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**Original Paper**

# Peripheral chondrosarcoma progression is accompanied by decreased Indian Hedgehog signalling

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## Abstract

**Hedgehog (HH) signalling is important for specific developmental processes, and aberrant, increased activity has been described in various tumours. Disturbed HH signalling has also been implicated in the hereditary syndrome, Multiple Osteochondromas. Indian Hedgehog (IHH), together with parathyroid hormone-like hormone (PTHLH), participates in the organization of growth plates in long bones. PTHLH signalling is absent in osteochondromas, benign tumours arising adjacent to the growth plate, but is reactivated when these tumours undergo malignant transformation towards secondary peripheral chondrosarcoma. We describe a gradual decrease in the expression of *Patched* (*PTCH*) and *glioma-associated oncogene homologue 1* (*GLI1*) (both transcribed upon IHH activity), and *GLI2* with increasing malignancy, suggesting that IHH signalling is inactive and PTHLH signalling is IHH independent in secondary peripheral chondrosarcomas. cDNA expression profiling and immunohistochemical studies suggest that transforming growth factor-$\beta$ (TGF-$\beta$)-mediated proliferative signalling is active in high-grade chondrosarcomas since TGF-$\beta$ downstream targets were upregulated in these tumours. This is accompanied by downregulation of energy metabolism-related genes and upregulation of the proto-oncogene *jun B*. Thus, the tight regulation of growth plate organization by IHH signalling is still seen in osteochondroma, but gradually lost during malignant transformation to secondary peripheral chondrosarcoma and subsequent progression. TGF-$\beta$ signalling is stimulated during secondary peripheral chondrosarcoma progression and could potentially regulate the retained activity of PTHLH.**
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**Keywords: Hedgehog; transforming growth factor-$\beta$; cartilaginous tumours; molecular pathways; tumour progression**

## Introduction

Hedgehog (HH) signalling plays an important role during embryonic and postembryonic development, where it regulates cell proliferation and/or differentiation [1]. Upon binding to HH, Patched (PTCH) relieves its inhibition of Smoothened (SMO), which activates glioma-associated oncogene homologue (GLI) transcription factor family members (GLI1–3). This leads to activation of target genes, including GLI1 and PTCH itself [2,3]. In the growth plate, Indian Hedgehog (IHH) regulates chondrocyte proliferation and differentiation in a tightly regulated paracrine feedback loop, together with parathyroid hormone-like hormone (PTHLH or PTHrP; Figure 1A) [4–6].

Deregulated IHH signalling has been implicated in patients with Multiple Osteochondromas [7–9], an autosomal dominant disorder characterized by the formation of cartilage-capped, benign, bony neoplasms on the outer surface of bones preformed by endochondral ossification [8,10]. *EXT1* and *EXT2* have been identified as tumour suppressor genes for Multiple Osteochondromas [11]. These genes are involved in the biosynthesis of heparan sulphate proteoglycans [12,13], multifunctional macromolecules involved in the diffusion of HH to PTCH [9,14]. Osteochondromas also occur as solitary lesions in a non-hereditary background [10]. The cartilage cap of osteochondromas morphologically recapitulates the epiphyseal growth plate [10].

A small fraction (<3%) of osteochondromas transforms into so-called secondary peripheral chondrosarcomas [15,16], which are classified histologically into three grades that correlate with prognosis [17]. Chondrosarcomas can recur at a higher histological grade [15], suggesting progression in malignancy with time.

Recent findings have linked upregulated HH signalling to various human diseases [2], such as Gorlin syndrome, in which HH signalling is constitutively active as a result of inactivating mutations in *PTCH* [18]. Moreover, constitutively active HH signalling has been found in several sporadic cancers,

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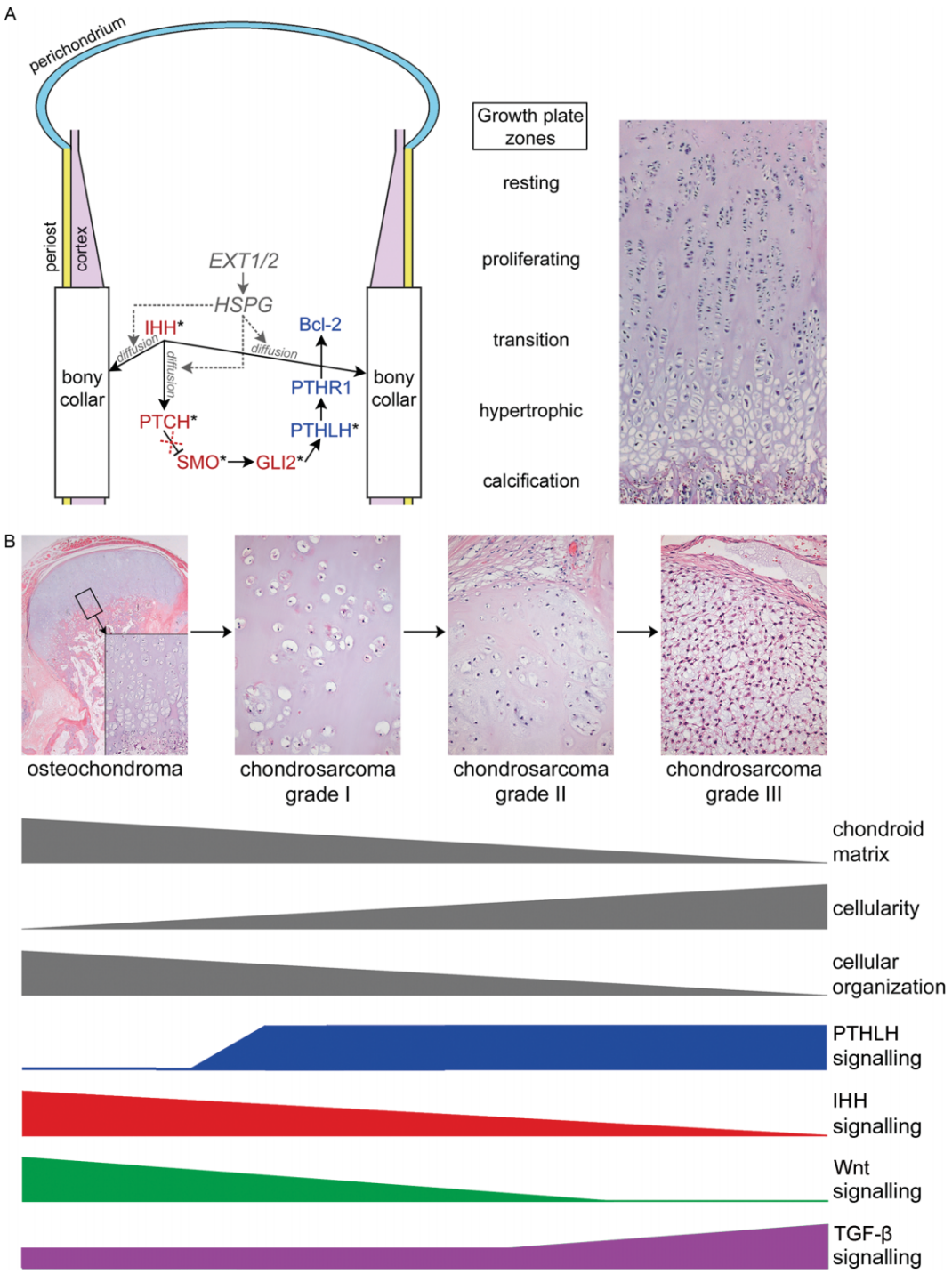


**Figure 1.** Schematic representations of signalling pathways in growth plate, osteochondromas, and chondrosarcomas. (A) IHH/PTHLH signalling in the postnatal growth plate. The IHH/PTHLH feedback loop is confined to the growth plate. In the transition zone, IHH will bind PTCH, relieving its inhibitory effect on Smoothened (SMO). SMO then activates the transcription factor GLI2, which subsequently moves to the nucleus to start transcription of IHH target genes. One of these targets is PTHLH. After PTHLH binds to its receptor PTHR1, Bcl-2 is upregulated, inhibiting chondrocyte differentiation (adapted from [4–6,34]). If EXT proteins are defective or absent, heparan sulphate proteoglycan expression at the cell surface may be altered or absent, affecting the diffusion of IHH to its receptor. IHH signalling molecules are indicated in red, PTHLH signalling molecules in blue, and molecules investigated by quantitative reverse transcriptase polymerase chain reaction (qPCR) by *. (B) Schematic representation of changes in extracellular matrix, cellularity, and the expression of signalling pathways in the course of malignant transformation of osteochondromas and subsequent progression of peripheral chondrosarcoma. The different tumour types are represented by their histology. Malignant transformation of osteochondromas and further progression of peripheral chondrosarcoma are characterized by a decrease in chondroid matrix and an increase in cellularity, and the strict organization seen in growth plate is lost. In osteochondromas, there is IHH, canonical Wnt, and TGF-$\beta$ signalling but no PTHLH signalling. During malignant transformation of tumour cells that have escaped from the tight control of IHH, PTHLH signalling is activated, but Wnt signalling decreases. During the progression of grade I towards grade III lesions, IHH signalling gradually diminishes and Wnt signalling is lost. The proliferative TGF-$\beta$ signalling pathway is upregulated during progression and activates target genes needed to acquire a more malignant phenotype

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including sporadic basal cell carcinoma [19], medulloblastoma [20], colon cancer [21], small-cell lung cancer [22], and prostate cancer [23]. Inactivation of HH signalling has been associated with developmental malformations such as cyclopia and holoprosencephaly [2].

The involvement of IHH signalling in tumourigenesis as well as endochondral ossification points to its possible involvement in osteochondroma and chondrosarcoma. We have previously demonstrated that molecules downstream in the IHH/PTHLH signalling pathway are not expressed in osteochondromas [24,25], suggesting that growth signalling is disturbed. Malignant transformation leads to re-expression of these signalling molecules [24,25].

Here, we aimed at elucidating the role of IHH signalling in the progression of peripheral chondrosarcomas, in a series of hereditary and sporadic osteochondromas, as well as chondrosarcomas. The same series was subjected to genome-wide expression analysis to identify other signal transduction pathways involved in chondrosarcoma progression.

## Material and methods

### Patient material

Fresh frozen samples were obtained from resected specimens (Table 1). Growth plates were acquired from resections or biopsies for orthopaedic clinical conditions not related to osteochondroma or chondrosarcoma. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines ('Code for Proper Secondary Use of Human Tissue', Dutch Federation of Medical Scientific Societies).

### RNA isolation

RNA was isolated from tumour samples that contained at least 70% tumour cells, as determined by haematoxylin and eosin stained frozen sections, as described previously [26]. From L-493, RNA was isolated from two different parts of the tumour (one part corresponding to a low-grade area and the other with high-grade morphology), to investigate possible differences in gene expression within one tumour.

**Table 1.** Clinicopathological and tumour data

| Sample | Material* | MO†/solitary | Location | Gender | Age (years) | Included in experiments‡ |
|---|---|---|---|---|---|---|
| GP-29 | GP | — | Knee | Female | 12 | Array, qPCR |
| GP-53 | GP | — | Hip | Female | 12 | Array, qPCR |
| GP-62 | GP | — | Femur | Female | 8 | Array, qPCR§ |
| GP-67 | GP | — | Unknown | Male | 12 | Array, qPCR |
| L-298 | OC | MO | Pelvis | Male | 24 | Array, qPCR |
| L-524 | OC | MO | Fibula | Male | 27 | Array |
| L-657 | OC | Solitary | Femur | Female | 12 | Array, qPCR§ |
| L-673 | OC | Solitary | Humerus | Male | 12 | qPCR |
| L-841 | OC | MO | Femur | Female | 14 | Array, qPCR§ |
| L-1235 | OC | MO | Radius | Female | 5 | Array, qPCR§ |
| L-1247 | OC | Solitary | Femur | Male | 30 | qPCR |
| L-114 | PCS-I | MO | Femur | Male | 39 | qPCR |
| L-144 | PCS-I | Solitary | Scapula | Male | 28 | Array |
| L-163 | PCS-I | Solitary | Tibia | Male | 49 | Array, qPCR |
| L-626 | PCS-I | Solitary | Femur | Male | 21 | Array, qPCR[d] |
| L-627 | PCS-I | Solitary | Scapula | Female | 31 | Array, qPCR |
| L-724 | PCS-I | Solitary | Ilium | Female | 82 | Array, qPCR |
| L-739 | PCS-I | Solitary | Pubis | Male | 37 | Array, qPCR |
| L-758 | PCS-I | MO | Femur | Male | 41 | Array, qPCR |
| L-868 | PCS-I | Solitary | Humerus | Male | 18 | Array, qPCR |
| L-1190 | PCS-I | MO | Humerus | Female | 16 | qPCR |
| L-1245 | PCS-I | Solitary | Fibula | Male | 25 | Array, qPCR |
| L-11 | PCS-II | Solitary | Ilium | Male | 49 | qPCR |
| L-76 | PCS-II | Solitary | Pelvis | Male | 47 | Array, qPCR |
| L-123 | PCS-II | Solitary | Femur | Male | 60 | Array, qPCR |
| L-224 | PCS-II | Solitary | Ilium | Male | 26 | qPCR |
| L-308 | PCS-II | MO | Scapula | Male | 42 | qPCR |
| L-493 | PCS-II | Solitary | Thorax | Male | 37 | Array, qPCR§ |
| L-1165 | PCS-II | Solitary | Pelvis | Female | 37 | Array, qPCR |
| L-281 | PCS-III | Solitary | Scapula | Female | 39 | Array, qPCR§ |
| L-300 | PCS-III | MO | Scapula | Female | 61 | Array, qPCR§ |

* GP = growth plate; OC = osteochondroma; PCS = peripheral secondary chondrosarcoma grade I, II or III.
† MO = Multiple Osteochondromas.
‡ Array = cDNA microarray; qPCR = quantitative reverse transcriptase PCR experiments.
§ Samples used for standard curve in the qPCR experiments.


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### Quantitative reverse transcriptase PCR (qPCR)

For first strand cDNA synthesis, 1 μg of total RNA was reverse transcribed using AMV reverse transcriptase (Roche, Penzberg, Germany) with 100 ng primer (dT) 15 (Roche) and 50 ng random primers (Invitrogen Carlsbad, CA, USA), according to the manufacturer's instructions.

qPCR reactions were performed as previously described [27] with PCR primers provided as shown in supplementary Table 1 (available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html). Expression levels were normalized to four genes (*CPSF6, GPR108, CAPNS1,* and *SRPR*) selected from expression profiling experiments of peripheral and central [28] cartilaginous tumours, with the least variation between all samples using the geNorm programme [29]. $Log_2$ transformed normalized data were analysed in SPSS 11.0 (SPSS Inc, Chicago, IL, USA). Expression levels in human growth plates were compared with those in osteochondromas, low-grade (grade I) and high-grade (grades II and III) chondrosarcomas using one-way analysis of variance with Bonferroni correction. Spearman's non-parametric correlation coefficients were computed for relations between expression levels and histological grade. Corrected $p$-values $\leq 0.05$ were considered significant.

### cDNA microarrays

A custom-made cDNA microarray was used in order to include more genes involved in chondrogenesis. The list of cDNA clones is available upon request. Array production, hybridization, and image acquisition procedures were performed as described previously [28]. In brief, tumour and growth plate samples were hybridized against a common reference panel of cell lines [28,30]. For hybridization, 1 μg of sample total RNA and 1 μg of reference panel total RNA were used to generate biotin- and fluorescein-labelled cDNA, using the Micromax TSA labelling kit (Perkin Elmer, Wellesley, MA, USA). Hybridized slides were scanned with a GeneTac LSV scanner (Genomic Solutions, Ann Arbor, MI, USA). Experimental quality was checked by labelling and hybridizing five samples in duplicate, either as experimental duplicates ($n = 2$) or as dye swaps ($n = 3$).

### Data analysis

Signal intensities were recorded and quantified with Genepix Pro 4.1 software (Axon Instruments, Union City, CA, USA). A Microsoft Excel macro [28] was previously created to select bona fide spots systematically, normalize these by dividing by the median of all bona fide spots, and log transform the normalized spots.

Analysis was performed for genes expressed in at least 70% of samples. Unsupervised hierarchical clustering (with 'complete linkage' and 'correlation') was performed with Spotfire Decisionsite™ software for functional genomics (Somerville, MA, USA). Two methods for group comparison were used. The first method (corrected $t$-test method) used the log-transformed ratios of the Excel macro. $P$-values were calculated by a two-sided Student's $t$-test with unequal variance and corrected for multiple testing using the step-up procedure with a false discovery rate of 10% [31]. The second method was the Limma (Linear models for Microarray Data) package [32], because of its excellent performance in microarray analysis. Limma used the raw Genepix data for within-array print-tip loess normalization of intensities after the same criteria for spot selection were applied, as described in the Excel macro [28]. Identities of differentially expressed, spotted clones were verified by sequencing the PCR products.

Differentially expressed genes were selected for verification by either qPCR or immunohistochemistry. The microarray series could be expanded with seven extra tumours (Table 1) for qPCR analysis.

### Immunohistochemistry

Expression of JUNB (antibody sc-8051, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, dilution 1 : 150) and PAI1 (*SERPINE1* protein; antibody No 3785, American Diagnostica, Stanford, CT, USA, dilution 1 : 350) was evaluated in 18 osteochondromas, 39 chondrosarcomas, and 12 human growth plates. Staining and scoring of 4 μm sections of formalin-fixed, paraffin-embedded material was performed as described [24]. Staining intensity was compared with internal positive controls: osteoblasts and osteoclasts for JUNB, blood vessels and connective tissue for PAI1.

Activity of TGF-$\beta$ signalling and Wnt signalling was investigated by the presence of either nuclear phosphorylated Smad2 (PS2 antibody, kindly donated by P ten Dijke [33], dilution 1 : 2000) expression or nuclear $\beta$-catenin (clone 14, BD Biosciences, Erembodegem, Belgium, dilution 1 : 800) expression. Osteoblasts served as positive internal control for PS2, and osteoblasts and blood vessels for $\beta$-catenin.

A $\chi^2$ test and Spearman's non-parametric correlation were used in the statistical analysis. Values of $p \leq 0.05$ were considered significant.

## Results

### qPCR to detect mRNA expression of IHH signalling molecules

Expression of seven genes involved in IHH signalling (Figure 1A) was assayed using qPCR, and compared with human growth plates (Table 2). Compared with growth plates, *PTCH* expression was decreased in low-grade ($p = 0.039$) and high-grade chondrosarcomas ($p = 0.001$) but not in osteochondromas ($p = 1.00$; Figure 2A). Of the *GLI* transcription factors, *GLI2* had the highest expression in the growth plate


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**Table 2.** Log2 transformed relative expression data of IHH signalling molecules in qPCR

| Genes | Growth plate ($n = 4$) Median† ± SD | Osteochondroma ($n = 6$) Median ± SD | Osteochondroma vs growth plate $p$-value‡ | Low grade PCS* n | Low grade PCS* Median ± SD | Low-grade PCS vs growth plate $p$-value | High grade PCS n | High grade PCS Median ± SD | High-grade PCS vs growth plate $p$-value |
|---|---|---|---|---|---|---|---|---|---|
| *IHH* | 10.94 ± 2.44 | 9.08 ± 2.62 | 1.000 | 9 | 10.34 ± 2.88 | 1.000 | 9 | 7.19 ± 2.65 | 0.166 |
| *PTCH* | 10.74 ± 0.54 | 10.22 ± 0.73 | 1.000 | 10 | 9.24 ± 1.38 | **0.039** | 10 | 7.80 ± 1.11 | **0.001** |
| *SMO* | 10.22 ± 0.49 | 10.33 ± 0.36 | 1.000 | 10 | 10.50 ± 0.66 | 1.000 | 10 | 9.73 ± 1.34 | 0.554 |
| *GLI1* | 9.80 ± 0.23 | 9.43 ± 0.75 | 1.000 | 10 | 8.49 ± 1.04 | 0.235 | 10 | 8.37 ± 1.35 | **0.043** |
| *GLI2* | 11.10 ± 0.93 | 9.87 ± 0.73 | 0.567 | 10 | 9.17 ± 1.23 | **0.015** | 10 | 8.62 ± 1.59 | **0.002** |
| *GLI3* | 9.65 ± 0.22 | 8.91 ± 0.71 | 1.000 | 10 | 9.04 ± 0.70 | 1.000 | 10 | 9.16 ± 1.00 | 0.475 |
| *PTHLH* | 7.44 ± 0.83 | 6.08 ± 1.11 | 0.620 | 10 | 5.68 ± 1.08 | 0.474 | 10 | 4.95 ± 1.89 | 0.206 |

* PCS = peripheral chondrosarcoma.
† Median = median log transformed expression level of the group.
‡ *P*-value after Bonferroni correction.

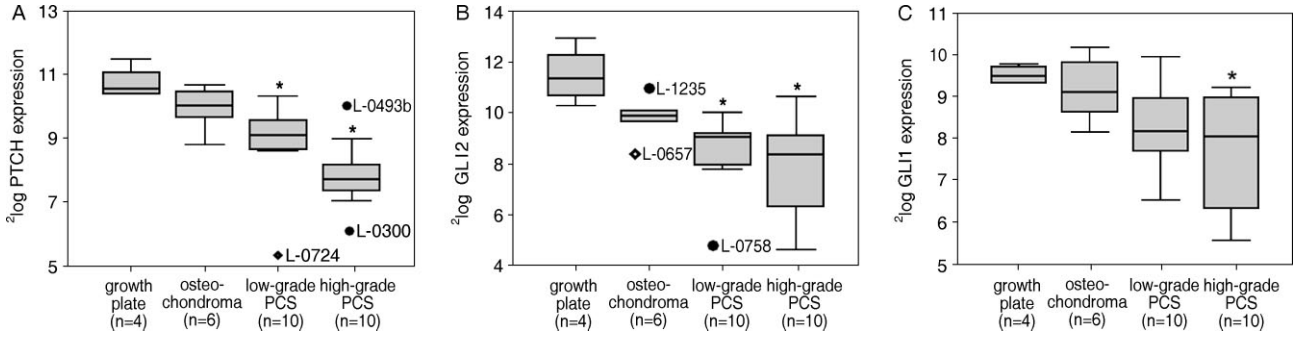


**Figure 2.** qPCR results of IHH signalling molecules. (A–C) Log-transformed relative mRNA expression levels in growth plate, osteochondromas, and low-grade and high-grade chondrosarcomas represented in boxplots of (A) *PTCH* expression, (B) *GLI2* expression, and (C) *GLI1* expression. *Significantly lower expression compared with growth plate ($p < 0.05$). Diamonds = extreme values; filled circles = outliers; PCS = peripheral chondrosarcoma

($p = 0.006$) and was reduced in low-grade chondrosarcomas ($p = 0.015$). Expression of both *GLI2* and *GLI1* was even lower in high-grade tumours than in growth plate ($p = 0.002$ and $p = 0.043$, respectively; Figure 2B and C). *GLI1* expression correlated with *GLI2* expression ($r = 0.584$, $p = 0.02$). *GLI3* expression was not affected in tumours (Table 2).

Increasing histological grade correlated negatively to the expression of *PTCH* ($r = -0.600$, $p = 0.001$), *GLI1* ($r = -0.458$, $p = 0.018$), and *GLI2* ($r = -0.457$, $p = 0.019$), suggesting gradual downregulation of IHH signalling during tumour progression (Figure 1B). Expression of *PTCH* ($r = -0.810$, $p < 0.001$), *GLI1* ($r = -0.524$, $p = 0.006$), and *GLI2* ($r = -0.421$, $p = 0.032$) correlated negatively with patient age (supplementary Figure 1, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html), gradually diminishing over 40 years. Using age as a covariate in the analysis, the difference in expression between growth plates and chondrosarcomas was only significant for *GLI2* ($p = 0.034$).

For the grade II chondrosarcoma L-493, two separate tissue samples were used. Expression data from the matrix-rich sample were similar to those of low-grade tumours, while results from the more cellular and myxoid sample corresponded to those of high-grade chondrosarcomas. *PTHLH* mRNA expression was present in all tumours. PTHLH protein expression data, as determined previously [24], was available for four osteochondromas and 14 chondrosarcomas, and correlated with the mRNA expression ($r = 0.53$, $p = 0.01$).

## Expression profiling

To identify signal transduction pathways that might be alternatives to IHH signalling, genome-wide cDNA microarray analysis was performed on 20 tumours and four growth plates. Hierarchical cluster analysis showed that technical duplicates and dye swaps cluster together (supplementary Figure 2, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html), assuring technical reproducibility and absence of dye bias as well as microarray quality. Duplicate spotted clones showed optimal correlation as well. The samples from L-493 with different histology did not cluster together, in contrast to technical duplicates from other samples, complying with the aforementioned qPCR results. Unsupervised clustering showed no distinct clusters of tumour samples, indicating that the different histological grades are characterized by more subtle changes in gene expression. Despite the good overall quality of the microarray, 20 cDNA clones of genes involved in IHH and PTHLH signalling were suboptimal and could


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**Table 3.** Log2 transformed normalized expression levels for verification of microarray data

| Genes | Growth plate ($n = 4$) Median‡ ± SD | Osteochondroma ($n = 6$) Median ± SD | PCS-I* ($n = 10$) Median ± SD | PCS-II ($n = 8$) Median ± SD | PCS-III ($n = 2$) Median ± SD | Correlation with microarray expression data† | |
|---|---|---|---|---|---|---|---|
| | | | | | | *r* | *p*-value |
| *ALDOA* | 7.50 ± 0.41 | 7.61 ± 0.51 | 8.47 ± 0.74 | 8.47 ± 0.25 | 7.89 ± 0.02 | 0.509 | 0.013 |
| *COX8A* | 8.39 ± 0.54 | 7.89 ± 0.61 | 8.55 ± 0.58 | 8.26 ± 0.67 | 8.19 ± 0.27 | 0.014 | 0.948 |
| *NDUFB8* | 11.73 ± 0.32 | 11.63 ± 0.71 | 11.98 ± 1.71 | 10.36 ± 0.81 | 10.39 ± 0.33 | 0.409 | 0.052 |
| *NDUFS3* | 8.50 ± 0.94 | 8.53 ± 0.79 | 8.36 ± 1.09 | 6.98 ± 0.69 | 7.13 ± 0.82 | 0.195 | 0.372 |
| *PLOD3* | 4.87 ± 0.44 | 5.18 ± 0.92 | 6.07 ± 0.74 | 6.42 ± 0.53 | 6.66 ± 0.38 | 0.579 | 0.004 |
| *FN1* | 11.36 ± 0.84 | 10.73 ± 1.13 | 10.61 ± 1.10 | 11.06 ± 1.29 | 13.50 ± 0.31 | 0.552 | 0.006 |

* PCS = peripheral chondrosarcoma.
† Correlation of qPCR expression data with microarray expression data available for four growth plates, four osteochondromas, eight grade I, five grade II, and two grade III chondrosarcomas.
‡ Median = median log transformed expression level of the group.

therefore not be optimally correlated to the qPCR results of these genes.

Limma analysis revealed 17 differentially expressed cDNA clones between osteochondromas and human growth plates (supplementary Table 2, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html). Fourteen clones were more highly expressed in osteochondromas, including multiple clones encoding the proto-oncogene jun B (*JUNB*) and several metallothionein 1 (*MT1*) genes.

We did not find any significantly differentially expressed genes in the comparison of osteochondroma and grade I chondrosarcoma.

A comparison of grade I and grade III chondrosarcomas for analysis of tumour progression identified 79 differentially expressed genes with the corrected *t*-test method and 32 with the Limma package (supplementary Table 3, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html). Eleven genes were present in both analyses. Several genes involved in TGF-$\beta$ signalling were upregulated in grade III chondrosarcomas: fibronectin 1(*FN1*); serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (*SERPINE1*); thrombospondin 1 (*THBS1*); and cyclin-dependent kinase inhibitor 1A (*CDKN1A*, *p*21, *CIP1*). TGF-$\beta$1 was upregulated in high-grade chondrosarcomas (supplementary Table 3, available at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2008.html). Several genes involved in extracellular matrix remodelling were upregulated; among the downregulated genes (corrected *t*-test $n = 23$; Limma $n = 5$) were those encoding the $\alpha$1 chains of type IX collagen(*COL9A1*) and type II collagen (*COL2A1*). Additionally, several genes encoding proteins involved in oxidative phosphorylation were downregulated in grade III chondrosarcomas. These included genes encoding NADH dehydrogenase (ubiquinone) 1 $\beta$ subcomplex, 8 (*NDUFB8*), a component of complex I, and cytochrome *c* oxidase subunit 8A (*COX8A*) of complex IV. Also, glycolytic enzyme aldolase C (*ALDOC*), part of the glycolysis, was downregulated in grade III chondrosarcomas compared with grade I lesions.

## qPCR verification of cDNA microarray results

To confirm the differences in mRNA expression of genes relevant to TGF-$\beta$ signalling, extracellular matrix, and oxidative phosphorylation found in the progression analysis, we performed qPCR on four genes representing these groups (Table 3). The qPCR results for *FN1, PLOD3*, and *NDUFB8* correlated with the microarray expression data ($p = 0.006$, $p = 0.004$ and $p = 0.052$, respectively). No correlation was found for *COX8A* ($p = 0.948$). *FN1* was upregulated in grade III chondrosarcomas compared with osteochondromas, and grade I and II chondrosarcomas ($p = 0.011$, 0.014, and 0.012, respectively). Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (*PLOD3*) expression was significantly positively correlated with increasing histological grade ($r = 0.506$, $p = 0.008$), while *NDUFB8* showed a negative correlation ($r = -0.391$, $p = 0.049$). Additionally, mRNA expression of NADH dehydrogenase (ubiquinone) Fe-S protein 3 (*NDUFS3*) of oxidative phosphorylation complex I, and aldolase A (*ALDOA*) of glycolysis were analysed. Similarly to *NDUFB8, NDUFS3* expression was negatively correlated with tumour progression ($r = -0.529$, $p = 0.005$). Expression of *COX8* and *ALDOA* decreased with progression. *ALDOA* was upregulated in grade I chondrosarcomas in comparison with osteochondromas ($p = 0.024$).

## Immunohistochemical analysis of protein expression

*SERPINE1* and *JUNB* microarray expression data were verified by immunohistochemistry (Table 4).

The sum score of PAI1 (*SERPINE1* protein) correlated with the mRNA expression ($r = 0.488$, $p = 0.047$) and with increasing histological grade ($r = 0.447$, $p < 0.001$, Figure 3A–C).

For 16 samples (two human growth plates, three osteochondromas, and five grade I, four grade II, and two grade III chondrosarcomas), also included


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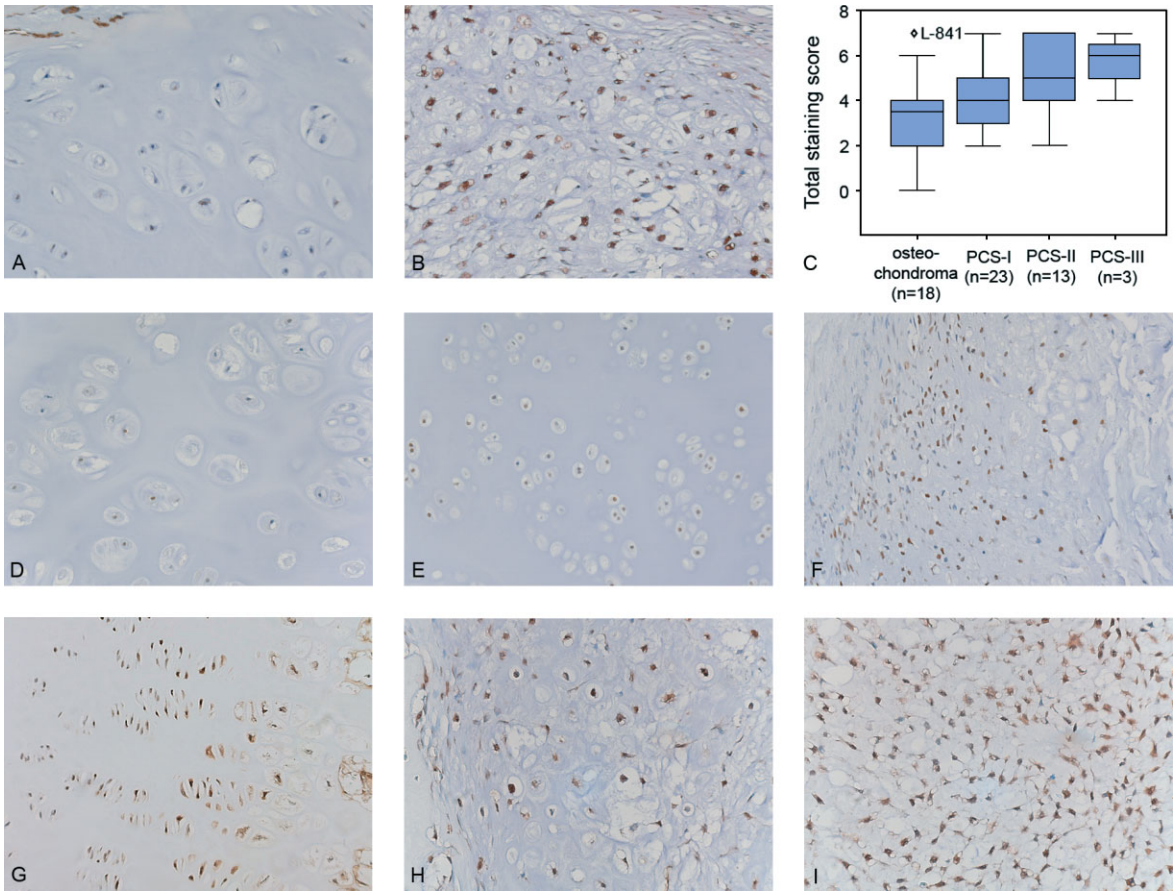


**Figure 3.** Immunohistochemical results. (A) Low-power view of an osteochondroma with weak PAI1 protein staining in less than 25% of the cells. Note the positive blood vessels, used as internal positive control; (B) a grade II chondrosarcoma with strong cytoplasmic PAI1 protein staining in 75–100% of the cells; (C) Boxplot of total scores from the semiquantitative scoring of PAI1 protein expression. There is a significant positive correlation between progression and PAI1 protein expression ($r = 0.447$, $p < 0.001$). (D) Low-power view of an osteochondroma demonstrating weak nuclear JUNB protein expression in 25–50% of the cells; (E) grade I chondrosarcoma with moderate nuclear JUNB protein expression in 75–100% of the cells; (F) strong nuclear JUNB expression was found in most cells in this grade II chondrosarcoma. (G) Low-power view of a human epiphyseal growth plate with strong nuclear and cytoplasmic phosphorylated Smad2 staining in the proliferating and hypertrophic chondrocytes, whereas the resting chondrocytes are negative; (H) peripheral chondrosarcoma grade II with strong phosphorylated Smad2 expression in the majority of tumour cells. The same was observed in grade III chondrosarcomas (I)

**Table 4.** Immunohistochemistry results for PAI1 and JUNB

| | PAI1 | | JUNB† | |
|---|---|---|---|---|
| | Positive* | Percentage | Positive* | Percentage |
| Growth plate | | | 1/10 (0/2) | 10 (0) |
| Osteochondroma | 9/18 (2/3) | 50 (67) | 5/13 (1/3) | 38 (33) |
| PCS grade I | 15/23 (5/8) | 65 (63) | 7/22 (3/5) | 32 (60) |
| PCS grade II | 12/13 (4/4) | 92 (100) | 7/12 (2/4) | 58 (50) |
| PCS grade III | 3/3 (2/2) | 100 (100) | 3/3 (2/2) | 100 (100) |

* Number of positive tumours/total number of tumours that could be evaluated. Data for the tumours for which cDNA array analysis was also available are shown in parentheses.

† Five osteochondromas and nine chondrosarcomas could not evaluated because the internal positive controls were negative.

in the microarray experiments, JUNB protein expression correlated with the mRNA expression ($r = 0.544$, $p = 0.016$). Expression analysis revealed differential expression of JUNB mRNA between growth plate and osteochondroma. When comparing growth plates and osteochondromas for JUNB protein expression, no difference was observed (Fisher's exact test, $p = 0.179$). The number of JUNB-positive tumours correlated significantly with increasing histological grade ($\chi^2$, linear-by-linear, $p = 0.005$, Figure 3D–F, Table 4).

In human growth plates, both cytoplasmic and nuclear expression of phosphorylated Smad2 was observed in proliferating and hypertrophic chondrocytes (Figure 3G). Expression was variable in resting chondrocytes. Nuclear and cytoplasmic expression of phosphorylated Smad2 was observed in all osteochondromas and chondrosarcomas (Figure 3H and I), implicating active TGF-$\beta$ signalling (Figure 1B).

Nuclear expression of $\beta$-catenin, was found for 9/17 (53%) osteochondromas, 4/21 (19%) grade I chondrosarcomas, and in the hypertrophic zone of 5/12 (42%) human growth plates, but not in 11 grade II and two grade III chondrosarcomas. This indicated that canonical Wnt signalling decreased with increasing malignancy ($\chi^2 p = 0.020$; Figure 1B). Twenty-two


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tumours also included in the frozen series demonstrated a positive correlation between PTCH expression and nuclear expression of $\beta$-catenin ($r = 0.567$, $p = 0.006$).

## Discussion

This study shows that mRNA expression of IHH downstream targets is gradually downregulated during tumour progression in peripheral chondrosarcoma compared with normal growth plates, from which its benign precursor, osteochondroma, originates. Expression of both *PTCH* and *GLI1*, two genes transcribed upon activation of HH signalling [3], correlated negatively with increasing histological grade. This was also found for *GLI2*, which transduces the IHH signal during endochondral bone development [34], but is not a known downstream target of IHH. Recently, GLI3 has been identified as a key effector of IHH signalling during cartilage development [35], where IHH antagonizes the repressor activity of GLI3 on PTHLH expression and proliferation. However, in chondrosarcomas, *GLI3* expression was similar to the expression seen in human growth plate (Table 2). Therefore, there was no indication that GLI3 plays a role in chondrosarcoma progression.

Recently, constitutively active IHH signalling was demonstrated in chondrosarcoma explants [36]. These contradictory results could be partly explained by the selection of housekeeping genes for qPCR analysis. This has been shown to be tissue type specific [29], which led us to choose genes based upon the expression array results, instead of choosing random, non-tumour-specific genes, like *GAPDH*, which was not stably expressed in cartilaginous tumours. Also, no discrimination was made by Tiet *et al* between peripheral and central-type chondrosarcomas, [36] even though it was previously shown that these subtypes clearly have a different genetic make-up [37,38] and other distinct pathways might be operative.

IHH signalling has been shown to be upstream of canonical Wnt signalling cascades required for osteogenic differentiation [39]. Wnt signalling appears to be involved in chondrogenic differentiation, since we could demonstrate decreased nuclear expression of $\beta$-catenin during chondrosarcoma progression (Figure 1B). This intriguing potential relation between IHH and Wnt signalling in chondrogenesis needs further studies.

*EXT1* and *EXT2* are of importance for the diffusion of IHH to its receptor [9,14,40]. Consequently, there is a strong indication that IHH signalling is important in osteochondroma formation. Here we have demonstrated that the expression of IHH signalling in osteochondromas did not differ from the expression found in growth plates. Morphologically, osteochondromas strongly resemble the epiphyseal growth plate [10], and different zones of endochondral ossification can sometimes be distinguished. It was not technically possible to dissect these different layers to investigate zone-specific expression of IHH signalling molecules. This probably also affects expression levels of genes that are only expressed in a specific layer.

Similar to growth plates, osteochondromas cease to grow after puberty [10]. Therefore, we investigated the putative relationship between mRNA levels of IHH signalling molecules in tumours and patient age, and demonstrated decreasing expression of *PTCH, GLI1*, and *GLI2* over a period of 40 years. However, the observation that diagnosis correlates with age [41], and the distinct expression profiles of low-grade and high-grade tumours of patients with the same age (including the two samples from L-493), suggest that expression of *PTCH, GLI1*, and *GLI2* does correlate with tumour progression.

Despite inactivation of IHH signalling, there is active, IHH-independent PTHLH signalling in chondrosarcomas (Figure 1B) [4,24]. This is similar to the finding in central chondrosarcomas [27], but not to murine growth plate, where IHH has been shown to directly regulate PTHLH expression [42]. Our results showed a discrepancy between PTHLH mRNA and previously published protein expression [24]. However *PTHLH* mRNA has a very short half-life [43], making it difficult to correlate protein and gene expression.

A good candidate to activate PTHLH signalling in the absence of IHH is TGF-$\beta$, which can regulate *PTHLH* expression independently of IHH [44]. Genome-wide expression profiling experiments revealed that tumour progression was indeed associated with upregulation of genes involved in TGF-$\beta$ signalling, including TGF-$\beta$1, comparable to results found in other tumours [45]. Expression of *FN1, SERPINE1*, and *THBS1* was upregulated in grade III chondrosarcomas (Tables 3 and 4). These genes are known downstream targets of TGF-$\beta$ signalling and are significant in regard to tumour invasion and metastasis [46]. Upregulation of the TGF-$\beta$ downstream cell cycle inhibitor CDKN1A has previously been noted at the protein level [24]. The changes observed in extracellular matrix-related genes could also be regulated by TGF-$\beta$. Taken together, these results implied that TGF-$\beta$ signalling is activated in high-grade chondrosarcomas and this conclusion was supported by the presence of nuclear phosphorylated Smad2 in these tumours. Phosphorylated Smad2 was also present in osteochondromas and human growth plates. For the latter the expression pattern was similar to that of unphosphorylated Smad2 in rat epiphyseal growth plate [47]. One can hypothesize that in chondrosarcomas TGF-$\beta$ activates specific target genes needed to acquire a malignant phenotype. The possibility cannot be excluded that some of these differentially expressed genes, like *THBS1* and *FN1*, are the result of increased vascularization in high-grade chondrosarcomas [48,49].

The diminished amounts of chondroid matrix in high-grade chondrosarcomas [15] and its lack of


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cellular organization in comparison with osteochondroma suggest loss of the IHH/PTHLH feedback loop (Figure 1). IHH signalling is a tightly controlled signalling pathway. We observed that osteochondroma cells are still controlled by IHH and undergo endochondral ossification. As the osteochondroma ages, IHH signalling decreases, which was also seen in the growth plate [50], and all tumour cells will eventually differentiate. One can hypothesize that tumour cells may escape from IHH control to transform into a malignancy, by switching to less controlled proliferative signalling pathways, such as TGF-$\beta$ signalling, thereby causing a cascade of events resulting in tumour progression (Figure 1B). To explore this hypothesis further, a model system would be helpful, but this is hampered by differences between humans and rodents, in which there is no closure of the growth plate at the end of sexual maturation [51].

Further analysis of the genome-wide expression studies revealed decreased expression of genes that encode proteins involved in oxidative phosphorylation and glycolysis in grade III chondrosarcomas compared with grade I chondrosarcomas. Glycolysis is upregulated in hypoxic environments, such as paucivascular osteochondromas and low-grade chondrosarcomas [10,15], giving cells a growth advantage [52]. High-grade chondrosarcomas show increased vascularization [48,49], which will raise oxygen availability and thereby abolish the need for glycolysis. This was reflected by the downregulation of *ALDOA* and *ALDOC* in grade III chondrosarcomas.

Oxidative phosphorylation was simultaneously downregulated with glycolysis. In otherwise non-invasive C2C12 myoblasts, depletion of mitochondrial DNA induces an invasive phenotype [53] and increases resistance to apoptosis, in part by upregulating Bcl-2 [54]. High-grade chondrosarcomas have high Bcl-2 expression [24] and an increased risk of recurrence and metastasis [15], consistent with an invasive phenotype.

The expression profiles of osteochondromas and grade I chondrosarcomas were indistinguishable. This distinction is also considered difficult at the histological level and is usually based on a combination of histological, radiological, and clinicopathological data [15]. Most likely the differences in gene expression are very subtle or only detectable at the protein level and cannot be detected with the method used.

Both qPCR and genome-wide expression profiling revealed similarities in gene expression between osteochondromas and human epiphyseal growth plates. However, *JUNB* (and to lesser extent *FOSB*) were more highly expressed in osteochondromas than in growth plates. JUNB and FOSB are members of the AP-1 transcription factor family, which has been implicated in endochondral ossification in mice [55,56]. Remarkably, we found very low levels of JUNB mRNA and protein in growth plates. JUNB can have a stimulatory effect on chondrocyte proliferation [55]. At the protein level, JUNB gradually increased during malignant transformation and further progression, which is consistent with its effect on proliferation. This increase in JUNB expression might also be regulated by TGF-$\beta$ [57].

In conclusion, IHH signalling controls the tight regulation of growth plate organization and is still active in osteochondroma. However, IHH signalling is gradually inactivated during peripheral chondrosarcoma progression when tumour cells adapt to a more malignant phenotype (Figure 1B). TGF-$\beta$ signalling can potentially regulate PTHLH signalling and concurrent remodelling of the extracellular matrix. Downregulation of genes involved in oxidative phosphorylation and glycolysis accompanies the more invasive phenotype of high-grade tumours.

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