0% en 10%), terwijl 71% van de graad III chondrosarcomen metastaseert. De daaraan gerelateerde 5-jaarsoverleving is respectievelijk 83%, 64% en 29% voor graad I, II en III chondrosarcomen. Wat de behandeling betreft, worden enchondromen vaak expectatief vervolgd, tenzij er sprake is van pijn of pathologische fracturen. Bij graad I chondrosarcomen wordt tegenwoordig intralesionale chirurgie toegepast, waarbij zoveel mogelijk tumorweefsel verwijderd wordt en de ontstane holte opgevuld wordt met cement of botchips. Adjuvant worden vaak de randen nabehandeld met fenolisatie of cryochirurgie om de maligne cellen die achtergebleven zijn te doden. Een agressiever beleid wordt gevolgd ten aanzien van de graad II en graad III chondrosarcomen, waarbij en-bloc resectie of soms zelfs amputatie noodzakelijk is.

Vraagstellingen in dit proefschrift

Het onderzoek aan chondrosarcomen beschreven in dit proefschrift is gebaseerd op drie vragen. De eerste vraag die beantwoord werd, is of er moleculaire markers zijn die onderscheid kunnen maken tussen benigne en maligne kraakbeentumoren. De tweede vraag is eenzelfde vraag naar markers die de prognose van kraakbeentumoren kunnen voorspellen, onafhankelijk van subjectieve histologische gradering. Het derde vraagstuk behelst de behandeling van chondrosarcomen en het ontwikkelen van nieuwe strategieën voor medicamenteuze therapie.

Benigne versus maligne kraakbeenvormende tumoren
In **hoofdstuk 2** werd bevestigd dat de criteria voor gradering van chondrosarcomen van Evans relatief subjectief zijn en dus afhankelijk van de patholoog die de gradering uitvoert. Achttien pathologen, geaffilieerd aan Eurobonet, een Europees netwerk waarbinnen de biologie en pathologie van bottumoren wordt bestudeerd, beoordeelden zestien casus. De variabiliteit tussen de beoordelingen bleek ruim te zijn, uitgedrukt in een kappawaarde van 0.78. De concordantie was het kleinst in het onderscheid tussen benigne en graad I chondrosarcomen, namelijk κ=0.54. In dezelfde studie werden van een grote groep van 20 enchondromen en 37 chondrosarcomen gegevens verzameld, waarvan tien jaar follow-up bekend was en die in een multidisciplinair verband gediagnosticeerd waren, door pathologen, radiologen en chirurgen. Hieruit bleek dat een accurate diagnose gesteld kon worden aan de hand van slechts twee histologische criteria, te weten invanging van pre-existent lamellair bot en mucoide/myxoide veranderingen van de extracellulaire matrix. Hiermee werden 54 van de 57 (94.7%) casus juist gediagnosticeerd (sensitiviteit 95% and specificiteit 95%). Wanneer de beslissing op vijf parameters gebaseerd werd, werd slechts één van de 57 gevallen verkeerd gediagnosticeerd. Deze vijf parameters waren: invanging, hoge celrijkdom, open chromatine in de kern, mucoide/myxoide degeneratie van de matrix en de leeftijd van de patiënt (ouder dan 45 jaar).

De Indian hedgehog (IHH), WNT en Transforming growth factor β (TGFB) signaaltransductie regelkringen spelen een belangrijke rol in de normale groei en differentiatie van kraakbeencellen in de groeischijf. IHH doet dit via het intracellulair (in de kraakbeencel) gelegen “Smoothened” (SMO) en “Patched” (PTCH), waardoor “Gliobastoma” (GLI) naar de celkern getransporteerd wordt. In de celkern bindt GLI aan het DNA en zorgt er voor dat er weer nieuwe SMO, PTCH en GLI worden afgeschreven waardoor het signaal versterkt wordt (Figuur 1.4). Het gaat uiteindelijk om het signaal dat door deze kraakbeencel via de binding van “parathyroid hormone like hormone” (PTHLH) wordt doorgegeven aan de parathyroid hormoon receptor (PTHR1). PTHR1 zorgt er vervolgens voor dat de kraakbeencellen in de prolifererende zone (Figuur 1.3) niet verder differentiëren, waardoor de botten in de lengterichting groeien. Voor het doorgeven van deze signalen door de extracellulaire matrix waarin de kraakbeencellen gelegen zijn, worden de lange eiwit-suikermoleculen gebruikt die in de matrix aanwezig zijn; de zogenoemde heparan sulfaat proteoglycanen (HSPG’s). Voor het verlengen van de suikerketens zijn twee genen belangrijk, EXT1 en EXT2. Uit genetische studies weten we dat bij patiënten met MO een van beide EXT-genen gemuteerd is en dus niet of minder goed werkt. Ook in perifere chondrosarcomen die solitair voorkomen, dus niet in verband met de erfelijke ziekte MO, is de expressie van het EXT-gen verminderd vergeleken met de expressie in de normale groeischijf. Interessant genoeg

blijkt de PTHLH signaaltransductiecascade echter niet aangedaan te zijn in hooggradige perifere chondrosarcomen. Gesuggereerd wordt dat er ofwel kraakbeencellen zijn die zelf PTHLH produceren of dat de functie van IHH overgenomen wordt door TGFB, dat juist in de hooggradige perifere chondrosarcomen hoger tot expressie komt. Ook is het mogelijk dat het aan IHH en TGFB gerelateerde WNT deze functie overneemt.

In **hoofdstuk 3** werden deze signaaltransductieroutes in centrale chondrosarcomen onderzocht. Het bleek echter dat, in tegenstelling tot in de perifere chondrosarcomen, in centrale chondrosarcomen EXT normaal is, zowel de DNA-volgorde als de afschrijving op mRNA-niveau. Tevens was IHH normaal actief, hoewel er toch afwijkingen in de distributie van de HSPG's in de matrix gevonden werden. Een van de HSPG's, syndecan-2, werd zelfs intracellulair in de kraakbeencellen gevonden in plaats van in de matrix. Meer onderzoek is nodig om deze bevinding te kunnen verklaren.
Door middel van β-catenine kleuring vonden we in graad I centrale chondrosarcomen een significant hogere activiteit van WNT dan in enchondromen (respectievelijk 48% en 17%) (Figuur 3.3). Deze verschillen zijn echter niet groot genoeg om β-catenine een rol te geven als immunohistologische marker bij het maken van onderscheid tussen beiden.

Markers om de prognose van chondrosarcomen te voorspellen
Veel onderzoeken hebben zich toegespitst op de vraag of er moleculaire markers zijn die de prognose van chondrosarcomen kunnen voorspellen. In **Hoofdstuk 2** werd naast variatie in het onderscheid tussen benigne en maligne kraakbeentumoren, ook een grote variatie in het graderen van hooggradige chondrosarcomen gevonden. Gradering is momenteel de enige basis voor het stellen van prognoses. De grote variatie hierin bevestigt de noodzaak van het ontdekken van objectieve moleculaire markers die het onderscheid tussen de drie verschillende graden kunnen maken en daarmee de keuze voor een bepaalde behandeling en de te verwachte prognose kunnen aanwijzen.

In dit proefschrift werd de vraag naar prognostische markers voor chondrosarcomen uitgediept. In **hoofdstuk 3** werd een associatie gevonden tussen histologische graad (volgens Evans) en de expressie (mate van aanwezigheid) van TGFB (Figuur 3.3). TGFB is verantwoordelijk voor velerlei signaaltransductie routes die bijvoorbeeld leiden tot proliferatie, overleving van cellen, de vorming van matrix (steunweefsel) rond cellen en het vormen van bloedvaten. Het is dus goed voor te stellen dat maligne tumoren beter in staat zijn deze eigenschappen te verzamelen dan benigne. Bij analyse van deze uitkomsten in het kader van de uitgebreide follow-up kon echter geen relatie tussen TGFB en een slechte prognose gelegd worden die onafhankelijk was van histologische graad.

Datzelfde gold voor *CDK4* en *MDM2* in **hoofdstuk 4**. Beide genen zijn gelegen op chromosoom 12q13, een locus waarvan uit eerdere studies bleek dat deze geamplificeerd is in centrale chondrosarcomen. Tevens hebben beide genen een functie in de celcyclus. De celcyclus is een soort voorgeprogrammeerd protocol dat de levensverwachting en voortplanting van een cel dicteert. Cellen doorlopen achtereenvolgens een M-fase (Mitose) waarin de kern wordt gedeeld en de cel wordt gesplitst. De periode tussen twee M-fases wordt de interfase genoemd. De interfase bestaat uit de G1-, S- en G2-fase (Figuur 4.3). Tijdens de S-fase (Synthese), repliceert de cel zijn DNA, wat essentieel is voor celdeling. Voorafgaand aan, én aansluitend op de S-fase zijn er twee fases waarin de cel kan groeien: de G1- en G2-fase (Gap (*ruimte*)). Gedurende deze gap-fases wordt er ook gecontroleerd of er geen fouten gemaakt zijn bij de replicatie en of alle voorbereidingen getroffen zijn om de cel te laten doorgaan met de volgende fase. Deze controle wordt onder andere uitgevoerd door CDK4 en MDM2.
In centrale chondrosarcomen werd een correlatie gevonden tussen zowel amplificatie van 12q13 en een verhoogde expressie van *CDK4*, als tussen de expressie van CDK4 en MDM2 en histologische graad (figuur 4.3). Echter, een relatie, onafhankelijk van histologische graad, tussen CDK4-expressie en prognose kon niet worden vastgesteld.

Het onderzoek naar de variatie in gradering van kraakbeentumoren, beschreven in hoofdstuk 2, bevestigt tevens dat conclusies uit studies naar nieuwe moleculaire markers altijd in het kader van uitgebreide follow-up getrokken moeten worden. Onderzoeksresultaten moeten dus gerelateerd worden aan objectieve gegevens zoals “metastasevrije periode”. Tevens benadrukt het de noodzaak van een goede archivering van studiemateriaal, zowel het patiëntenmateriaal als de bijbehorende follow-up data.

Nieuwe strategieën voor de behandeling van chondrosarcomen
Chondrosarcomen reageren niet op conventionele radiotherapie en chemotherapie. Dat betekent dat chirurgie de enige curatieve behandelingsmodaliteit is. Curatief wil zeggen dat als doel van de behandeling genezing wordt beoogd, in tegenstelling tot palliatief waarbij pijnbestrijding en het behoud van kwaliteit van leven als doel gesteld wordt.
Dit impliceert dat patiënten bij wie het chondrosarcoom ontstaan is op een plaats die niet toegankelijk is voor chirurgie met vrije marges (bijvoorbeeld in de bekken- of de schedelbasisbotten), en patiënten met tumoren die gemetastaseerd zijn naar organen zoals de longen, per definitie niet curatief behandeld kunnen worden waardoor ze een zeer slechte prognose hebben. Recent echter lijken er goede resultaten te zijn voor deze tumorlokalisaties bij bestraling met protonen, waarbij de stralingsbundel heel precies gericht kan worden en er minimale schade aan de omliggende weefsels ontstaat. De laatse doelstelling van dit proefschrift was het ontwikkelen van nieuwe strategieën voor de medicamenteuze behandeling van chondrosarcomen.

Deze doelstelling werd op twee manieren aangepakt: volgens op hypotheses gebaseerde methodes (hoofdstuk 3, 4 en 6) en volgens een op screening gebaseerde methode (hoodstuk 5). Nieuwe strategieën verkregen uit beide methodes werden *in vitro* getest op levende chondrosarcoomcellen. Tevens werd één strategie verder bestudeerd in een chondrosarcomen muizenmodel, *in vivo*.

In **hoofdstuk 3** werd onderzocht of de activiteit van IHH, die in centrale chondrosarcomen aanwezig bleek te zijn, als aangrijpingspunt voor therapie kon dienen. Zes chondrosarcoom-cellijnen werden daartoe behandeld met cyclopamine. Cyclopamine remt de activiteit van de IHH signaaltransductieroute door het blokkeren van intercellulair SMO. Het is momenteel in ontwikkeling als behandeling van onder andere glioblastomen en medulloblastomen (twee soorten hersentumoren). Echter, in slechts een van de zes chondrosarcoom-cellijnen werd de celgroei geremd. Verder onderzoek naar de effectiviteit van cyclopamine bij de behandeling van kraakbeentumoren lijkt derhalve niet zinvol.

Een ander aangrijpingspunt voor de behandeling van chondrosarcomen is beschreven in **hoofdstuk 4**, te weten de verhoogde expressie van *CDK4*-in hooggradige chondrosarcomen. *In vitro* werd met behulp van short hairpin RNA (shRNA) alle *CDK4* activiteit in drie chondrosarcoom-cellijnen weggevangen. Deze shRNA-stukjes werden in de cellen gebracht met behulp van lentivirussen. Dit leidde in alledrie de cellijnen tot remming van de celgroei. Eenzelfde effect werd bewerkstelligd door het herintroduceren van het *p16*-gen in deze cellen. Het p16-eiwit heeft een remmende rol in de celcyclus waarin ook CDK4 betrokken is. In hooggradige centrale chondrosarcomen is

de genetische informatie van het *p16* gen vaak verdwenen (gedeleteerd). Door het herintroduceren van p16, eveneens met behulp van lentivirussen, werd de celgroei geremd in de drie chondrosarcoom cellijnen. Beide strategieën zijn echter alleen toepasbaar in het laboratorium en niet op patiënten. Van het middel Flavopiridol is bekend dat het CDK's remt *in vivo*. Flavopiridol zou dus door het remmen van CDK4 een rol kunnen spelen bij de behandeling van chondrosarcomen. Meer onderzoek is nodig om dit verder uit te diepen.

Uit de vakliteratuur en de studie beschreven in **hoofdstuk 6** blijkt dat cyclo-oxygenase-2 (COX-2) ook verhoogd tot expressie komt in hooggradige chondrosarcomen, wat een interessant aangrijpingspunt is voor therapie. Tevens werd in enchondromatosis gerelateerde tumoren een hogere COX-2-expressie gevonden dan in solitaire tumoren. COX-remmers zijn geregistreerd als analgetica en ontstekingsremmers en er is veel onderzoek gedaan naar hun veiligheid. Celecoxib en NS-398, twee selectieve COX-2-remmers, bleken *in vitro* celgroei te remmen in drie van de vier chondrosarcoom cellijnen. Vervolgens werd de behandeling met celecoxib in een chondrosarcoom muizenmodel onderzocht. Bij immunoincompetente muizen werden menselijke chondrosarcoomcellen onder de huid ingespoten. Deze cellen vormen een xenograft en zo kan de groei van de tumor tijdens het experiment, vanaf de buitenkant van de muis, gevolgd worden. De muizen werden behandeld met ofwel een celecoxib profylaxe, dat wil zeggen dat de behandeling één week voordat chondrosarcoomcellen ingespoten werden, gestart werd, ofwel de behandeling werd gestart vier weken nadat de cellen ingespoten waren. Beide groepen werden nog onderverdeeld in lage en hoge dosis celecoxib. De profylaxegroep boekte vooral in de eerste vier weken van het experiment goede resultaten en de tumoren bleven kleiner dan in de controlegroep. Na week zes begonnen de tumoren die met een lage concentratie celecoxib behandeld werden (zowel de profylaxe- als de behandelinggroep) harder te groeien dan de controlegroep. De tumoren die profylactisch met een hoge dosis celecoxib werden behandeld, bleven gedurende het hele experiment kleiner dan die in de controlegroep.
Er lijkt dus een beschermend effect te zijn van een hoge concentratie celecoxib profylaxe, wat een optie zou kunnen zijn voor de behandeling van enchondromen en osteochondromen bij EC- en MO-patiënten ter voorkoming van maligne ontaarding. Echter, meer onderzoek naar de plotselinge groeiversnelling bij lage celecoxibconcentraties is nodig voordat therapie bij patiënten gerechtvaardigd kan worden.

In **hoofstuk 5** werd op een screenende wijze gezocht naar nieuwe strategieën voor de behandeling van chondrosarcomen. Hiertoe werd gebruik gemaakt van "kinome profiling" met behulp van de nieuwe Pepchip®-techniek. Het kinoom is de verzameling van kinases in het menselijk lichaam. Een kinase is een enzym dat een fosfaatgroep kan aanbrengen op een ander eiwit of

een ander molecuul (fosforylering). Door fosforylering wordt het doeleiwit geactiveerd of geïnactiveerd. Deze schakelfunctie kan zo chemische reacties in de cel aansturen en vormt een belangrijke factor in de interne signaaltransductie. Dat maakt kinases een uitermate goed aangrijpingspunt voor behandeling. De fosfaatgroep die wordt gebruikt door een kinase is vaak afkomstig van een molecuul adenosine-tri-fosfaat (ATP) en op dit principe berust de Pepchip®, waarbij activiteit van de kinases gemeten wordt door het inbouwen van radioactief fosfaat.
In het onderzoek beschreven in **hoofdstuk 5** werd activiteit van kinases gemeten in vier chondrosarcoom cellijnen en negen primaire kweken van chondrosarcoompatiënten. Activiteit van de Src-familie van kinases en van PDGFRβ werd gevonden in alle celkweken. Src is een belangrijke regulator in verschillende intracellulaire processen, zoals embryogenese en celgroei. Genetische mutaties in het Src-gen leiden tot een verhoogde graad van maligniteit van colorectaal carcinomen. In **hoofdstuk 5** werden vervolgens Src-remmer dasatinib, bekend van de behandeling van leukemieën, en PDGFRβ-remmer imatinib, bekend van de behandeling van gastro-intestinale stromaceltumoren, getest op hun celgroeiremmende eigenschappen op chondrosarcoomcellen. Hoewel chondrosarcomen ongevoelig bleken te zijn voor imatinib, werd met dasatinib in zeven van de negen celkweken een groeiremmend effect waargenomen. Hiermee is dasatinib geïdentificeerd als een potentiële behandelingsoptie voor inoperabele chondrosarcomen en deze bemoedigende data verdienen in de toekomst verdere aandacht in *in vivo* modellen voor chondrosarcoomgroei, alsmede in klinische trials.

Naast de Src-familie werden met behulp van de Pepchip® ook nog AKT, Aurorakinase B en CDK2 geïdentificeerd als actief in chondrosarcoom celkweken. Elk van deze kinases is interessant voor verdere studies naar nieuwe behandelingsstrategieën voor het chondrosarcoom. Van AKT is in andere studies aangetoond dat het een rol speelt in de overleving van chondrosarcoomcellen. AKT speelt een rol in verschillende pathofysiologische processen zoals het ontstaan van diabetes mellitus, Alzheimer en carcinogenese. AKT-activiteit kan geremd worden met behulp van Enzastaurine. Momenteel worden *in vitro* experimenten uitgevoerd die gebaseerd zijn op deze bevinding.
Aurorakinase B speelt een belangrijke rol bij de celdeling. Het zorgt ervoor dat het DNA op de juiste manier uit elkaar getrokken wordt tijden de mitose. Overexpressie van Aurorakinase B kan leiden tot aneuploidie: een onevenwichtige verspreiding van het genetische materiaal tussen twee kernen. Aneuploidie wordt veel gevonden in hooggradige chondrosarcomen. Pyrazoloquinazolines als AZD-1152 zijn in staat Aurorakinase B te remmen. Flavopiridol, dat in **hoofdstuk 4** als potentiële therapie voor chondrosarcomen genoemd werd als CDK4-remmer, heeft ook een remmende werking op CDK1 en 2. Het feit dat CDK1 en 2-remming en daarmee Flavopiridol in

hoofdstuk 5 opnieuw als mogelijke strategie voor behandeling gevonden wordt, versterkt het belang van deze bevindingen.

Samenvattend hebben zowel de hypothese-gerelateerde als de screenende methode meerdere voorzetten gegeven voor vervolgstudies, welke hopelijk zullen leiden tot nieuwe therapeutische strategieën voor de behandeling van inoperabele en gemetastaseerde chondrosarcomen.

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Curriculum Vitae

Yvonne Schrage werd geboren op 8 mei 1982 te Spijkenisse. Na het behalen van het gymnasiumdiploma aan het "Erasmiaans gymnasium" te Rotterdam in 2000, begon zij in september 2000 met de studie biomedische wetenschappen aan de Universiteit Leiden. In september 2001 begon zij aan de studie geneeskunde aan diezelfde universiteit. Zij liep stage op de afdeling Pathologie onder begeleiding van Prof. Dr. P.C.W. Hogendoorn met als onderwerp de karakterisatie van de matrix van myxoide tumoren. In augustus 2004 behaalde zij haar bachelordiploma biomedische wetenschappen en haar doctoraaldiploma geneeskunde. Van oktober 2004 tot en met november 2008 voerde zij als assistent-in-opleiding het in dit proefschrift beschreven promotieonderzoek uit op de afdeling Pathologie van het Leids Universitair Medisch Centrum, onder supervisie van Dr. J.V.M.G. Bovée en Prof. Dr. P.C.W. Hogendoorn. Zij ontving in 2007 de Best Basic Science Award van de European Musculoskeletal Oncology Society voor onderdelen van het in dit proefschrift beschreven onderzoek. In januari 2009 is zij gestart met de coschappen om de studie geneeskunde af te ronden.

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