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The role of EXT and growth signalling pathways in osteochondroma and its progression towards secondary peripheral chondrosarcoma

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Decreased EXT expression and intracellular accumulation of HSPG in osteochondromas and peripheral chondrosarcomas

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Abstract
Mutational inactivation of *EXT1* or *EXT2* is the cause of hereditary Multiple Osteochondromas. These genes function in heparan sulphate proteoglycan (HSPG) biosynthesis in the Golgi apparatus. Loss of heterozygosity of the *EXT1* locus at 8q24 is frequently found in solitary osteochondromas, whereas somatic mutations are rarely found. We investigated the expression of *EXT1* and *EXT2* (quantitative RT-PCR) and of different HSPGs (immunohistochemistry) in solitary and hereditary osteochondromas and in cases with malignant progression to secondary peripheral chondrosarcoma, in relation to possible mutations and promoter methylation. The mutation status of patients with Multiple Osteochondromas correlated with decreased *EXT1* or *EXT2* expression found in their resected tumours. We could not show somatic point mutations or promoter hypermethylation in 17 solitary tumours; however *EXT1* expression was decreased in 15 cases, whereas *EXT2* was not. Intracellular accumulation of syndecan-2 and heparan sulphate-bearing isoforms of CD44 (CD44v3) was found in most tumours, which concentrated in the Golgi apparatus as shown by confocal microscopy. This contrasted with the extracellular expression found in normal growth plates. In conclusion, mutational inactivation of either *EXT1* or *EXT2*, leads to loss of mRNA expression of the corresponding gene. We hypothesize that loss of *EXT* expression disrupts the function of the EXT1/2 complex in HSPG biosynthesis, resulting in the intracellular accumulation of HSPG core proteins that we found in these tumours.

Keywords: osteochondroma; chondrosarcoma; heparan sulphate proteoglycan; EXT1; EXT2; growth plate; bone neoplasm

Introduction

Osteochondromas are the most common benign bone tumours, arising at the external surface of bones preformed by endochondral ossification [1]. They consist of a bony stalk covered by a cartilage cap, which histologically shows resemblance with the epiphyseal growth plate. About 15% of osteochondromas occur as Multiple Osteochondromas (MO) [2,3], a familial skeletal disorder with an autosomal dominant mode of inheritance [4,5]. One to three per cent of hereditary and <1% of solitary osteochondromas transform towards their malignant counter part, secondary peripheral chondrosarcoma [1,3,6].

For MO two genes, exostosis (multiple)-1 (*EXT1*) and exostosis (multiple)-2 (*EXT2*), located at 8q24 and 11p11-12, respectively [7-9], have been identified. Of the Multiple Osteochondromas families 44-66% show linkage to *EXT1* [10,11] and 27% to *EXT2* [11].

The protein products of *EXT1* and *EXT2*, exostosin-1 and –2 (EXT1 and EXT2), are type II transmembrane glycoproteins and are as a Golgi-localized hetero-oligomeric complex involved in the heparan sulphate proteoglycan (HSPG) biosynthesis [12,13]. Proteoglycans are proteins that bear chains of glycosaminoglycans (polymers of specific disaccharide repeats), like heparan sulphates (HS) [14]. After formation of a linkage region on the HSPG protein core, the EXT-like genes, *EXTL2* and *EXTL3*, are thought to initiate HS polymerization [15,16]. Elongation of the HS chain is catalysed by the EXT1/EXT2 complex [12].

HSPGs are large multifunctional macromolecules, involved in several growth signalling pathways, anchorage to the extracellular matrix (ECM), and sequestering of growth factors (reviewed by Knudson et al. [17] and Selleck et al. [14]). Several HSPGs have been identified, like the syndecan and glypican families, perlecan, and CD44 isoforms containing HS bearing variable exon 3 (v3) [17,18]. In the growth plate, HSPGs have been shown to be involved in regulation of chondrocyte proliferation and differentiation through Indian Hedgehog (IHH)/ parathyroid hormone-like hormone (PTHLH, PTHrP) and Fibroblast growth factor (FGF) signalling [19-21].

Altered expression of *EXT* genes in osteochondromas and chondrosarcomas, e.g. because of mutations and/or loss of heterozygosity (LOH), is hypothesized to disturb HSPG synthesis, consequently affecting IHH/PTHLH and FGF signalling in these lesions. We previously demonstrated that in osteochondromas IHH signalling is not affected [22], whereas both PTHLH and FGF signalling are absent [23,24].

Interestingly, in solitary lesions LOH and clonal rearrangement of 8q24 are as frequent as in hereditary tumours [25-28], while somatic mutational inactivation of an *EXT1* allele has been described in only two solitary osteochondromas [29,30] and one solitary chondrosarcoma [31]. LOH at the *EXT2* locus has been reported in one solitary osteochondroma [27] and somatic mutations are absent.

In the present study we compared both solitary and hereditary osteochondromas and secondary peripheral chondrosarcomas and investigated the *EXT* genes at the genetic (mutations and methylation) and expression level (quantitative reverse transcriptase-polymerase chain reaction [RT-PCR]). In addition, we studied the expression of HS chains and HSPG protein cores (immunohistochemistry).

Table IV.I. Tumour and clinical data (fresh frozen tissues) and *EXT* status

ID	Material	MO/ solitary	Location	Gender	Age (years)	*EXT* mutation analysis* (type of mutation)	LOH 8q24†	LOH 11p11.2-13†	*EXT1* expression‡	*EXT2* expression‡
GP-29§	GP		Knee	female	12				1.11	1.12
GP-53§	GP		Hip	female	12				1.05	0.67
GP-62§	GP		Femur	female	8				1.17	1.14
GP-67	GP		Unknown	male	12				0.73	1.17
								average expression GP:	1 ± 0.19	1 ± 0.25
L-298§,¶	OC	MO	Pelvis	male	24	*EXT1* exon 3: c.1121G>A, p.W374X (ns)	ni	ni	**0.13**	**0.10**
L-841§,¶	OC	MO	Femur	female	14	*EXT2* deletion exon 6+7 (del)	ni	ni	**0.34**	**0.11**
L-1235	OC	MO	Radius	female	5	*EXT1* deletion exon 4-11 (del) *EXT2* exon 10: c.1641C>T, p.D547D (uv)	ni	ni	0.55	**0.25**
L-657§,¶	OC	solitary	Femur	female	12	none in *EXT1/2*	yes	no	**0.32**	1.54
L-673§,¶	OC	solitary	Humerus	male	12	none in *EXT1/2*	no	no	**0.08**	0.93
L-1247§,¶	OC	solitary	Femur	male	30	none in *EXT1/2*	ni	ni	**0.05**	0.55
L-114§	PCS-I	MO	Femur	male	39	*EXT2* exon 5: c.764T>C, p.L255P (uv)	yes	yes	**0.08**	1.42
L-758§,¶	PCS-I	MO	Femur	male	41	*EXT2* deletion exon 6+7 (del)	ni	ni	**0.15**	**0.31**
L-1190§,¶	PCS-I	MO	Humerus	female	16	*EXT2* exon7: c.1080-2a>g (splice)	ni	ni	**0.29**	**0.09**
L-163§,¶	PCS-I	solitary	Tibia	male	49	none in *EXT1/2*[c]	yes	no	**0.03**	0.35
L-626§,¶	PCS-I	solitary	Femur	male	21	none in *EXT1/2*	yes	no	**0.25**	0.58
L-627§,¶	PCS-I	solitary	Scapula	female	31	*EXT2* exon 2: c.499A>T, p.I167F (uv)	ni	ni	**0.16**	**0.08**
L-724§,¶	PCS-I	solitary	Ilium	female	82	none in *EXT1/2*	ni	ni	**0.21**	1.11
L-739§,¶	PCS-I	solitary	Pubis	male	37	*EXT2* exon 4: c.710C>T, p.S237L (pm)	ni	ni	0.71	0.73
L-868§,¶	PCS-I	solitary	Humerus	male	18	none in *EXT1/2*	ni	ni	0.38	1.14
L-1245¶	PCS-I	solitary	Fibula	male	25	none in *EXT1/2*	ni	ni	**0.10**	0.57
L-308¶	PCS-II	MO	Scapula	male	42	*EXT1* exon 6: c.1469delT, p.L490fsX (fs)	yes	yes	**0.18**	0.59
L-11¶	PCS-II	solitary	Ilium	male	49	none in *EXT1/2*[c]	yes	no	**0.06**	**0.30**
L-76¶	PCS-II	solitary	Pelvis	male	47	*EXT1* exon1: c.947A>G, p.N316S (uv)[c]	no	no	**0.18**	**0.32**
L-123§,¶	PCS-II	solitary	Femur	male	60	none in *EXT1/2*	no	yes	**0.11**	**0.12**
L-224§,¶	PCS-II	solitary	Ilium	male	26	none in *EXT1/2*	ni	ni	**0.11**	0.58
L-493§,¶	PCS-II	solitary	Rib	male	37	none in *EXT1/2*	yes	yes	0.59	0.74
L-1165§,¶	PCS-II	solitary	Pelvis	female	37	none in *EXT1/2*	ni	ni	**0.09**	0.46
L-281§,¶	PCS-III	solitary	Scapula	female	39	none in *EXT1/2*	yes	yes	N/A	**0.18**
L-300§	PCS-III	MO	Scapula	female	61	none in *EXT1/2*	yes	yes	0.64	1.54

GP = growth plate; OC = osteochondroma; PCS = peripheral secondary chondrosarcoma grade I, II or III.
* Mutation nomenclature was according to the Nomenclature Working Group [34]; ns=non-sense; del=deletion; uv = unclassified variant; fs = frame shift; pm = polymorphism.
† Results of mutation and LOH analysis from previous studies [25,35]; ni = not investigated.
‡ Relative expression of *EXT1* and *EXT2* as fraction of the average expression in growth plates. Fold changes > 3 are indicated in bold; N/A = not available. Expression data were first normalized to the expression of four normalization genes as described previously [22,36].
§ Paraffin-embedded material available for immunohistochemical series.
¶ Included in methylation specific PCR experiment.

Material and methods

Material

Tumour tissue was obtained from the cartilage cap of resected specimens. Post-natal growth plates were acquired from resections or biopsies for orthopaedic clinical conditions not related to osteochondroma or chondrosarcoma. Embryonic growth plates were obtained from legal autopsies of premature births or spontaneously aborted fetuses. In all growth plates resting, proliferating and hypertrophic chondrocytes could be distinguished.

Tumour-related data were obtained by review of pathology specimens and reports by two pathologists specializing in bone tumour diagnosis (PCWH and JVMGB). For secondary peripheral chondrosarcoma a pre-existing osteochondroma was documented at either gross pathology or radiographically [32]. Histological grading of chondrosarcoma was performed according to Evans et al. [33]. Multiplicity of osteochondromas was considered the criterion for MO since a family history was not always available [23]. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue in The Netherlands", Dutch Federation of Medical Scientific Societies).

Fresh frozen tissue for DNA and RNA isolation

Material was available from four post-natal growth plates, six osteochondromas and 19 secondary peripheral chondrosarcomas (table IV.I). DNA and RNA were isolated as described previously [25,37]. The tumour percentage, determined at haematoxylin and eosin stained frozen sections, was at least 70%.

Three tumours (L-11, L-76 and L-163) were previously screened for mutations [25]. Only L-76 (solitary grade II chondrosarcoma) demonstrated a constitutional *EXT1* DNA alteration, but this alteration did not affect the enzymatic activity of *EXT1* [38]. Twelve tumours were previously studied for LOH at the *EXT1* and *EXT2* loci (table IV.I) [25,35].

Formalin-fixed, paraffin embedded tissue for immunohistochemistry

Material was available for 64 tumours from 63 patients and 20 normal growth plates (pre-natal n = 8, post-natal n = 12) (table IV.II). Twenty tumours and three growth plates were also included in the frozen tissue series (table IV.I). Four hereditary grade I chondrosarcomas were retrieved from other laboratories [24] using the national pathology database (PALGA), while all other cases originated from the local archives.

Mutation analysis

All eight patients with MO and 14 solitary tumours were screened for DNA alterations in the coding sequence of *EXT1* or *EXT2* by direct sequencing [39]. DNA was isolated from resected tumours except for three Multiple Osteochondromas patients for whom DNA was isolated either from peripheral blood after informed consent (n = 2) or from muscle tissue originating from the resection specimen (n = 1). If no alterations were found, a multiplex ligation-dependent probe amplification assay designed for *EXT1* and *EXT2* was performed to identify possible large deletions, as described previously [40].

Methylation specific PCR

EXT1 CpG island promoter methylation was investigated in four hereditary tumours with a known mutation but unknown LOH status, and 17 solitary lesions using methylation-specific PCR as described previously [41]. HL-60 (acute promyelocytic leukemia cell line) and MCF-7 (breast cancer cell line) (both obtained from ATCC, Manassas, VA, USA) were used as respectively positive and negative controls.

Table IV.II. Tumour-related data of formalin-fixed paraffin embedded series

	Osteochondroma	**Peripheral chondrosarcoma**	**Growth plate**
	(*n* = 22)	**(*n* = 42)**	**(*n* = 20)**
Male vs. female*	16 vs 6	20 vs 22	9 vs 9
Median age at diagnosis in years	20	37	5
Range*	(4-39)	(14-82)	(4mo GA-16yrs)
Histological grade			
Grade I		26	
Grade II		13	
Grade III		3	
Multiple osteochondromas (known *EXT* mutations)	11(10)	13(7)	

* Patients with inconclusive data were omitted
GA = gestational age

Quantitative RT-PCR (qPCR)

For first strand cDNA synthesis, 1 μg of total RNA was reverse transcribed by AMV-RT (Roche, Penzberg, Germany) with 100ng Primer (dT) 15 (Roche) and 50ng Random Primers (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was diluted 1:25 in water and stored until further use at -20°C.

PCR-primers and *CD44v3* TAQman probe (supplementary table IV.I) were designed with Beacon Designer 3 software (Premier Biosoft International, Palo Alto, CA, USA). PCR conditions are available upon request. Reactions were performed using qPCR Corekits for SybrGreen or TAQman probes (Eurogentec, Seraing, Belgium) and run on an iCycler (BioRad, Hercules, CA, USA). All samples were measured in duplicate. Expression levels were normalized to four genes (*CPSF6*, *GPR108*, *CAPNS1,* and *SRPR*) that were selected based on their stable expression in profiling experiments of cartilaginous tumours [22,36]. Data analysis was performed as described previously [36].

EXT1 and *EXT2* were expressed in all control tissues tested (growth plates, fetal mesenchymal cells, placenta, normal mammary, colon, muscle, leucocytes, osteosarcoma, mammary cancer and two colon cancer cell lines; data not shown). Highest expression was found in growth plates and fetal mesenchymal stem cells. *EXT1* and *EXT2* are described to be expressed in the proliferating and prehypertrophic chondrocytes of the growth plate [42]. Since osteochondromas histologically resemble the growth plate [1], expression profiles of solitary and hereditary osteochondromas, low-grade (grade I) and high-grade (grade II and III) chondrosarcomas were compared with growth plates. We used one-way analysis of

variance with Bonferroni correction and data were visualized with box plots in SPSS 10.0 (SPSS Inc, Chicago, IL, USA). Correlation between expression and histological grade was investigated with Pearson correlation. Corrected *p*-values ≤0.05 were considered significant.

Immunohistochemistry

Details of antibodies are described in supplementary table IV.II [43-45]. Immunohistochemical reactions were performed as described previously [46]. The commercially available antibody against perlecan (Neomarkers, Fremont, CA, USA) did not give reproducible results in our hands on formalin-fixed, paraffin-embedded material, despite testing numerous protocols.

The 10E4 antibody reacts with an epitope that occurs in native HS chains [44]. Six samples expressing native HS chains (three growth plates, two solitary osteochondromas and three grade I chondrosarcomas) were pre-treated with heparitinase (heparan sulphate lysase, EC 4.2.2.8, Seikagaku, Kogyo Co. Ltd, Tokyo, Japan) to test the specificity of 10E4 staining [44]. Heparitinase cleaves HS chains specifically at the 2-acetamido and 2-sulfamido-2-deoxy-D-glucosyl-α(1-4) D-glucuronate linkages. A specific antibody (3G10) against cleaved HS chains has previously been generated, enabling investigation of expression of HS chains independent of size and complexity [44]. Unfortunately, this 3G10 antibody was shown not to work reliably on decalcified tumour material and was therefore not used in this study.

Two pairs of observers evaluated the slides independently. Sections were scored on presence (positive, negative) and location (cytoplasmic, membrane, ECM) of the staining. In all 22 osteochondromas and 29/42 chondrosarcomas proliferating cells and hypertrophic/calcifying cells could be evaluated separately. In the remaining 13 chondrosarcomas hypertrophic/calcifying cells were absent. Observers were blinded towards all clinico-pathological data.

Confocal microscopy to detect intracellular localization

One osteochondroma and four chondrosarcomas with peri-nuclear CD44v3 expression were selected for fluorescent double staining using CD44v3 and the 58K Golgi protein antibodies (supplementary table IV.II). Staining was detected using Alexa Fluor® 488 or 647 secondary antibodies (Molecular Probes, Invitrogen) and scanned with an LSM 510 confocal fluorescence microscope (Zeiss, Jena, Germany), as described previously [47].

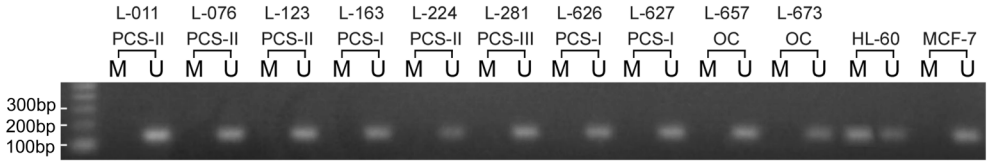

Figure 4.1. Methylation-specific PCR for *EXT1* promoter in osteochondromas and chondrosarcomas. The presence of a PCR band under lane M indicates methylated *EXT1* promoter, whereas an unmethylated *EXT1* promoter is indicated by the presence of a PCR band under lane U. None of the tumours demonstrated a PCR product for methylated *EXT1* promoter. The cell lines HL-60 and MCF-7 served as controls for methylated and unmethylated *EXT1* promoter, respectively. PCS = peripheral secondary chondrosarcoma grade I, II or III; OC = osteochondroma.

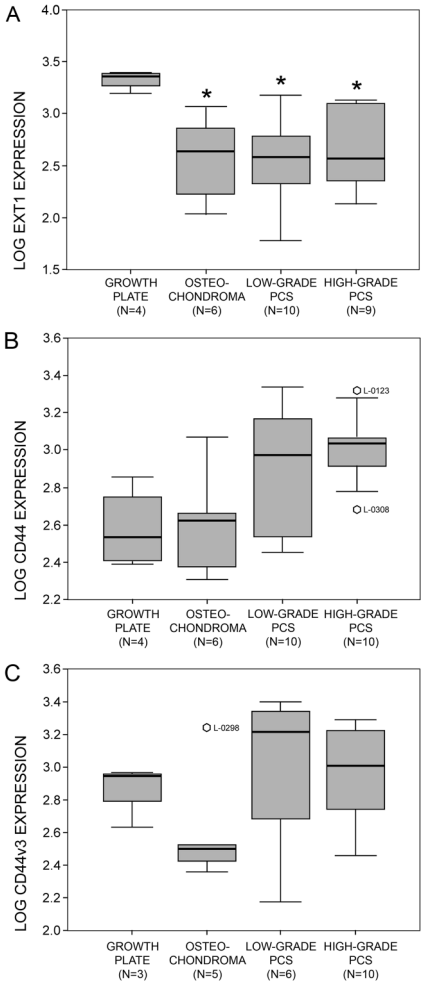

Figure 4.2. mRNA expression data. Log transormed relative mRNA expression levels in growth plates, osteochondromas, low-grade and high-grade chondrosarcomas represented in box plots of *EXT1* (A), all CD44 isoforms (B) and heparan sulphate bearing variable exon 3 of *CD44* (*CD44v3*) (C). There is a significant positive correlation between increasing histological grade and the expression of *CD44* isoforms (r = 0.562, p =0.001). * Expression significantly lower than in growth plate (p < 0.05). PCS = peripheral chondrosarcoma. L-0298 is an outlier.

Results

Mutation analysis

Seven of eight patients with MO patients studied demonstrated a mutation in *EXT1* or *EXT2* (table IV.I). Apart from two unclassified variants (uv), no mutations were found in solitary tumours. The uv c.499A>T, p.I167F in *EXT2*, found in L-627 was absent in the patient's peripheral blood DNA and in 93 healthy controls. The uv c.710C>T, p.S237L in *EXT2*, in L-739 has been reported recently in a moderately affected patient with MO and his unaffected father [48]. This DNA alteration was found in 1 of 93 healthy controls and therefore considered a rare polymorphism.

Methylation specific PCR for EXT1 promoter methylation

EXT1 promoter methylation was detected in DNA from HL-60 cells, whereas it was absent in all 17 solitary and four hereditary tumours (figure 4.1).

qPCR for EXT genes and CD44

Table IV.I shows the relative mRNA expression of *EXT1* and *EXT2* as fraction of the average expression in growth plates. There was no difference in *EXT1* or *EXT2* expression between solitary and hereditary tumours (p = 0.313 for *EXT1* and p = 0.301 for *EXT2*). Expression of *EXT1* was significantly less in solitary (p = 0.001) and hereditary (p = 0.024) osteochondromas, as well as in low-grade (p = 0.011) and high-grade (p = 0.025) peripheral chondrosarcomas, than in growth plates (figure 4.2A), independently of patient age. *EXT2* expression was not decreased in osteochondromas (p = 0.536) and chondrosarcomas (p = 0.723) compared with growth plates. In four of five hereditary tumours with an *EXT2* mutation, a significant reduction of *EXT2* expression (p = 0.002)(table IV.I) was found in comparison with growth plates. Tumours with low expression of *EXT2* also had low *EXT1* expression.

Expression of all *CD44* isoforms increased with malignant transformation of osteochondroma and subsequent increasing histological grade (r = 0.562, p = 0.001, figure 4.2B). This was not seen for the expression of HS bearing variable exon 3 of *CD44* (*CD44v3*) (r = 0.241, p = 0.256) (figure 4.2C).

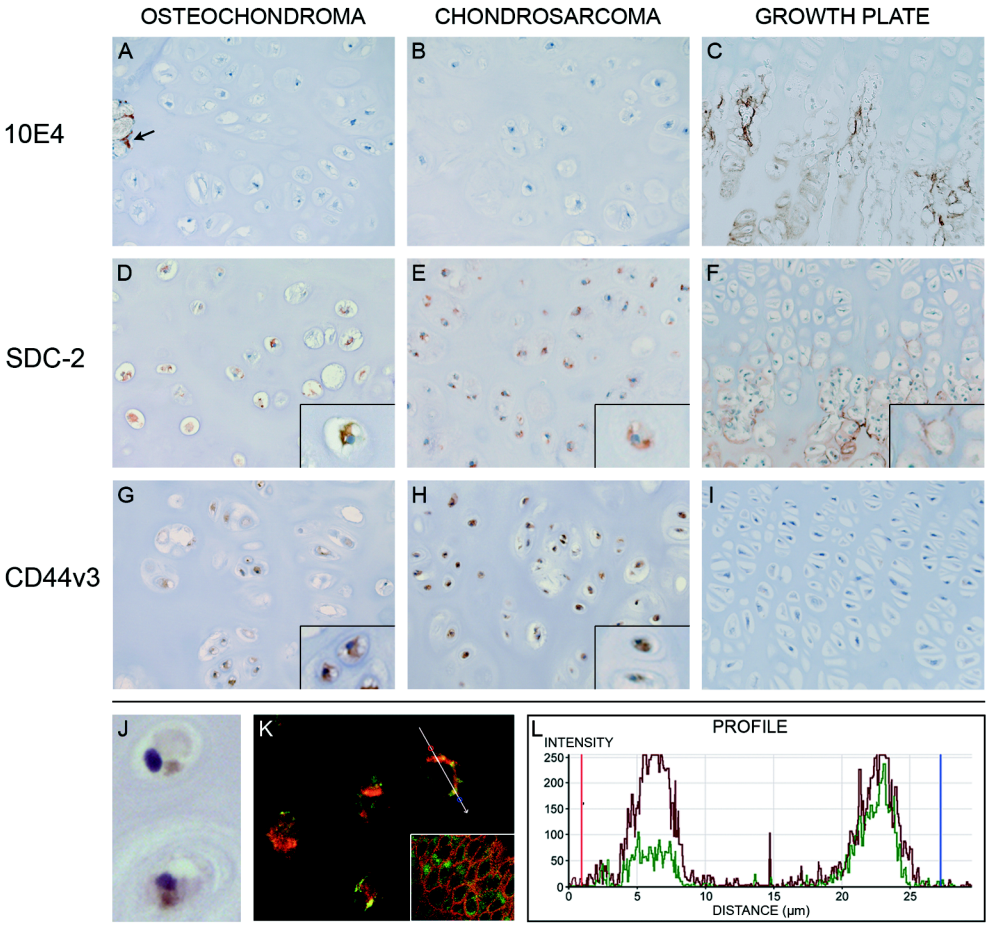

Figure 4.3. Expression of heparan sulphates and HSPG core proteins. Absence of expression of native HS chains in osteochondroma (A) and chondrosarcoma (B), while growth plate (C) demonstrates extracellular expression in the late hypertrophic and calcifying zones. Osteoclasts (arrow in A) served as positive internal controls. Cytoplasmic staining for Syndecan-2 was observed in osteochondroma (D) and chondrosarcoma (E), while in the growth plate (F) extracellular expression is found in the late hypertrophic and calcifying zones. CD44v3 staining was found in the cytoplasm of osteochondroma (G) and chondrosarcoma (H) with a perinuclear, Golgi-like staining in 63% of the tumours (J). Growth plate demonstrated absent or only very weak CD44v3 expression in the hypertrophic zone (I). Two-colour immunofluorescence of CD44v3 (red) and 58K Golgi protein (green) in grade I peripheral chondrosarcoma is shown in (K). The CD44v3 expression is retained in the cytoplasm. Note the presence of both CD44v3 and 58K Golgi protein positive areas (yellow) in the cytoplasm of two cells, indicative of colocalization. (L) Colocalization is shown as an intensity profile for one cell (see vector line between red and blue marker in K). Inset: membranous CD44v3 colocalization in tonsil.

Immunohistochemistry

For all antibodies, no correlation was found between expression and malignancy, tumour grade, patient age, gender or presence or absence of *EXT* mutations. Also, was there no difference between solitary and hereditary tumours, or between tumours with normal and low *EXT1* and *-2* expression levels. Therefore, the tumours were analyzed as one group. Table IV.III summarizes the results of the immunohistochemical analysis.

In the majority of tumours (39 of 50 [78%]) expression of native HS chains was not detected (figure 4.3A and B). Eleven (22%) tumours expressed native HS chains in the ECM around hypertrophic and calcifying cells. Five growth plates (33%) expressed native HS chains in the ECM of hypertrophic and calcifying chondrocytes (figure 4.3C). ECM staining vanished after heparitinase pre-treatment, indicating staining specificity.

Sixty-two per cent of tumours demonstrated extracellular syndecan-2 expression, whereas all 15 growth plates were positive (figure 4.3F) ($p < 0.001$). In addition, 78% demonstrated cytoplasmic expression of syndecan-2 (figure 4.3D and E), compared with 6/15 (40%) growth plates, where cytoplasmic expression was restricted to the hypertrophic zone ($p < 0.001$).

Table IV.III. Results Immunohistochemistry

		Growth plate		Osteochondroma		Peripheral chondrosarcoma		*Growth plate vs tumours*
Antigen	**Location**	**positive***	**%**	**positive***	**%**	**positive***	**%**	***p*-value**
Native HS	ECM hypertrophic/ calcifying cells	5/15	33	3/21	14	8/29	28	0.495‡
Syndecan-2	ECM hypertrophic/ calcifying cells	15/15	100	14/20	70	15/27	56	<0.001†
	Cytoplasm	6/15	40	12/20	60	35/40	88	<0.001†
CD44s	ECM hypertrophic/ calcifying cells	5/13	38	3/22	14	0/29	0	0.007‡
CD44v3	ECM hypertrophic/ calcifying cells	0/7	0	1/19	5	5/28	18	0.416‡
	Cytoplasm	9/16	56	18/19	95	39/40	98	<0.001‡
	+ peri-nuclear/ Golgi-like	0/9	0	8/18	44	28/39	72	<0.001†

* The number of positive samples/total number of samples that could be evaluated
† Pearson's χ^2
‡ Fisher's Exact test

Only 3/22 (14%) osteochondromas expressed the CD44 standard isoform (CD44s) compared with 5/13 growth plates (38%) ($p = 0.007$). Since none of the chondrosarcomas were positive, the correlation between increasing histological grade and CD44 expression, demonstrated by qPCR at the mRNA level, was not confirmed at the protein level. Fifty-seven tumours (97%) demonstrated moderate to strong cytoplasmic expression of CD44v3. The expression ranged from very focal to diffuse in both proliferating and hypertrophic cells (figure 4.3G and H). In contrast, focal weak cytoplasmic CD44v3 staining was restricted to 9/16 (56%) growth plates ($p < 0.001$) (figure 4.3I). Remark-ably, 36/57 (63%) CD44v3 positive tumours demonstrated a perinuclear, dot-like, staining (figure 4.3J), which was absent in growth plates ($p < 0.001$).

Syndecan-3 expression was absent in a pilot of 12 tumours and four growth plates and therefore the series was not further extended. Perichondrial cells demonstrated membranous staining of native HS chains, syndecan-2 and CD44s in a subset of growth plates as well as osteochondromas, while CD44v3 demonstrated additional weak cytoplasmic staining.

Confocal microscopy to detect intracellular localization

In all five tumours tested, CD44v3 colocalized with 58K Golgi protein, marker of the Golgi apparatus, confirming retention of the HSPG in the Golgi apparatus (figure 4.3K-L). Also cytoplasmic expression outside the Golgi apparatus is observed. In contrast, in normal control tissue (tonsil), membranous CD44v3 expression was found in the basal epithelial layer.

Discussion

Mutations in the tumour suppressor genes *EXT1* and *EXT2* are expected to result in a dysfunctional EXT1/EXT2 complex that is hypothesized to disturb HS biosynthesis in the growth plate leading to osteochondroma formation. We investigated in both hereditary and solitary osteochondromas and peripheral chondrosarcomas whether mutations or promoter methylation affect mRNA expression of *EXT1* and *EXT2* and the expression of HS chains and HSPG protein cores.

Seven of eight patients with MO tested demonstrated DNA alterations. The mutations resulted in downregulation of either *EXT1* or *EXT2* mRNA in six out of seven hereditary tumours, as was also reported previously by others [30]. Most germ-line mutations are non-sense, frame shift or splice-site mutations [49], leading to a premature stop codon which will result in non-sense mediated decay and thus loss of RNA.

In contrast, in DNA isolated from 17 solitary cases we only found one unclassified variant. Also in literature, somatic mutations are extremely rare [29-31], while loss or rearrangement of the 8q24 region is almost as frequent as in hereditary osteochondromas[28]. Remarkably, similar to hereditary cases, *EXT1* was downregulated in all three solitary osteochondromas and 11 of 14 solitary chondrosarcomas. None of the three solitary osteochondromas showed low *EXT2* expression. Loss at the *EXT2* locus has been described in only one solitary osteochondroma [27]. Hence, *EXT2* seems less important for the development of solitary osteochondroma than *EXT1*.

We investigated CpG island promoter methylation as alternative mechanism for inactivation of *EXT1* in solitary tumours. Approximately 50% of genes that cause familial cancers through germ-line mutations undergo methylation-associated silencing in sporadic cancers [50]. Others demonstrated *EXT1*, but not *EXT2*, promoter hypermethylation in leukemia, but not in 67 chondrosarcomas [41,51]. This concurs with our results that none of the solitary tumours displayed *EXT1* promoter methylation. Thus, point mutations or small deletions and promoter methylation are excluded as cause of the loss of *EXT1* expression in solitary osteochondromas. We very recently demonstrated somatic homozygous deletion of *EXT1* in 7 of 8 solitary osteochondromas using array-based comparative genomic hybridization (array-CGH)[52]. This explains the absence of somatic point mutations and the loss of *EXT1* expression in solitary cases as demonstrated in the present study.

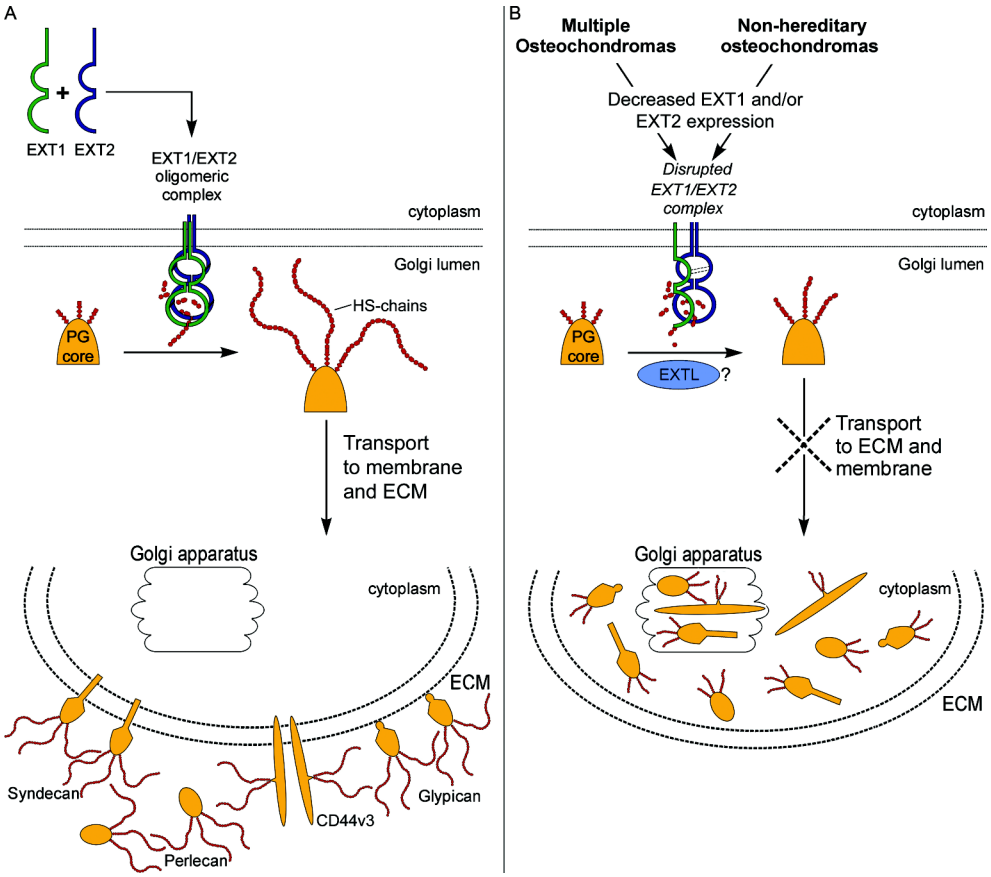

Figure 4.4. Schematic representation of function of the EXT1 /EXT2 complex in HS biosynthesis in normal tissue (A) and tumours (B). (A) In normal tissue, EXT1 and EXT2 form a hetero-oligomeric complex, involved in the heparan sulphate (HS) biosynthesis in the Golgi apparatus. The EXT1/EXT2 complex polymerizes HS disaccharides onto proteoglycan core proteins to form HS proteoglycans (HSPGs). The HSPGs are subsequently transported to the cell membrane or extracellular matrix (ECM). Adapted from 12,13. (B) Hypothesized model of aberrant HS biosynthesis in osteochondromas and chondrosarcomas. We demonstrated diminished *EXT1* and/or *EXT2* expression in both hereditary and solitary tumours. An aberrant EXT1/EXT2 protein complex will probably not be able to fully polymerize HS disaccharides. EXTL-genes might be able to polymerize shorter chains. Our results suggest that the abnormal HSPGs will not be properly transported to the cell membrane and ECM, but are retained in the Golgi apparatus and cytoplasm of the tumour cell.

Interestingly, *EXT2* expression was decreased in eight of 15 tumours with low *EXT1* expression. Diminished expression of both proteins has been reported in osteochondroma cell cultures [30], suggesting similar expression regulation. Moreover, *EXT2* is not redundant to *EXT1* [12] and HS biosynthesis cannot be restored when *EXT2* is transfected in *EXT1*-deficient cell lines [12,53]. Thus, functional HS biosynthesis requires both gene products.

To evaluate the effect of *EXT* downregulation we investigated the expression of HS chains and HSPGs. The cytoplasmic staining of syndecan-2 and CD44v3 we found suggests

intracellular accumulation of HSPGs concentrated in the Golgi apparatus. Cytoplasmic staining was weak or absent in the control growth plates. Moreover, in other tumours membranous expression of these proteins is described [54,55]. CD44v3 demonstrated perinuclear dot-like staining and using immunofluorescent double staining we could confirm the accumulation of CD44v3 in the Golgi apparatus. The EXT1/2 complex elongates HS chains to HSPG protein cores in the Golgi apparatus [12] before HSPGs are transported to the cell surface. Others have reported diminished and abnormal distribution of the HSPG perlecan in 11 osteochondromas[29,56]. We hypothesize that the disrupted function of the EXT1/2 complex due to loss of *EXT* expression, results in improperly synthesized HS chains. This may lead to an inadequate transport of HSPGs to the cell surface, resulting in intracellular accumulation concentrated in the Golgi apparatus (figure 4.4).

In a minority (22%) of the tumours, however, HS chains were still detected in the ECM despite diminished *EXT1* and *EXT2* mRNA expression. Apparently, the tumours have sufficient glycosyltransferase activity left to synthesize native HS chains. Since neither protein demonstrates high catalytic activity in absence of the other [12] it is unlikely that the non-affected protein can synthesize these HS chains. Alternatively, these HS chains may be elongated by EXTL1 and EXTL3, since these possess some glycosyltransferase activity [16]. HSPGs are important for proper growth plate signalling [20,57,58]. Murine and chick express syndecan-2 and -3 in the proliferating zone of the growth plate [45,59]. We found syndecan-2 expression only in the late hypertrophic zone, whereas syndecan-3 expression was absent. Thus, the expression of HSPG core proteins seems to display interspecies variation.

CD44 isoforms act as HSPGs when they express HS bearing variable exon 3 (v3) [18]. Misregulation of CD44, resulting in expression of different splice variants, is related to poor prognosis in many cancers [18]. This is consistent with the increased mRNA expression we found of all CD44 isoforms in high-grade chondrosarcomas.

EXT genes and HS chains have been demonstrated to act on the diffusion of IHH [20,57], which is part of a signalling cascade of importance in the normal growth plate. IHH signalling molecules are still expressed in osteochondromas [22,60], but are downregulated during chondrosarcoma progression [22,61]. PTHLH signalling, downstream of IHH, is absent in osteochondroma [23,24]. Further functional *in vitro* studies will be necessary to investigate the activity of IHH signalling in osteochondromas, where HS chains and HSPGs are no longer present at the cell surface.

In conclusion, we demonstrated in both hereditary and solitary osteochondromas and peripheral chondrosarcomas that mutational inactivation of either *EXT1* or *EXT2*, leads to loss of mRNA expression of the corresponding gene. We hypothesize that this loss of *EXT* expression will disrupt the function of the EXT1/2 complex in the HSPG biosynthesis, leading to the intracellular accumulation of HSPG core proteins that we found in these tumours.

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Supplementary data

Supplementary table IV.I. Primers and probe used in quantitative PCR experiments

Gene symbol	Gene name	Chromosomal location	Reference sequence	Forward primer	Reverse primer	Product length (bp)
EXT1	Exostoses (multiple) 1	8q24.12-q24.13	NM_000127	TGACAGAGACAACACCGAGTATGA	GCAAAGCCTCCAGGAATCTGAAG	119
EXT2	Exostoses (multiple) 2	11p12-p11	NM_000401	CAGTCAATTAAAGCCATTGCCCTG	GGGATCAGCGGGAGGAAGAG	149
CD44	CD44 antigen	11p13	NM_000610	CCTTTGATGGACCAATTACCATAAC	GGTAGATGTCTTCAGGATTCGTTCT	95
CD44v3	variable exon 3 CD44		NM_000610	CCTTTGATGGACCAATTACCATAAC TAQman probe CD44v3: ACCGTGATGGCACCCGCTATGTCC	ATCTTCATCATCATCAATGCCTGA	493
Normalization genes						
CPSF6	cleavage and polyadenylation specific factor 6	12q15	NM_007007	AAGATTGCCTTCATGGAATTGAG	TCGTGATCTACTATGGTCCCTCTCT	89
SRPR	signal recognition particle receptor	11q24.3	NM_003139	CATTGCTTTTGCACGTAACCAA	ATTGTCTTGCATGCGGCC	70
GPR108	G protein-coupled receptor 108	19p13.3	XM_290854	AGATGCCCCTTTTCAAGCTCTAC	GCCATGAGCCAGTGGATCTTG	122
CAPNS1	calpain, small subunit 1	19q13.12	NM_001749	ATGGTTTTGGCATTGACACATG	GCTTGCCTGTGGTGTCGC	66

Supplementary Table 2. Antibodies and protocols for immunohistochemical experiments.

Antigen	Manufacturer	Mono/ poly-clonal	Positive control	Internal positive control	Staining	antibody concen-tration	antigen retrieval
CD44 (7D8)	G. David [43]	Mono	normal skin	osteocytes	membrane, ECM	10 μg/ml	none
CD44v3	Labvision, Fre-mont CA, USA	Mono	tonsil	none	membrane, cytoplasm	1:15	citrate
HS-chains (10E4)	G. David [44]	Mono	normal skin	vessel walls, osteoclasts	membrane, ECM	5 μg/ml	none
Syndecan-2 (10H4)	G. David [45]	Mono	normal growth plate	none	membrane, ECM	35 μg/ml	none
Syndecan-3 (1C7)	G. David [43]	Mono	normal colon	none	membrane	5 μg/ml	none
58K Golgi protein	Abcam Ltd, Cambridge, UK	Poly	tonsil	all cells	perinuclear	1:100	citrate