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Original Paper

# Absence of IHH and retention of PTHrP signalling in enchondromas and central chondrosarcomas

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## Abstract

Enchondromas and conventional central chondrosarcomas are, respectively, benign and malignant hyaline cartilage-forming tumours that originate in the medulla of bone. In order to gain a better understanding of the molecular process underlying malignant transformation of enchondroma, and to investigate whether there is a biological difference between conventional central cartilaginous tumours and those of enchondromatosis or with phalangeal localization, a series of 64 enchondromas (phalanx,  $n = 21$ ; enchondromatosis,  $n = 15$ ) and 89 chondrosarcomas (phalanx,  $n = 17$ ; enchondromatosis,  $n = 13$ ) was collected. Indian Hedgehog (IHH)/parathyroid hormone related peptide (PTHrP) signalling, an important pathway in chondrocyte proliferation and differentiation within the normal growth plate, was studied by immunohistochemical analysis of the expression of PTHrP, PTHR1, Bcl-2, p21, cyclin D1, and cyclin E. Quantitative real-time PCR for IHH, PTCH, SMO, and GLI2 was performed on a subset of tumours. The data show that IHH signalling is absent in enchondromas and central chondrosarcomas, while PTHrP signalling is active. There was no difference in the expression of any of the molecules between 35 enchondromas and 26 grade I central chondrosarcomas, indicating that PTHrP signalling is not important in malignant transformation of enchondroma. Higher expression of PTHR1 and Bcl-2 was associated with increasing histological grade in chondrosarcoma, suggesting involvement in tumour progression. No difference was found between samples from enchondromatosis patients and solitary cases, suggesting no difference in PTHrP signalling. A small subset of phalangeal chondrosarcomas demonstrated down-regulation of PTHrP, which may be related to its more indolent clinical behaviour. Thus, in both enchondromas and central chondrosarcomas, PTHrP signalling is active and independent of IHH signalling, irrespective of the presence or absence of enchondromatosis.

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**Keywords:** bone; bone neoplasm; chondrosarcoma; enchondroma; enchondromatosis; Ollier disease

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## Introduction

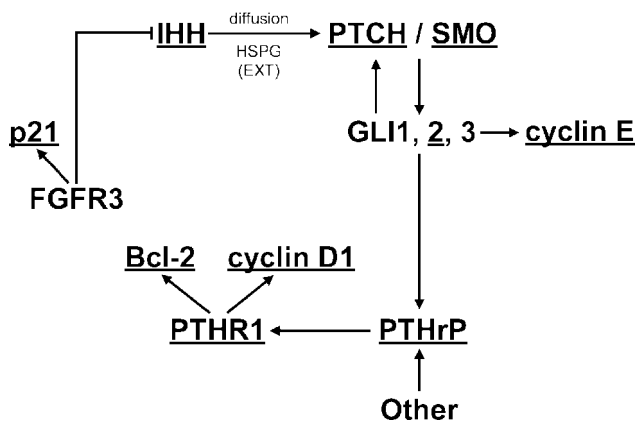
Chondrosarcoma of bone is a malignant tumour characterized by the formation of hyaline cartilage. The majority (83%) arise centrally within the medullary cavity of bone (primary conventional central chondrosarcomas) [1]. Transformation of enchondroma, a benign cartilaginous tumour, towards secondary central chondrosarcoma is thought to be very rare (<1% of enchondromas) [1,2], and the mechanism underlying malignant transformation is unknown. In about 40% of central chondrosarcomas, remnants of a previous, often undetected, enchondroma are found next to the chondrosarcoma, implying that these cases are strictly not primary, but secondary chondrosarcomas. Since these remnants are difficult to detect, it may be that all central chondrosarcomas are secondary [3].

About 50% of enchondromas are found in small bones of the hands and feet, while chondrosarcomas

are rare at this location [2] and, in contrast to elsewhere, only rarely metastasize [4]. This raises the question of whether cartilaginous tumours with phalangeal localization are biologically different, or whether localization determines prognosis.

While most enchondromas are solitary, patients with enchondromatosis (Ollier disease, Maffucci syndrome) have multiple enchondromas, scattered all over the skeleton, often with unilateral predominance [5]. The percentage of malignant transformation in patients with enchondromatosis is much higher (25–30% per patient) than in patients with solitary enchondromas [1,2]. For enchondromas with phalangeal localization, as well as enchondromas from enchondromatosis patients, more worrisome histological features are tolerated [1,2], since they clinically behave in a more indolent fashion.

The aims of our study were, firstly, to gain a better understanding of the molecular process underlying



**Figure 1.** Signalling within the normal human growth plate. Indian Hedgehog (IHH), produced by pre-hypertrophic chondrocytes, binds after diffusion mediated by heparan sulphate proteoglycans (HSPG), to its receptor Patched (PTCH). This results in the release of Smoothed (SMO) from PTCH, which allows GLI molecules to act as transcriptional activators of target genes [6]. These include genes from the IHH pathway itself, such as PTCH and GLI [6,7]. Two other target genes are cyclin E [8], involved in control of the transition of G1 to S-phase, and parathyroid hormone related protein (PTHrP) [9]. PTHrP binds to its receptor (PTHR1) in the late proliferating zone, resulting in up-regulation of the anti-apoptotic protein Bcl-2 [10]. This inhibits further differentiation, thereby limiting IHH-producing cells, closing the negative feedback loop. Moreover, PTHR1 directly induces activation of the cyclin D1 promoter [11]. FGFR3 activation leads to repression of IHH/PTHrP signalling, and to up-regulation of the cell cycle inhibitor p21 [12]. The molecules investigated in this study are underlined

malignant transformation of enchondroma and, secondly, to investigate possible biological differences between conventional central cartilaginous tumours and those within the context of enchondromatosis or with phalangeal localization.

Within the normal growth plate, the Indian Hedgehog (IHH)/parathyroid hormone related peptide (PTHrP, PTHLH) negative feedback loop plays an important role in the regulation of chondrocyte growth and differentiation [6–12] (Figure 1). We investigated molecules involved in PTHrP signalling by comparing the protein expression of these signalling molecules

between the different subsets of tumours and assessed whether putative differences in this signalling pathway are associated with different clinical behaviour. Since commercially available antibodies for IHH signalling molecules have been shown previously not to work reliably in our hands [13], expression was studied with quantitative RT-PCR in a subset of tumours to evaluate their role in tumourigenesis and tumour progression.

## Materials and methods

### Pathological material

Formalin-fixed, paraffin wax-embedded material was used from 153 tumours (Table 1). Cases were collected from (consultation) files of the Leiden University Medical Center.

Patient data (Table 1) were obtained by review of pathological specimens and reports, clinical charts, and radiographs. Enchondromatosis was found in 28 patients. Histological grading for non-phalangeal cases was performed according to Evans *et al* [14]. Clinico-pathological features of phalangeal chondrosarcomas have been described previously [4]. All tissue 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).

### Immunohistochemistry

Antibodies, controls, and antigen retrieval are described in Table 2. Antibodies were tested for their susceptibility to formalin fixation on appropriate tissues fixed for 1, 3, 7, and 40 days, respectively, but none revealed diminished staining reactivity. Internal positive controls were used (Table 2) to exclude absence of staining due to decalcification. Negative controls were performed using isotype controls. Immunohistochemical staining was performed as described previously [13].

**Table 1.** Patient and tumour related data

	Enchondroma		Central chondrosarcoma	
	Phalanx n = 21	Non-phalanx n = 43	Phalanx n = 17	Non-phalanx n = 72
Male vs female	13 vs 8	24 vs 19	8 vs 9	37 vs 35
Median age at diagnosis, years	25.4 (range 12.3–74.3)	37.8 (range 4.3–68.8)	61.6 (range 8–83.4)	50 (range 17.8–78.7)
Histological grade				
Grade I	—	—	—*	30
Grade II	—	—	—*	30
Grade III	—	—	—*	12
Enchondromatosis (Maffucci/Ollier)	7 (0/7)	8 (0/8)	3 (0/3)	10 (2/8)
Median follow-up, months	87.5 (range 2–226) <sup>†</sup>	83.5 (range 1–170) <sup>‡</sup>	96 (range 15–249) <sup>§</sup>	59.5 (range 2–212) <sup>  </sup>

\* Histological grading in chondrosarcoma of the phalanx proved not useful [4].

Data available from <sup>†</sup> 14, <sup>‡</sup> 26, <sup>§</sup> 15, and <sup>||</sup> 64 patients.

Table 2. Details of the antibodies used and the immunohistochemical protocols employed

Antigen	Manufacturer	Mono/polyclonal (type)	Staining	Positive control	Internal positive control	Dilution	Antigen retrieval
PTHrP	Oncogene	Polyclonal (rabbit IgG)	Cytoplasm	Normal skin	None (occasionally osteoclasts)	1:25	Trypsin (30 min, 37°C)
PTHrI	Upstate	Monoclonal (mouse IgG1)	Nucleus	Normal skin	Osteoblasts, vessel walls	1:100	Citrate (120 min, 95°C)
Bcl-2 (clone 124)	Roche	Monoclonal (mouse IgG1)	Cytoplasm	Normal tonsil	Osteoclasts, lymphocytes	1:500	Citrate (120 min, 95°C)
Cyclin D1	Neomarkers	Monoclonal (mouse IgG1)	Nucleus	Normal tonsil	Occasionally endothelial cells	1:500	Citrate (120 min, 95°C)
Cyclin E	Neomarkers	Monoclonal (mouse IgG2A)	Nucleus	Placenta	None	1:100	Citrate (120 min, 95°C)
p21 <sup>WAF1/CIP1</sup>	Calbiochem	Monoclonal (mouse IgG1)	Nucleus	Normal colon	None (occasionally vessels and osteoclasts)	1:400	Citrate (120 min, 95°C)

### Evaluation and scoring

Three observers (LBR, PCWH, and JVMGB) evaluated the slides independently and discrepancies were discussed. All observers were blinded towards clinicopathological data. Scoring was performed as described previously [13]. In short, staining intensities (0 = negative, 1 = weak, 2 = moderate, and 3 = strong intensity) and the percentage of positive cells (0 = 0%, 1 = 1–24%, 2 = 25–49%, 3 = 50–74%, and 4 = 75–100% positive) were assessed. The slides were scored positive if the combined values of the staining intensity and the percentage of positive cells were greater than 3 for PTHrP, PTHR1, and Bcl-2, and greater than 0 for p21, cyclin D1 and cyclin E [13,15,16]. For cyclin E and cyclin D1, lacking an absolute internal control, cases were only scored negative if immunohistochemical data were available for at least four other antibodies.

### RNA isolation and quantitative PCR (qPCR)

RNA was isolated from 10 tumours [two phalangeal enchondromas (enchondromatosis patients), one solitary grade I chondrosarcoma, five grade II chondrosarcomas (three from enchondromatosis patients), and two solitary grade III chondrosarcomas] and seven normal samples (four growth plates and three normal resting cartilage samples) as described previously [17].

One microgram of total RNA was converted to complementary DNA (cDNA) by using AMV reverse transcriptase (Roche Applied Science). qPCR was performed for *IHH*, *PTCH*, *SMO*, *GLI2*, and four normalization genes (*CPSF6*, *GPR108*, *HNRPH1*, and *SRPR*; primers and qPCR conditions are available on request). The latter were selected from expression profiling experiments of enchondromas, central chondrosarcomas (different grades), normal epiphyseal growth plates, and resting cartilage samples, demonstrating the least variation between all samples (unpublished data). qPCR amplification was performed according to the manufacturer's protocol. For each gene, a standard curve, consisting of a mixture of eight samples, was included to calculate the relative starting quantity of each gene, which was used in normalization and statistical analysis.

Geometric averaging of the candidate normalization genes [18] was performed to acquire reliable normalization of the qPCR experiments. This method provides a normalization factor (NF) that is representative for the amount of mRNA in each sample. Expression levels in the tumours were related to those of four normal growth plates, where IHH signalling is known to be active [9].

### Statistical analysis

Comparison of immunohistochemical data between two different groups was analysed using Pearson chi-square/Fisher's exact test, two-sided. Correlation between staining and histological grade was tested

**Table 3.** Scoring results for immunohistochemical staining in enchondromas and chondrosarcomas

		PTHrP		PTHRI		Bcl-2		Cyclin D1		Cyclin E		p21	
		pos*	% pos <sup>†</sup>	pos	% pos	pos	% pos	pos	% pos	pos	% pos	pos	% pos
EC	Solitary	32/32	100	7/19	37	4/25	16	9/18	50	0/17	0	27/30	90
	EC-tosis	6/6	100	2/4	50	1/4	25	1/2	50	0/2	0	3/3	100
	Total	38/38	100	9/23	39	5/29	17	10/20	50	0/19	0	30/33	91
EC-P	Solitary	14/14	100	10/14	71	9/13	69	9/13	69	1/13	7	14/14	100
	EC-tosis	5/5	100	3/4	75	2/3	67	2/3	67	0/1	0	3/3	100
	Total	19/19	100	13/18	72	11/16	69	11/16	69	1/14	7	17/17	100
All EC	Solitary	46/46	100	17/33	51	13/38	34	18/31	58	1/30	3	41/44	93
	EC-tosis	11/11	100	5/8	63	3/7	43	3/5	60	0/3	0	6/6	100
	Total	57/57	100	17/36	47	16/45	36	21/36	58	1/33	3	47/50	94
CS-I	Solitary	26/26	100	17/25	68	6/22	27	14/23	61	1/21	5	19/20	95
	EC-tosis	2/2	100	1/1	100	1/2	50	1/1	100	—	—	1/1	100
	Total	28/28	100	18/26	69	7/24	29	15/24	63	1/21	5	20/21	95
CS-II	Solitary	25/25	100	21/25	84	19/22	86	18/22	81	1/22	5	19/21	90
	EC-tosis	4/4	100	3/3	100	1/3	33	2/3	67	0/2	0	3/3	100
	Total	29/29	100	24/28	86	20/25	80	20/25	80	1/24	4	22/24	92
CS-III	Solitary	10/10	100	8/10	80	5/8	63	4/8	50	1/7	14	7/7	100
	EC-tosis	2/2	100	1/1	100	1/1	100	1/1	100	0/1	0	1/1	100
	Total	12/12	100	9/11	82	6/9	67	5/9	56	1/8	13	8/8	100
CS-P	Solitary	11/14	79	5/9	56	5/10	50	5/10	50	0/2	0	10/10	100
	EC-tosis	1/2	50	1/2	50	1/1	100	1/1	100	—	—	1/1	100
	Total	12/16	75	6/11	55	6/11	55	6/11	55	0/2	0	11/11	100
All CS	Solitary	72/75	96	51/69	74	35/62	56	41/63	65	3/52	6	55/58	95
	EC-tosis	9/10	90	6/7	86	4/7	57	5/6	83	0/3	0	6/6	100
	Total	81/85	95	57/76	75	39/69	57	46/69	67	3/55	5	61/64	95

\* pos: number of positive samples/number of samples that could be evaluated.

† % pos: percentage of positive samples.

EC = enchondroma; CS = chondrosarcoma; EC-tosis = enchondromatosis; -P = located in the phalanx

with chi-square, linear by linear. Correlation with outcome was analysed using the log-rank test. To correct for multiple testing,  $p$  values  $\leq 0.01$  were considered significant. Relative RNA expression levels from the different tumour groups were compared with growth plates by one-way ANOVA with Bonferroni correction, after log transformation. A  $p$  value  $\leq 0.05$  was considered significant.

## Results

### Immunohistochemistry

PTHrP signalling molecules are present in enchondromas and central chondrosarcomas. p21 and PTHrP were positive in almost all samples, whereas PTHR1, Bcl-2, and cyclin D1 showed more variation (Table 3). Cyclin E was only minimally positive in 4 of 88 samples and therefore not used in further statistical analysis. A variable number of samples, ranging from 11 (PTHrP) to 29 (cyclin D1), could not be evaluated due to detachment from the glass slides.

### Statistical analysis of immunohistochemistry

#### Enchondroma vs chondrosarcoma grade I

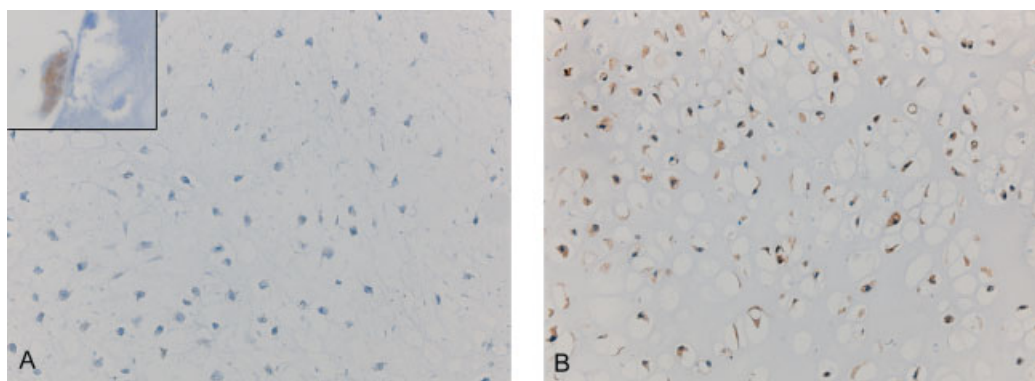
No significant difference was found for any of the molecules investigated between 35 non-phalangeal

solitary enchondromas and 26 solitary conventional central chondrosarcomas, grade I ( $p = 0.066-1.00$ ); between the 21 enchondromas of the phalanx and 17 chondrosarcomas of the phalanx [ $p = 0.035$  (PTHrP)– $1.00$ ]; or between 15 enchondromas and 13 chondrosarcomas in patients with enchondromatosis ( $p = 0.267-1.00$ ).

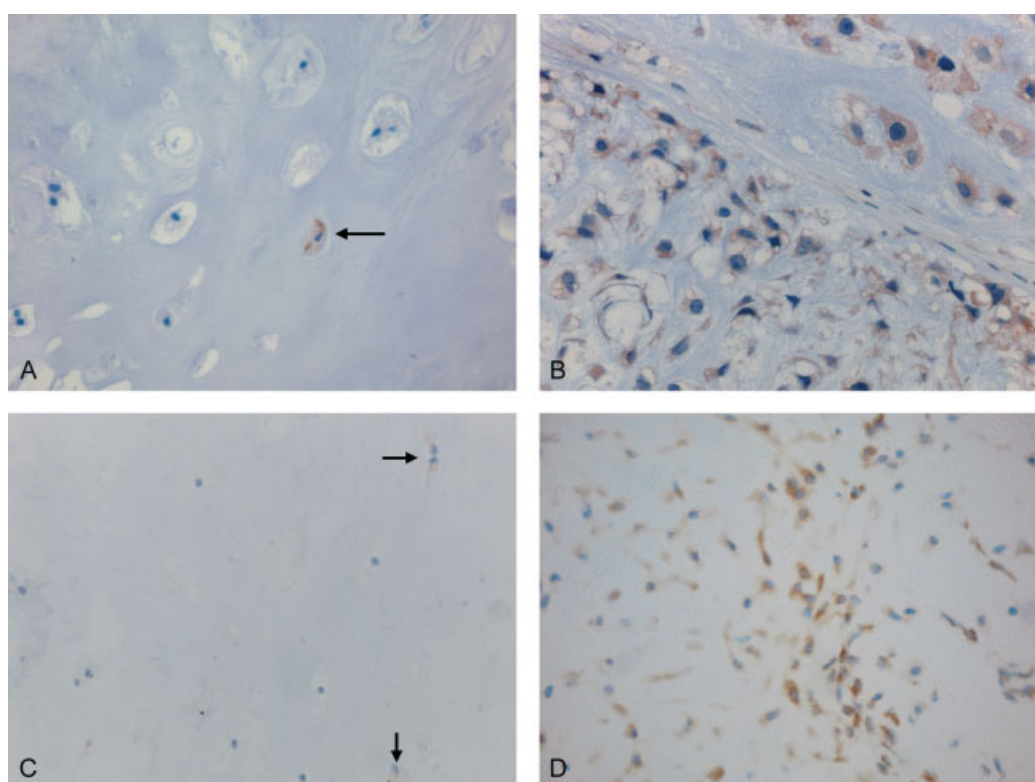
#### Phalangeal localization vs localization elsewhere

Phalangeal chondrosarcomas were compared with grade II conventional central chondrosarcomas located elsewhere, based on similar histological features [4]. Only PTHrP differed significantly, being expressed in 12 of 16 (75%) phalangeal chondrosarcomas compared with 100% ( $n = 25$ ) of grade II chondrosarcomas located elsewhere ( $p = 0.010$ ) (Figure 2).

In addition, expression of Bcl-2 was found in 9 of 13 (69%) solitary phalangeal enchondromas compared with 4 of 25 (16%) solitary enchondromas located elsewhere ( $p = 0.001$ ). A trend was found towards higher expression of PTHR1 in all phalangeal enchondromas [13 of 18 (72%) positive] compared with all enchondromas elsewhere [9 of 23 (39%) positive] ( $p = 0.072$ ).



**Figure 2.** Lack of PTHrP expression in a subset of phalangeal chondrosarcomas. (A) PTHrP expression is absent in a phalangeal chondrosarcoma, with a positive internal control (inset), while (B) strong expression of PTHrP is found in grade II chondrosarcoma



**Figure 3.** Immunohistochemical staining for Bcl-2 and PTHr1 in low- and high-grade conventional central chondrosarcoma. PTHr1 (A, B) and Bcl-2 (C, D) staining shows low intensity and a low percentage of positive cells in grade I conventional central chondrosarcomas (A and C, arrows), while increased staining is found in high-grade conventional central chondrosarcomas (B and D, grade III)

#### Enchondromatosis vs solitary cases

Expression was compared both for all tumours together and for each subtype separately (eg enchondroma; enchondroma of the phalanx; chondrosarcoma grade I, II, and III). No difference was observed for any of the proteins.

#### Low-grade vs high-grade conventional central chondrosarcomas

Analysing all samples, increased PTHr1 and Bcl-2 expression correlated with increasing histological grade ( $p = 0.002$  and  $p = 0.000$ , respectively) (Figure 3). For Bcl-2, this was mainly based on the

increased percentage of positive cells ( $p = 0.000$ ), while for PTHr1, both the intensity and the percentage of cells increased (both  $p = 0.000$ ). Histological grade correlated with disease-free or metastasis-free survival ( $p < 0.0000$  for both parameters), but none of the molecules was an independent significant predictor of outcome.

#### Correlation between expression of the different proteins

A positive correlation was found between Bcl-2 and PTHr1 ( $p = 0.000$ ).

**Table 4.** Average mRNA expression of IHH, PTCH, SMO, and GLI2 per subgroup, relative to the average expression in the human growth plate

Subtypes	IHH	PTCH	SMO	GLI2
Resting cartilage (n = 3)	0.28	0.10*	1.51	0.70
Human growth plate (n = 4)	1.00	1.00	1.00	1.00
EC-P (n = 2)	0.48	0.48	0.54	0.22
CS-I (n = 1) <sup>†</sup>	0.20	0.01	—	0.21
CS-II (n = 5)	0.49	0.06*	0.73	0.23*
CS-III (n = 2)	0.51	0.06*	0.63	0.15*

\* Significant difference compared with growth plate.

<sup>†</sup> Statistical test could not be performed on this group since it contains only one sample.

EC = enchondroma; CS = chondrosarcoma; -P = located in the phalanx.

### qPCR

Expression of IHH, PTCH, GLI2, and SMO was present in the growth plate specimens. Expression of PTCH, which can be used as a read-out system for HH activity, and GLI2 was dramatically decreased in the tumours compared with the normal growth plate (Table 4). Although the sample size is small, PTCH expression was lower in normal cartilage ( $p = 0.019$ ) and chondrosarcoma grade II ( $p = 0.000$ ) and III ( $p = 0.000$ ), and GLI2 expression was lower in chondrosarcoma grade II ( $p = 0.020$ ) and III ( $p = 0.013$ ) compared with growth plates. No differences were found comparing solitary and enchondromatosis-related samples.

### Discussion

In this study, we have demonstrated active PTHrP signalling in both enchondroma and central chondrosarcoma by protein expression of PTHrP, PTHR1, Bcl-2, and cyclin D1. Expression of PTCH RNA was very low or absent. Activation of IHH signalling leads to the activation of target genes, including PTCH and GLI [6,7]. Therefore, transcriptional activation of PTCH is used as a reporter for Hedgehog signalling [6]. The absence of PTCH thus indicates that IHH signalling is not active in enchondroma and central chondrosarcoma. In *Drosophila*, cyclin E is downstream of Hedgehog [8]. We detected cyclin E expression in only four samples, indicating that this molecule is not very important in the development of enchondromas and/or chondrosarcomas, which corroborates the absence of IHH signalling. Thus, PTHrP signalling in enchondroma and central chondrosarcoma is not activated by IHH but by other, as yet unknown, mechanisms. TGF-beta is a good candidate since it was reported to induce PTHrP [19] and is expressed in chondrosarcoma [20].

Our findings confirm the importance of PTHrP signalling in cartilage neoplasia, as previously shown in chondroblastoma [15] and secondary peripheral chondrosarcoma [13]. Since no difference was found between enchondroma and chondrosarcoma, it seems

that PTHrP signalling does not play a vital role in malignant transformation of enchondroma towards secondary central chondrosarcoma.

About 17% of chondrosarcomas develop within the cartilaginous cap of a pre-existing benign osteochondroma (secondary peripheral chondrosarcomas) [21]. In osteochondromas, PTHrP signalling is absent [13], most likely due to mutational inactivation and/or loss of EXT genes [22]. Upon malignant transformation, PTHrP and Bcl-2 expression is up-regulated [13]. This makes the absence of PTHrP signalling specific for osteochondromas, since PTHrP signalling is active in enchondromas and chondroblastomas [15].

We have confirmed that higher Bcl-2 expression is associated with progression towards high-grade central chondrosarcoma, as suggested by our previous results [13]. Bcl-2 negatively controls programmed cell death in growth plate chondrocytes both *in situ* and *in vitro* [10]. Expression of PTHR1, using a monoclonal antibody, correlated with increasing histological grade, which was not found with the polyclonal antibody used in our earlier pilot series [13].

In the present study, PTHrP was expressed in almost all samples, while previously [13] expression seemed to increase with grade. However, this was done using a much smaller sample size and a different batch of the polyclonal antibody. Although no distinction between central and peripheral tumours was made, expression of PTHrP and PTHR1 has also been examined by others, showing up-regulation with grade [23,24]. Thus, in enchondroma and central chondrosarcoma, PTHrP signalling is active and seems to increase with histological grade, in parallel with the increased proliferative activity.

One of our goals was to investigate whether phalangeal cartilaginous tumours are biologically different from other sites. Phalangeal chondrosarcomas display locally aggressive behaviour with very low metastatic potential (<2%) [4]. Remarkably, only 4 of 142 tumours were PTHrP-negative and all four were chondrosarcomas of the phalanx. This down-regulation of PTHrP signalling, which is also found in osteochondroma [13], may be specific for at least a subset of phalangeal chondrosarcomas and may be related to their more indolent behaviour. Combined with the slightly lower proliferation rate and slightly lower percentage of p53 overexpression in phalangeal chondrosarcomas reported previously [4], these data support the suggestion that chondrosarcomas of the phalanx are indeed biologically different.

Expression of Bcl-2 and PTHR1 was higher in solitary phalangeal enchondromas than in solitary enchondromas elsewhere. Since PTHR1 and Bcl-2 expression correlates with increasing histological grade in non-phalangeal chondrosarcomas, these results are probably associated with the more worrisome histological features tolerated in phalangeal enchondroma, but leading to a diagnosis of chondrosarcoma at other localizations [1,2].

Finally, no differences were found between solitary and enchondromatosis-related tumours. An activating mutation has been reported in PTHR1 in two enchondromatosis patients [25]. The mutation would lead to up-regulation of IHH signalling [25]. Here we demonstrate absence of IHH signalling in two enchondromas and three chondrosarcomas from enchondromatosis patients. In addition, we previously screened 31 patients and could not find any PTHR1 mutations [26]. These data again indicate that PTHR1 is not the culprit for enchondromatosis.

p21 was expressed in the majority of tumours. p21 inhibits chondrocyte proliferation, reducing the number of IHH-expressing cells in the growth plate [12]. There may be a role for p21 in the down-regulation of IHH signalling that we observed.

In conclusion, we have demonstrated that IHH signalling is absent in enchondromas and central conventional chondrosarcomas, indicating that although this pathway is important in normal chondrocyte growth and differentiation, it is not involved in enchondromas and chondrosarcomas. However, PTHrP signalling is active in both enchondromas and conventional central chondrosarcomas, confirming its importance in growth and differentiation of neoplastic cartilage and suggesting activation independent of IHH signalling.

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