Molecular profiling of solitary and Ollier disease-related central chondrosarcomas: An investigation of DNA aberrations, mRNA and protein expression

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An investigation of DNA aberrations, mRNA and protein expression

Cover: A radiographic image of a hand from a patient with Ollier disease (top left), a macro picture of a central chondrosarcoma (top right), an immunohistochemical staning of a central chondrosarcoma (bottom left), and a graphic representation of the genomic aberrations of a grade III central chondrosarcoma (bottom right).

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CONTENTS

Chapter 1

General introduction

CONTENTS

I. Bone tumors

Bone sarcomas are rare neoplasms with an incidence of around the 1-2 new cases per 100.000 individuals per year. The most common primary malignant bone tumors are osteosarcomas, followed by chondrosarcomas and Ewing sarcomas. Both osteosarcomas and Ewing sarcomas have the highest incidence in younger patients, up to twenty years of age, whereas chondrosarcomas are more prevalent at around the second to fourth decade of life (Table 1.1).

Table 1.1: Overview of the incidence and age of presentation of the most common primary bone tumors

a) Benign bone tumors

Tumor	Incidence	Peak occurrence
Osteochondroma	45%	< 20 years
Giant cell tumor	20%	20 - 45 years
Enchondroma	~10%	10 - 50 years
Osteoid osteoma	10%	5 - 25 years
Aneurysmal bone cyst	7	0 – 20 years
Chondromyxoid fibroma	2%	10 - 30 years
Osteoblastoma	1%	10 - 35 years
Chondroblastoma	1%	10 - 20 years

Incidence is percentage of all benign bone tumors

b) Bone sarcomas

Tumor	Incidence	Peak occurrence
Osteosarcoma	21%	10 - 20 and 50 - 70 years
Chondrosarcoma	20%	30 - 60 years
Ewing sarcoma	5%	10 - 30 years
Malignant Fibrous Histiocytoma	5%	> 30 years
Fibrosarcoma	2%	35 – 60 years
Adamantinoma	extremely rare	10 – 40 years

Incidence is percentage of all malignant primary bone tumors

Clinical features of bone tumors are often non-specific, and as a consequence of this they often are not detected in the early phases. Symptoms pointing to bone tumors are pain, swelling and a general discomfort. They can also be detected through spontaneous fractures, as a consequence of the fact that the bone structure is altered by the tumor.

II. Chondrosarcoma

Chondrosarcomas are, after osteosarcomas, the most prevalent bone sarcomas. They are characterized by the production of cartilage instead of bone. The incidence in males and females is equal, and it mainly occurs in adults of 30-60 years [1]. Most of these tumors occur in the long bones. Within the group of chondrosarcomas different subtypes are discerned [2-4] (Table 1.2).

Conventional central chondrosarcomas can arise *de novo* in the medulla of the bone (primary), or from an enchondroma (secondary) [5]. This group comprises about 80% of all conventional chondrosarcomas. The second most prevalent chondrosarcomas are the secondary peripheral chondrosarcomas, which per definition arise in the cartilage cap of an osteochondroma [6].

Table 1.2: Clinio-pathological subtypes of chondrosarcoma of bone

Subtype	Incidence (% of all chondrosarcomas)
Conventional	80-85 %
Central	83% of conventional chondrosarcomas
	60-98 % primary
	2-40 % secondary
Peripheral	17% of conventional chondrosarcomas
	100 % secondary
Dedifferentiated	6-10 %
Mesenchymal	2 %
Juxtacortical	2 %
Clear cell	1 %

Osteochondroma is a cartilage-capped bony projection arising on the external surface of bone, and contains a marrow cavity that is continuous with that of the underlying bone [7]. The cartilage is organized histologically in the same manner as the normal human growth plate. In enchondromas no specific growth pattern is discerned. The peripheral and central chondrosarcomas look histologically similar and in both three grades of malignancy are discerned (Table 1.3; Figure 1.1) [2].
Grading is one of the most important prognostic predictors. In high-grade tumors the risk of metastasis is increased. Where as grade I chondrosarcomas seldom metastasize, 10-33% of grade II and ~70% of grade III chondrosarcomas metastasize [2,8]. However, this method of grading is somewhat subjective and therefore it would be helpful to find a molecular marker predicting outcome. Apart from grading also the localization is of importance for prognosis, as is summarized in chapter 2.

Table 1.3: Histological grading of conventional chondrosarcomas according to Evans et al. [2]

Grade	Histological features
I	Marked preponderance of small densely-straining nuclei
	Background varies from chondroid to myxoid
	Calcification and bone formation are frequent
	Multiple nuclei within one lacuna, sometimes infrequent
	Occasionally small number of larger, somewhat pleomorphic nuclei are present
II	Proportion of nuclei is of moderate size
	Low mitotic rate (less than 2 mitoses per 10 high power fields)
	Increased cellularity, specifically toward periphery of tumor lobules
	Nuclei are paler and have visible intranuclear detail
	Background in more cellular areas tends to be more myxoid
III	Two or more mitoses per 10 high power fields in most active areas
	Increased cellularity in periphery of tumor lobules
	Larger nuclei in areas with increased cellularity as compared to grade II chondrosarcomas
	Spindle cell shaped cells in high cellular areas, no appreciable chondroid/myxoid matrix

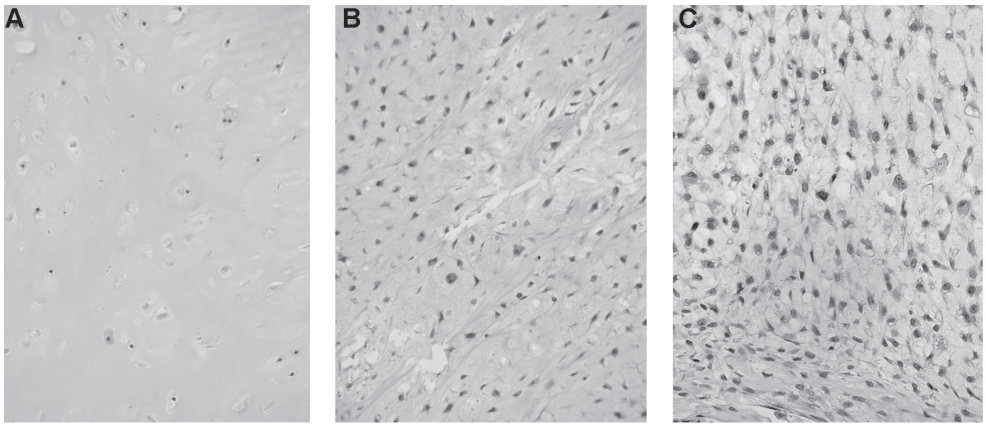

Figure 1.1: *Histology chondrosarcoma grade I (A), II (B), and III (C)*

III. Conventional central chondrosarcoma

About 80% of the conventional chondrosarcomas are originating centrally in the medulla cavity of the bone central. In contrast to enchondromas that frequently occur in the small bones of hands and feet, chondrosarcomas are extremely rare at this phalangeal localization. The most prominent affected sites are femur (30%), pelvis (30%) and humerus (15%) [1]. In the literature the incidence of secondary central chondrosarcomas varies significantly, ranging from 1-2 % to 40% [3,9] depending on if one requires evidence of a pre-existing enchondroma or evidence of an enchondroma next to a chondrosarcoma (see also chapter 2).

Genetics of conventional central chondrosarcoma

Little is known regarding the way conventional central chondrosarcomas arise and only few studies have been performed that make the distinction between central and peripheral chondrosarcomas. Most central chondrosarcomas are near-diploid [10]. Genetic studies revealed only few genomic alterations in enchondromas and low-grade central chondrosarcomas. In high-grade central chondrosarcomas more complex aberrations are found [11,12]. However, most alterations appear to be random. Studies using cytogenetics, loss of heterozygosity (LOH) or mutational analysis revealed that some chromosomes or chromosomal regions seem to be non-randomly affected. Structural alterations of chromosome 9, mainly focused on 9p, seem to be more common in for central chondrosarcomas [10]. Chromosomal region 9p12-22, which was found to be targeted in at 3 of 12 central chondrosarcomas, contains the tumor suppressor gene *CDKN2A*, also known as p16 [10]. This gene is deleted in several types of cancer and could therefore be a candidate gene for central chondrosarcomas. Also band 17p13, containing the tumor suppressor *TP53*, has been studied and loss or mutations were identified, mainly in high-grade tumors. Without distinguishing central and peripheral chondrosarcomas, loss of chromosome arms 6q, 10p, 11p, 11q or 22q seemed to correlate with grade and loss of 13q was found to be a prognostic factor for metastasis [12].

Protein expression studies
Immunohistochemistry studies on chondrosarcomas have been done quite extensively. In most cases again no distinction was made between central and peripheral chondrosarcomas and prognostic factors were not found (summarized in chapter 2).

IV. Enchondroma
Enchondroma is the benign counterpart of central conventional chondrosarcomas. The incidence of enchondromas is not known, since they often are asymptomatic, and only found after a fracture or bone scans on other grounds, such as screening for some metastasis of epithelial cancer. The risk of malignant transformation is <1%.
Histologically, the distinction between enchondroma and chondrosarcoma is mainly based on comparison of cytology, architecture and growth patterns. Whereas the enchondromas are often encased by normal lamellar bone at the outside of the tumor (encasement), chondrosarcomas show infiltration of tumor tissue in the normal bone (entrapment) [9,13]. However, the distinction is not always easy and molecular markers for diagnostic purposes would be helpful.
Radiological analysis is helpful in making the distinction between enchondromas and chondrosarcomas. Plain X-ray is unable to make this distinction [14], whereas magnetic resonance (MR) improves tissue characterization of cartilaginous tumors and may assist in identifying low-grade chondrosarcoma. Gadolinium-enhanced MR can be used [15], but better results are obtained using fast contrast-enhanced MR imaging, that may assist in differentiation between benign and malignant cartilaginous tumors in adults [16].

V. Enchondromatosis
While most enchondromas and/or conventional central chondrosarcomas are solitary, some occur multiple in the context of a syndrome (enchondromatosis) [17]. The two best known are Ollier disease [18,19], characterized by the presence of multiple enchondromas, and Maffucci syndrome [20], characterized by the presence of multiple enchondromas and

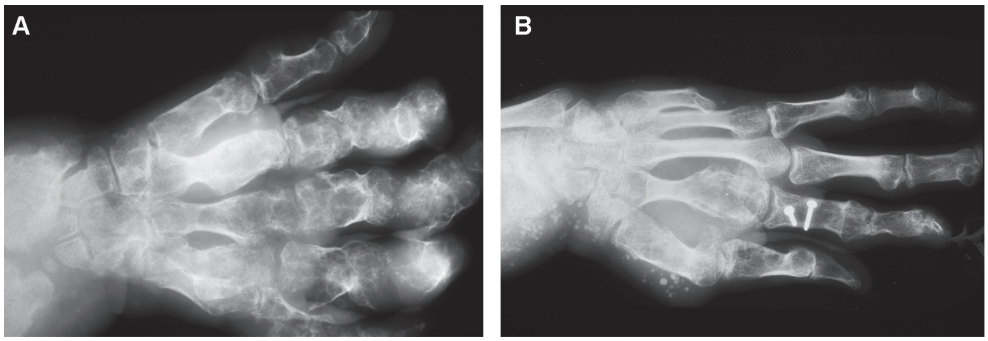

Figure 1.2: *Radiographs of hands from 2 patients with enchondromatosis. A) Hand from patient with Ollier disease with multiple enchondromas, B) Hand from patient with Maffucci syndrome, containing enchondromas and calcified thrombi in soft tissue haemangiomas. Source: Committee on Bone Tumors, The Netherlands*

haemangiomas (benign vascular lesions) (Figure 1.2). None of the syndromes is hereditary, though rare cases in which more than one person in the family is affected have been reported. In patients with enchondromatosis it is frequently seen that only one side of the body is affected. This could point in the direction of an early mutation event in embryogenesis, resulting in mosaicism.
Patients with enchondromatosis have an increased risk of malignant transformation and the chance of developing a chondrosarcoma can be as high as 35%, compared to < 1% in patients with a solitary enchondroma [17]. Enchondromas in patients with enchondromatosis behave less aggressively and slightly different criteria for malignancy are thus applied: more worrisome features are accepted in enchondromas [17] from patients with enchondromatosis, such as increased cellularity and more cytological atypia.

Genetics
Genetic studies on enchondromatosis tumor samples are rare. LOH of chromosomal regions 13q14 and 9p21 [21] were found in a chondrosarcoma of one patient and deletion of 1p in another patient [22]. Also a mutation in the *PTHR1* gene, called p.R150C PTHR1, has been described in two of six patients with Ollier disease, one as a germline mutation and one most likely somatic [23].
In addition to the well-known syndromes, Ollier disease and Maffucci syndrome, some other, less well-defined subtypes of enchondromatosis have been described:
1) Spondyloenchondromatosis / spondyloenchondrodysplasia
Spondyloenchondromatosis [24-27] is a syndrome defined by the presence of multiple enchondromas and the presence of platyspondyly (flattened vertebral body shape with reduced distance between the endplates). The patients have short stature, short trunk, and short limbs, and the hands and feet are only mildly involved. In contrast to Ollier disease and Maffucci syndrome spondyloenchondromatosis is thought to be hereditary, following an autosomal recessive pattern.
2) Metachondromatosis
Metachondromatosis [28] is characterized by a combination of multiple enchondromas and osteochondromas. It follows an autosomal dominant inheritance pattern.
3) Generalized enchondromatosis
Generalized enchondromatosis [29,30] is described by evenly distributed enchondromas with severe involvement of hands and feet, mild platyspondyly and skull deformity. It was found to be familial in one case, following an autosomal recessive pattern.
4) Others
Other subtypes have been described, and they are subdivided based upon varying involvement of hands and feet, platyspondyly or vertebral lesions [26,30,31].

VI. Phalangeal enchondromas and chondrosarcomas
Most enchondromas are located in the long bones and about 50% of these tumors are found in the hands and feet [32,33], where they most frequently involve the proximal phalanx, followed by the middle phalanx, and the metacarpals. In these cases deformations may arise.

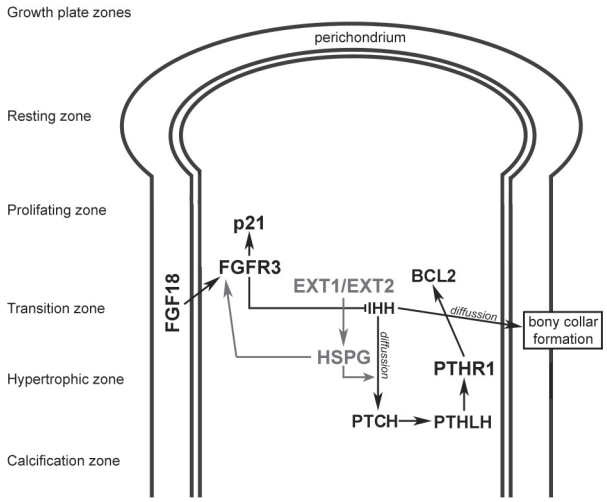

Figure 1.3: *IHH/PTHLH signaling in the postnatal growth plate. Chondrocytes in the transition zone secrete Indian Hedgehog (IHH) protein, which diffuses to its receptor Patched (PTCH) in the hypertrophic zone. This increases secretion of Parathyroid Hormone Like Hormone (PTHLH), located in the hypertrophic zone [36], which diffuses to its receptor. Terminal differentiation is inhibited by direct or indirect upregulation of BCL2, prolonging cell survival [37]. In this way, PTHLH regulates chondrocyte differentiation by delaying the progression of chondrocytes towards the hypertrophic zone and allowing longitudinal bone growth [38]. EXT1 and EXT2 are expressed in the proliferative and transition zone [39] and are required for the formation of Heparan sulphate proteoglycans (HSPGs), expressed in all zones of the growth plate [40-45]. HSPGs play a role in the high affinity binding of ligands to their receptor and possibly the diffusion of the molecules. High affinity binding of the ligand FGF18 [46] to the receptor (FGFR3, but also FGFR1 and FGFR2), expressed in the proliferation zone, requires HSPGs. Upon binding cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) is activated [47], inhibiting chondrocyte proliferation, and thereby the IHH expressing cells [48].*
Adapted from L. Hameetman et al [49].

Conventional central chondrosarcomas of the phalanx are extremely rare and they only rarely metastasize (<2%)[34]. Therefore, a different histological cut-off between enchondroma and low-grade chondrosarcoma is applied to these tumors. Thus, enchondromas of the phalanx can look histologically like a chondrosarcoma, but are still diagnosed as enchondromas because of the phalangeal localization. However, the reason for this favorable outcome of phalangeal chondrosarcomas is not known. Either these tumors are biologically different or the location (and smaller size of these tumors due to early detection, different vascularization or lower temperature) could account for their low property to metastasize [34,35].

VII. IHH/PTHLH signaling

The pathogenesis of enchondromas and central chondrosarcomas is not known. It has been suggested that they arise from cartilage residues of the growth plate. In the normal growth plate a tight signal regulation takes place, organizing the proliferation and differentiation of the chondrocytes. One of the important signaling pathways is the Indian Hedgehog/ Parathyroid Hormone Like Hormone IHH/PTHLH negative feedback loop [36,38,50-52] (Figure 1.3). This signaling pathway is suggested to be altered in tumors of patients with

multiple osteochondromas, a hereditary syndrome caused by mutations in the *EXT* genes (see chapter 2).
Binding of IHH to its receptor PTCH will result in the release of Smoothened (SMOH) by PTCH. Now the transcription factors GLI-Kruppel family members 1-3 (GLI1-3) are stabilized and transcription of several genes including *PTCH*, *GLI1-3* and *Cyclin E* (*CCNE*) is upregulated [51,53]. Downstream of IHH PTHLH (PTHrP) signaling is found. This signaling regulates the pace of chondrocytes differentiation by delaying the progression of chondrocytes towards the hypertrophic zone, allowing longitudinal bone growth [38] completing the negative feedback loop. In addition, the transition from G1 to S phase is a crucial step in the growth and is regulated by, amongst others, Cyclin D1, Cyclin E and p21 (CDKN1A). In chondrocytes this transition is partly influenced by IHH (Cyclin E) and PTHR1 (Cyclin D1) [50-52].

VII. Aim of the study and outline of the thesis

Enchondromas and conventional central chondrosarcomas are cartilage producing bone tumors as outlined above. Among these tumors two differently behaving subtypes are discerned, enchondromatosis-related and phalangeal enchondromas/chondrosarcomas.
The purposes of the studies presented in this thesis were to:
further elucidate the multi-step genetic model in central chondrosarcoma.
by identifying the molecular change(s) underlying malignant transformation of enchondroma we aim at finding molecular markers that may aid in the difficult differential diagnosis between enchondromas and low-grade chondrosarcomas.

1) by identifying the molecular change(s) involved in the progression from low-grade towards high-grade chondrosarcoma we hope to find prognostic markers, independent of histological grade. This is of interest since progression of grade can be observed with recurrent chondrosarcomas. In this thesis we looked at progression by comparing primary chondrosarcomas of different grades to each other.
2) investigate if there are molecular differences between enchondromatosis-related tumors and solitary tumors, to see if we can identify the molecular defect underlying enchondromatosis and further understand the less aggressive behavior of enchondromas in patients with enchondromatosis. We looked genome wide both at RNA expression levels and genomic aberrations. In addition a more hypothesis-driven approach was chosen, based on the IHH and PTHLH signaling pathways. These pathways are involved in normal cartilage growth and differentiation, which are though to play a role in osteochondromas and secondary peripheral chondrosarcomas and was alleged to be affected in Ollier disease.

investigate if there is a biological difference between phalangeal enchondromas and chondrosarcomas compared to enchondromas and chondrosarcomas located elsewhere, since phalangeal tumors only rarely metastasize, although the histological appearance suggests malignancy. The reason for this favorable outcome of phalangeal chondrosarcomas is not known. Either these tumors are biologically different or the location (and smaller size of these tumors) may be causative for their favorable prognosis.

Chapter 2 contains a more detailed review regarding the diagnosis and prognosis of both conventional central chondrosarcomas and secondary peripheral chondrosarcomas. The genetics of the different subtypes and an inventory of what was known in the literature of molecular markers for chondrosarcomas at the start of this study are summarized.

Since the loss of chromosomal region 9p21-22 was found more frequently in central chondrosarcomas as compared to peripheral chondrosarcoma in **chapter 3** we investigated the candidate gene *CDKN2A* located in 9p21.

In **chapter 4** the presence of a reported *PTHR1* mutation in patients with enchondromatosis was investigated. The reported mutation was shown to lead to an upregulation of the IHH/PTHLH signaling. This was puzzling since in osteochondromas the IHH/PTHLH signaling is downregulated most likely as a consequence of *EXT* inactivation.

Chapter 5 describes the expression of the IHH/PTHLH signaling pathway in several subtypes of enchondromas and conventional central chondrosarcomas. This pathway most likely plays an important role in osteochondromas and peripheral chondrosarcomas, which closely resemble central chondrosarcomas. In this article also samples from patients with Ollier disease and enchondromas and chondrosarcomas of the phalanx were included, to get a more complete overview of the IHH/PTHLH signaling in the different subtypes

The results of a genome wide approach to unravel central cartilaginous tumorigenesis are discussed in **chapter 6** and **7**. In **chapter 6** expression profiles found in enchondromas, the different grades of chondrosarcomas and those present in Ollier disease are compared**.** The genomic alterations, studied with a ~1 Mb spaced array CGH, are described in **chapter 7** in which they are combined with array expression studies from **chapter 6.**

All results are summarized and discussed in **chapter 8**.

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Chapter 2

Diagnosis and prognosis of chondrosarcoma of bone

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The mechanisms of chondrosarcoma development are just beginning to be unraveled. The distinction between benign and low-grade malignant cartilaginous tumors is difficult and is based mainly on radiological and clinicopathological features. In this review, the conventional chondrosarcomas are subdivided into central and secondary peripheral chondrosarcomas, based on their different genetic and clinicopathological background. Thus far, no diagnostic markers have been identified for central tumors. BCL2 is a good diagnostic marker that can be used in the distinction between osteochondroma and low-grade secondary peripheral chondrosarcoma. For the prognosis of chondrosarcomas, the best and most commonly used marker at present is histological grade. Several molecular markers, recapitulated in this paper, have been tested to see if they aid in determining diagnosis and predicting prognosis, but most are not independent of histological grade.

The prevalence of primary malignant bone tumors is estimated 1: 100.000 within the general population, 17-24% of which are chondrosarcomas [1] .[2] Chondrosarcoma of bone is a slowly growing malignant tumor characterized by the formation of cartilage instead of bone. The tumors have an equal sex incidence and principally occur in adults of 30-60 years. Chondrosarcomas are found mostly in the long bones (33%), pelvic bones (27%) and in ribs and scapula (10%). The most adequate treatment is surgery, since the tumors are highly resistant to chemotherapy and radiotherapy [3]. The final diagnostic assessment is the result of a multidisciplinary approach involving the clinical, radiological and histopathological data. Chondrosarcomas can be classified based upon microscopic features. The most common is conventional chondrosarcoma (80-85%). Dedifferentiated chondrosarcoma (10%), mesenchymal chondrosarcoma (2%), juxtacortical chondrosarcoma (2%) and clear cell chondrosarcoma (1%) are rare.

Conventional chondrosarcomas

Conventional chondrosarcomas can be subclassified in two ways. One subdivision is made between primary (without evidence of a pre-existing benign lesion such as enchondroma or osteochondroma) and secondary (evidence of a pre-existing benign lesion is present) chondrosarcomas. Another subdivision can be made according to their location in the bone. Most chondrosarcomas (83%) arise centrally within the medullary cavity of bone (primary conventional central chondrosarcomas, or secondary central chondrosarcomas if they develop from a pre-existing enchondroma), while a minority (17%) consist of secondary peripheral chondrosarcomas, developing at the surface of bone secondarily within the cartilaginous cap of a pre-existing osteochondroma (cartilage-capped bony protuberance developing from juxta-epiphyseal regions of long bones) [2,4].

Both central and secondary peripheral chondrosarcoma have similar cytonuclear features and three grades of malignancy are discerned [5]. Increasing histological grade is correlated with an increased risk of metastases and reduced survival time [5,6]. The fact that recurrences of chondrosarcomas can exhibit a higher grade of malignancy compared to the previous lesion suggests that these tumors may progress in grade [5,7].

Despite the fact that both central and peripheral chondrosarcomas have similar, if not indistinguishable, cytonuclear features, they do have a different genetic etiology.

Diagnosis of chondrosarcoma

The distinction between enchondroma or osteochondroma and low-grade chondrosarcoma is difficult both at the radiological level (in case of central chondrosarcomas) [8] and the histological level (for both central and peripheral chondrosarcomas) [9,10]. Thus far, the differential diagnosis between benign and low-grade chondrosarcomas is usually based on a combination of clinical, radiological and histological features. A correct diagnosis is essential for selecting therapy. Benign lesions can have a wait-and-see policy - since the risk of malignant transformation is low (about 1%) - or local treatment. In contrast, complete wide en bloc excision, which may be mutilating, is required for malignant tumors.

Radiology

Using (dynamic) magnetic resonance imaging (MRI), a fairly reliable assessment of the differential diagnosis between osteochondromas and low-grade secondary peripheral chondrosarcomas can be provided by estimating the thickness and evaluating the characteristic staining of the cartilaginous cap [11]. For the distinction between enchondromas and low-grade central chondrosarcomas clinical symptoms and radiographic features are of help but both lack specificity [8,12,13]. Fast contrast-enhanced MRI can be used to confirm the difference between benign lesions and all conventional high-grade chondrosarcomas, since in contrast to benign and borderline cases, high-grade chondrosarcomas have a highly vascularised pattern [14]. Analogously, at the histological level, enchondromas consist of an avascular cartilaginous matrix without overt induction of neovascularisation. In contrast, low-grade chondrosarcomas have a fibrovascular stroma surrounding the avascular cartilaginous nodules [15,16]. These features thus form the basis for dynamic contrast-enhanced MRI [14].

Histology

At the histological level, the distinction between enchondroma and low-grade central chondrosarcoma is mainly based on growth patterns and cytomorphological features. Host-bone entrapment (defined as the permeation of tumor around preexisting lamellar host bone), tumor encasement (defined as new shells of lamellar bone at the periphery of cartilage nodules), high cellularity , marked nuclear pleomorphism an irregular cell distribution are the main histological determinators [9,16]. The presence of mucoid matrix degeneration in 20% or more of the lesion, and the presence of host-bone entrapment almost certainly indicates malignancy [16].

Additional techniques

Several studies have been reported on potential markers for distinguishing benign precursor lesions (enchondroma, osteochondroma) from low-grade chondrosarcomas. Although some give significant different expression levels for benign and malignant lesions, a problem is that the distinction between the expression levels may not always be so accurate. Factors reported to show a significantly increased expression in chondrosarcoma grade I as compared with their benign counterparts are parathyroid hormone like hormone (PTHLH) and BCL2 [17], both restricted to peripheral tumors [18], and platelet derived growth factor (PDGF)-α receptor and Ki-67 [19]. These molecular markers may be especially helpful in small biopsies that are not big enough to assess the growth patterns as described above [9]. No molecular diagnostic markers are known for the distinction between enchondromas and central chondrosarcomas.

A major drawback of most molecular genetic reports on chondrosarcomas so far, however, is that no clear distinction is made between central and secondary peripheral chondrosarcomas. A distinction is, however, essential because of their different genetic mechanisms of development. This could mean that potential diagnostic factors that are declared unfit for the assessment of malignancy, may be important diagnostic factors specifically for central or

peripheral tumors. The genetics of these two different clinico-pathological subtypes will therefore be discussed separately.

Central chondrosarcoma

Most chondrosarcomas are central (intramedullary) chondrosarcomas. They can be primary or they can develop secondarily to a benign precursor lesion, enchondroma, which is estimated to occur in less than 1% of enchondromas [2]. Most enchondromas are solitary, while in patients with nonhereditary Ollier disease or Maffucci syndrome, multiple enchondromas are found, scattered all over the skeleton, often with a unilateral predominance [20]. Maffucci syndrome is characterized by the simultaneous occurrence of multiple enchondromas and soft tissue vascular lesions. The percentage of malignant transformation in patients with Ollier disease or Maffucci syndrome is much greater than for solitary enchondroma and has been estimated at 25-30% [2,21].

Origin of central chondrosarcomas

There is considerable debate in the literature about the incidence of secondary central chondrosarcomas and the percentage ranges from 1-2% [2] to approximately 40% [10]. This wide range is most likely explained by how secondary central chondrosarcoma are defined. The incidence is low when the definition is applied that a pre-existing enchondroma has to have been detected previously. A point of discussion is, however, that enchondromas do not always give complaints and often remain undetected or are only found by chance, for example, with X-ray after fracture. A much greater incidence of secondary central chondrosarcoma is found if one defines central chondrosarcomas as secondary when the remains of a pre-existing enchondroma are present at the time of diagnosis, histologically or radiologically, next to a central chondrosarcoma [10]. Not all known secondary chondrosarcomas do, however, show evidence of the previous enchondroma. For example, Brien et al. [10] described a patient known to have an enchondroma. When the patient returned with complaints, the benign lesion had transformed into a chondrosarcoma, without it showing any evidence of the pre-existing enchondroma. This could imply that a substantial number, if not all, central chondrosarcomas are actually secondary.

Genetics

Not many genetic aberrations are known to be specific for low-grade central chondrosarcomas. DNA flow cytometry data that make a distinction between central and secondary peripheral chondrosarcomas show that central chondrosarcomas are predominantly peridiploid [22]. Loss of heterozygosity (LOH), comparative genomic hybridization and karyotyping show that a broad range of genomic alterations can be found but most are probably random. However, there are indications that chromosomes 9 and 22 are more often affected (Figure 2.1) [22].

Recently, an activating mutation in the *parathyroid hormone receptor type 1* (*PTHR1*) gene (R150C PTHR1) has been found in two patients with Ollier disease, of which in one case the mutation was also found in the father of the patient. This may suggest a hereditary component

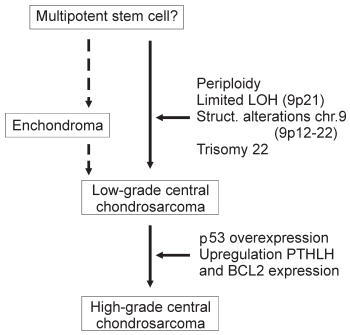

Figure 2.1: *Multistep genetic model for central cartilaginous tumorigenesis*

in Ollier disease. The mutation could not be found in any healthy individuals, nor in 50 cases of sporadic chondrosarcomas. This single base-pair substitution results in a constitutive active receptor leading to increasing cAMP signaling, resulting in slowed chondrocyte differentiation [23]. PTHR1 is part of a negative feedback loop involved in the maturation and differentiation of cartilage, which includes other proteins such as Indian Hedgehog (IHH) and BCL2. In high-grade central chondrosarcomas overexpression of PTHLH and BCL2 is seen, suggesting that the IHH/PTHLH pathway could perhaps indeed be affected (Figure 2.1) [17].
Other genes that have been tested for the presence of mutations in central chondrosarcomas are *p53* and *p16*. The *p53* gene has been found to be genetically affected in some chondrosarcomas, mainly high-grade. They can show mutations and/or LOH on chromosome 17p13 at the p53 locus [24-28]. The *p16* gene, located on chromosome 9p21, has been studied extensively in central chondrosarcomas. Although both cytogenetics and LOH point to the 9p21 region as an important candidate locus for central chondrosarcoma development [29], mutations were absent and methylation (a mechanism of gene downregulation) of the *p16* gene combined with absent p16 protein expression is found in only a subset of central chondrosarcomas [30-32].

Secondary peripheral chondrosarcoma

Secondary peripheral chondrosarcomas develop within the cartilaginous cap of a pre-existing sporadic or hereditary osteochondroma. The hereditary cases represent patients with multiple exostoses (MO), an autosomal-dominant disease with genetic heterogeneity. These patients have multiple osteochondromas, deformities of the forearm and disproportionate short statue. The development of secondary peripheral chondrosarcomas is estimated to occur in < 1% of solitary osteochondromas and in 1-3% of cases of MO [33,34].

Genetics

Multiple osteochondromas is a genetically heterogeneous disorder and, so far, two genes have been identified, *EXT1* (8q24) and *EXT2* (11p11-p12) [35-38]. Additionally, linkage to MO has been found at chromosomal region 19p [39] but the gene has never been identified and LOH is absent at this locus [22,40,41]. Most mutations found in patients with MO result

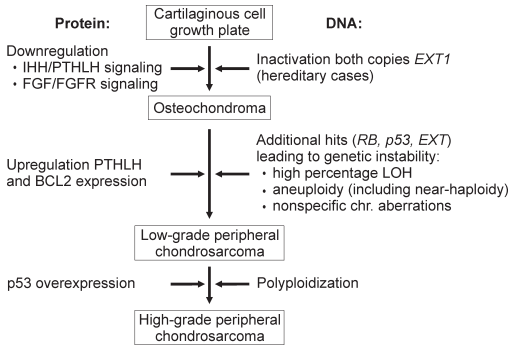

Figure 2.2: *Multi-step genetic model for peripheral cartilaginous tumorigenesis*

in truncation or a nonfunctional protein, inactivating the gene. In addition, both copies of an *EXT* gene need to be inactivated for an osteochondroma to develop in hereditary cases (Figure 2.2) [41,42]. These genes are therefore hypothesized to be tumor-suppressor genes.
For sporadic cases, however, only one somatic mutation in a sporadic osteochondroma has been described [43,44], although LOH of the *EXT* genes is found in chondrosarcomas derived from sporadic osteochondromas.
Peripheral chondrosarcomas developing secondary to osteochondroma demonstrate DNA aneuploidy with DNA indices ranging from 0.56 to 2.01, equivalent to respective half and double portions of genetic material. Only mild aneuploidy is found in osteochondromas [44]. Near haploidy, which is very uncommon for human tumors, is restricted to low-grade tumors. It is hypothesized that additional genetic hits in the cartilaginous cells of osteochondroma lead to genetic instability, characterizing malignant progression towards low-grade secondary peripheral chondrosarcoma, which includes near-haploidy resulting in the detection of a high percentage of LOH [22]. High-grade peripheral chondrosarcomas display polyploidization of near haploid precursor clones, as confirmed by LOH and FISH analysis (Figure 2.2) [22,44]. This extensive LOH may be considered as a hallmark of peripheral chondrosarcomas, but obscures the detection of specifically targeted loci. Moreover, cytogenetic analysis did not reveal any specific aberrations in peripheral chondrosarcomas [29,45].

Proteins

The *EXT* gene products are involved in the biosynthesis of heparan sulfate [46-48]. Heparan sulfate is important for the anchorage of the cells to the matrix and is involved in high affinity binding of growth factors. Mutation of an *EXT* gene effects heparan sulfate synthesis and may influence the distribution of IHH, a member of the negative feedback loop that includes other proteins such as PTHLH (-receptor), fibroblast growth factor (FGF) (-receptor) and BCL2, and may be involved in the regulation of proliferation and differentiation of chondrocytes in the normal growth plate [49,50].
These putative downstream targets of EXT, proteins from the IHH/PTHLH (PTHLH, PTHR1, BCL2) and the FGF pathway (FGF2, FGFR1, FGFR3, p21) were demonstrated to be mostly

absent in osteochondromas (Figure 2.2). Upregulation of these proteins was seen in peripheral chondrosarcomas and the expression increased with histological grade. Upregulation of BCL2 and PTHLH was shown to specifically characterize the progression from osteochondroma towards low-grade secondary peripheral chondrosarcomas (Figure 2.2) [17]. Moreover, BCL2 was recently confirmed as a valuable immunohistochemical marker for the distinction between osteochondroma and low-grade secondary peripheral chondrosarcoma, in which moderate-to-strong or diffuse cytoplasmic staining is highly suggestive (95%) of malignancy [51]. Unfortunately, BCL2 expression does not characterize malignant progression of enchondroma towards low-grade central chondrosarcoma and to date such markers are still lacking.

Prognostic factors in chondrosarcoma

Thus far, the best prognostic parameter in both primary conventional and secondary peripheral chondrosarcoma is histological grade. Since chondrosarcomas are heterogeneous, adequate sampling of the tumor is essential [52]. Because histological grading can be difficult on small samples, many studies have attempted to find molecular markers that can help distinguish good and poor prognosis in chondrosarcoma. Unfortunately, most literature on potential prognostic factors does also not distinguish central and peripheral chondrosarcoma subtypes.

Clinical and microscopical prognostic indicators

The best known prognostic indicators for chondrosarcomas are based on clinicopathological criteria. The major factors are tumor localization and size, histological subtype of chondrosarcoma and histological grade. Size of the tumors can be an indication of the likelihood of recurrence [6] and the chance of development of metastases (Table 2.1) [3]. The assessment of tumor subtype is also important since the different subtypes have different prognosis. For instance, dedifferentiated chondrosarcomas have an ominous prognosis, with 90% of patients dying with distant metastases within 2 years [53]. In contrast, juxtacortical chondrosarcomas have a very good prognosis, if the tumor has been adequately removed [54]. Grade is of importance because the risk of metastasis increases with increasing histological grade [5,7]. Incomplete excision or excision with minor margins results in a higher chance of recurrence. This recurrence can be of the same or higher grade than the primary tumor [5,7].

In addition, anatomical location is of importance [19,55], since location in pelvis has a much worse chance of curative treatment [3,56] than location in the extremities. Moreover, chondrosarcomas in phalanges have an excellent prognosis since they rarely metastasize, despite ominous histological features [57]. Extension of the tumor in soft tissues is also a negative risk factor, since this often leads to local recurrence.

Molecular prognostic indicators

Several attempts have been made to find molecular markers that can indicate the aggressiveness of the tumor and the chance of local recurrence or metastasis (Table 2.2). Most of these markers are, however, not independent of tumor grade in a multivariate statistical analysis and do therefore not have any additional prognostic value.

Table 2.1: Clinico-pathological data investigated in cartilaginous tumors

Marker	Type of CS (no of cases)	Correlation	Remarks	Ref
Inadequate margins	conv. (191) dediff. (20)	Survival		[3]
Inadequate margins	conv. (108)	Metastases Recurrence Survival		[82]
Inadequate margins, tumor size >10 cm	conv. (153)	Recurrence	Multivariate analysis	[6]
Size	conv. (191) dediff. (20)	Metastases		[3]
Grade	conv. (114)	Metastases Recurrence	Only in univariate analysis	[64]
Grade	conv. (191) dediff. (20)	Age Metastases Recurrence Survival		[3]
Grade	conv. (42)	Survival		[83]
Grade, recurrence, extracompartmental spread	conv. (153)	Survival	Multivariate analysis	[6]
Grade, location	(28)	Recurrence		[55]
Grade, location extremities	conv. (67)	Recurrence Survival	Independent factor for disease-free and overall survival in multivariate analysis	[19]
Location pelvis	conv. (191) dediff. (20)	Metastases Recurrence Survival		[3]
Tumor extension	conv. (114)	Metastases Recurrence Survival	Metastases only significant in univariate analysis	[64]

conv.: conventional; CS: chondrosarcoma; dediff.: dedifferentiated.

Angiogenesis in chondrosarcoma

Important candidates for prognostic markers were thought to be molecules involved in angiogenesis. Histologically, low-grade chondrosarcomas have a fibrovascular stroma surrounding the avascular cartilaginous nodules, while in high-grade chondrosarcomas vascularisation is more prominent including both fibrovascular stroma and vessels in direct apposition to tumor cells [15].

Angiogenesis, as seen in high-grade chondrosarcomas, requires invasion by endothelial cells. It has been suggested that vascular endothelial growth factor - whose expression appears to be restricted to tumor cells of high-grade chondrosarcomas (Table 2.2) - is required for vessels to penetrate the cartilage [15]. Moreover, localized proteolytic modification of the extracellular matrix is required, for instance by matrix metalloproteinases (MMP). The activity of MMPs is related to invasiveness and metastatic potential of tumors. It is suggested that increased expression of MMP-1, -2, -9 and -13 and a decrease in MMP-3 and -8 expression may be associated with a high-grade malignant phenotype in cartilaginous tumors (Table 2.2)[58-

64]. Proteolytic activity is, however, determined by the overall balance between the activity of MMPs and the activity of tissue inhibitors of MMPs (TIMPs). TIMP-1 and -2 expression has also been demonstrated in chondrosarcoma [58,62,65-67] and a high ratio of MMP-1 to TIMP-1 is associated with poor outcome (Table 2.2)[58,65].

Other molecular prognostic factors

In addition, several other factors have been tested, like heat shock protein 72 [68], FGFs [17], plasminogen activators (PA) [55,64,69] and cathepsins (Table 2.2) [64,64,67]. For instance, cathepsin B is an enzyme involved in lysosomal proteolysis and degradation of antigens correlating with poor prognosis when overexpressed (Table 2.2) [64]. However, it is difficult to define cathepsin B overexpression.

Genetic prognostic indicators

A recently reported prognostic factor is loss of chromosomal region 13q. Investigation of 59 chondrosarcomas showed it to be an independent prognostic factor for metastasis (relative risk = 5.2), regardless of tumor grade or size [67]. In this study, other genomic aberrations such as 6q, 10p, 11p, 11q, 22q also showed a correlation with impaired metastasis-free survival. However, significance was lost in a multivariate statistical analysis when other factors, like tumor grade and size, were included in the comparison [70].

DNA flow cytometry data do also have some prognostic value [71-79]. The tumours showing aneuploidy are mainly high-grade chondrosarcomas and thus have a worse prognosis (Table 2.2). Therefore, aneuploidy is not a prognostic factor independent of histological grade.

The complex and aspecific cytogenetic aberrations that increase with histological grade interfere with the identification of specific prognostic factors. The result is that different lesions show different karyotypes and/or mutations with some found more often than others. A good example is *p53* which has been found mutated in a subset of the chondrosarcomas, mainly related to high-grade or dedifferentiated chondrosarcomas, but not in all (Table 2.2) [24,25,27,28,68,72,80,81].

Summary and conclusions

Although the mechanisms of chondrosarcoma development have been the subject of many studies in the past, their multistep genetic pathways are just beginning to unravel. Based upon their location, the tumors can be subdivided in two groups: central and peripheral, which demonstrate different mechanisms of development. Most of the multistep genetic model for central chondrosarcoma development remains to be elucidated. In contrast, more is known about the multistep model for secondary peripheral chondrosarcomas, arising in the cartilaginous cap of osteochondroma. For development of osteochondromas both copies of the *EXT* genes need inactivation. In addition, secondary peripheral chondrosarcomas demonstrate genetic instability, including near-haploidy. High-grade peripheral chondrosarcomas show polyploidization of these near-haploid precursor clones. Thus far, the distinction between benign and low-grade malignant cartilaginous tumors has been difficult and is mainly based on radiological and clinico-pathological features. It would be helpful to

Table 2.2: Prognostic molecular markers investigated in cartilaginous tumors

Marker	Type of CS (no of cases)	Correlation	Remarks	Ref
BMP-2, -4, -6, -7, -RIB, -RII	conv. (12) dediff. (8)		BMPRII is significant correlated with the dediff. tumor type	[84]
High expression FGF2, FGFR1, FGFR3, PTHrP, BCL2	conv. (49)	Grade	A difference has been made between central and peripheral CS	[17]
High expression PTHLH, BCL2	conv. (12) dediff. (2)	Grade		[18]
hsp 72	? (37)	Grade		[85]
Expression CTGF(low), PCNA(high)	conv. (18)	Grade	Expression CTGF > 30% has better survival	[86]
High expression Ki-67	conv. (29)	Grade Recurrence Survival	Also in multivariate analysis	[68]
High expression Ki-67	conv. (25)	Grade		[87]
High expression Ki-67	conv. (39)	Grade Recurrence Survival		[82]
High expression Ki-67, PDGF-AA	conv. (67)	Grade		[19]
High expression PDGF-alpha receptor	conv. (67)	Grade Survival	High expression associated with shorter survival	[19]
Collagen I, II, III, IV, V and IX	conv. (15)		Type I, III and V increased with increasing histological grade	[88]
Cts B	conv. (114)	Grade Recurrence		[64]
Cts L	conv. (114)	Grade Recurrence Survival		[64]
Cts B, H, K and S	conv. (12)		No correlation found	[67]
Cts L	conv. (12)	Grade		[67]
Expression MMP-1, 2, 9 and TIMP-1	conv. (11)		Matrix staining more present in tumors with higher malignancy	[60]
Expression MMP-13, bFGF(=FGF2)	conv. (16)		MMP-13 expression regulated by bFGF	[59]
Expression MMP-2	conv. (114)		Overexpression is feature of larger/more advanced lesions	[64]
Expression MMP-2, MMP-14	conv. (12)	Recurrence	No statistical analysis	[52]
Expression MMP-2, TIMP-1, and 2	conv. (22) mes. (1)		No correlation found	[61]
High expression MMP-2, TIMP-2, MMP-14	conv. (34) dediff. (8) mes. (7) cc. (5)	Grade		[62]
MMP9	conv. (114)	Grade Recurrence	More in male patients	[64]
High ratio MMP1/TIMP1	conv. (7) dediff. (5) mes. (1) cc. (1)	Recurrence Metastases		[65]
High ratio MMP1/TIMP1	conv. (29)	Recurrence Survival	Independent factor for survival	[58]
Expression TIMP-2	conv. (12)	Grade Recurrence	No statistical data	[52]

Table 2.2, continued

Marker	Type of CS (no of cases)	Correlation	Remarks	Ref
VEGF, intracartilage and pericartilage vessels	conv. (19) myx. (1) mes. (1)	Grade	No statistical data, pericartilage vessels in all, increased with grade; intracartilage vessels and VEGF mainly in high grade	[15]
Microvessel densities	conv. (32)	Grade	More vessels in high grade tumors	[89]
nm23 (NDP kinase)	conv. (22)	Grade	Higher expressed in high grade tumors	[90]
Expression p21(waf1/cip1)	conv. (49)	Grade Survival	Dependent of grade, expression increases with grade. central and peripheral CS separated	[17]
Expression p21(waf1/cip1)	conv. (14)	Grade	Less frequent in higher grade tumors	[91]
p53 overexpression	conv. (7) dediff. (4)	Grade	Expression increasing with grade and in dediff. CS	[72]
p53 overexpression	conv. (29)	Grade Survival	Expression increasing with grade	[68]
p53 overexpression	dediff. (8)	Grade	Expression increasing with grade	[80]
p53 overexpression and mutations	conv. (126) myx. (32)	Grade Metastases	Expression and presence of mutations increase with grade	[28]
p53 mutations	conv. (9)		4 CS had a mutation in p53	[81]
p53 overexpression and mutations	conv. (10) myx. (2) cc. (3)	Grade	Only found in 1 conv. grade III and 1 CC. grade III	[24]
p53 overexpression and mutations	conv. (15) dediff. (15) myx. (12) mes. (3) cc. (1)		Associated with high grade and dediff. subtype	[27]
p53 overexpression and mutations	conv. (15) dediff. (3) mes. (1)	Grade	Associated with high grade and dediff. subtype	[25]
Expression P-glycoprotein	conv. (11) dediff. (7) cc. (1)		Present in almost all tested tumors	[92]
Expression u-PA	conv. (114)	Age Grade Recurrence		[64]
u-PA, PA inhibitor (PAI)-2	(28)	Metastases Survival		[55]
u-PA, tissue- type PA (t-PA), PAI	conv. (14) dediff. (10)	Grade	Significant higher expression in high grade sarcomatous components in dediff. compared with conv. CS	[69]
Expression c-myc, c-myb, c-Ha-ras and c-fms	conv. (8) mes. (1)		Two CS showed c-myc amplification	[93]
Abl	conv. (15) dediff. (1)	Grade	High expression in low grade	[94]
Aneuploidy	conv. (9)	Grade	More aneuploidy in high grade	[71]
Aneuploidy	conv. (7) dediff. (4)	Grade	All high grade conv. and dediff. aneuploid	[72]
Aneuploidy	(11)	Grade	More aneuploidy in high grade	[73]
Aneuploidy	conv. (36) dediff. (1)	Recurrence	More aneuploidy in high grade	[95]

Table 2.2, continued

Marker	Type of CS (no of cases)	Correlation	Remarks	Ref
Aneuploidy	conv. (45)	Grade Recurrence Survival	More aneuploidy in high grade	[75]
Aneuploidy	conv. (13)		High grade more likely to be aneuploid	[76]
Aneuploidy	conv. (26) dediff. (3)	Grade	More aneuploidy in high grade	[77]
Aneuploidy	conv. (191) dediff. (20)	Grade Metastases	More aneuploidy in high grade	[3]
Aneuploidy	conv. (140)		No correlation found	[78]
Aneuploidy	conv. (16)		No correlation found	[79]
PTEN mutations	all (40)		No correlation found	[96]
LOH 10q	conv. (18)		No correlation found	[97]
LOH 13q, 17q	conv. (28)	Grade		[26]
LOH chr. 6q, 10p, 11p, 11q, 13q, 22q	conv. (53) dediff. (6)	Metastases	Only significant in multivariate analysis for LOH chr. 13q	[70]
p14 ARF mutations	conv. (18) dediff. (3) myx. (1)		No correlation found	[31]
p16 genetic alterations	conv. (17) dediff. (3) myx. (1) mes. (1)	Grade?	No statistical data	[32]

Abl: Abelson murine leukemia virus oncogene; BMP: Bone morphogenic protein; cc.: Clear cell; conv.: Conventional; CS: Chondrosarcoma; Cts: Cathepsin; dediff.: Dedifferentiated; FGF: Fibroblast growth factor; LOH: Loss of heterozygosity; mes.: Mesenchymal; MMP: Matrix metalloprotease; myx.: Myxoid; NDP: Nucleoside diphosphate; PA: Plasminogen activator; PAI: PA inhibitor; PCNA: Proliferating cell nuclear antihen; PDGF: Platelet-derived growth factor; TIMP: Tissue inhibitor of MMP; u-PA: Urokinase PA; VEGF: Vascular endothelial growth factor.

have molecular markers to aid the pathologist in this differential diagnosis. BCL2 is a good diagnostic marker that can be used in the distinction between osteochondroma and low-grade secondary peripheral chondrosarcoma. Thus far, no diagnostic markers have been identified for central tumors. For the prognosis of chondrosarcomas the best available marker is the histological grade. Many molecular and genetic markers were tested on chondrosarcomas but most are not independent of histological grade and therefore do not have additional prognostic value, except in small lesions not big enough to ascertain the histological grade by using the present criteria.

Expert opinion

The difference between benign and low-grade malignant cartilaginous tumors is difficult and is so far mainly based on clinico-pathological and radiological features. Unfortunately, most of the research on chondrosarcomas does not distinguish between central and secondary peripheral chondrosarcomas, probably because of their similar cytonuclear features. Although peripheral chondrosarcomas are rare, the distinction is, however, important because they have a different genetic etiology. Thus, it is imperative to distinguish between central and secondary peripheral chondrosarcomas, since it is likely that diagnostic and prognostic markers

may also be different for the two subtypes, as is demonstrated by BCL2 being a diagnostic marker for peripheral but not for central tumors. Eventually, a similar pathway may turn out to be affected at different levels, for which the EXT downstream negative feedback loop, involved in the formation and maturation of chondrocytes within the normal growth plate, is a good candidate.

Five year view

In the next 5 years, more molecular and genetic markers will probably be tested. Many of these markers will be chosen hypothetically, based on their involvement in what is presently known about the development and maturation of chondrocytes in physiological circumstances. In addition, other pathways involved in the development of chondrosarcomas and their benign lesions may be discovered with the use of new high-throughput techniques such as cDNA microarray. This will probably lead to an improved understanding of the biology of chondrosarcomas, which may in turn lead to better diagnostic and prognostic markers.

Key issues

- The distinction between benign and low-grade malignant chondrosarcomas can be difficult and is currently based on radiologic and clinicopathologic features
- Conventional chondrosarcomas are classified in three histological grades, correlating with prognosis
- Based on their location in bone, conventional chondrosarcomas should be subdivided into central (medullary cavity) and peripheral (surface of bone, within osteochondroma) chondrosarcomas
- Peripheral and central chondrosarcomas have a different genetic mechanism
- BCL2 is a good diagnostic tool for the identification of progression from osteochondromas to low-grade secondary peripheral chondrosarcomas
- A diagnostic tool to distinguish enchondromas from low grade central chondrosarcomas is still lacking

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Chapter 3

Molecular analysis of the INK4A / INK4A-ARF gene locus in conventional (central) chondrosarcomas and enchondromas: Indication of an important gene for tumor progression

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Loss of heterozygosity (LOH) at chromosomal band 9p21 is one of the few consistent genetic aberrations found in conventional central chondrosarcoma. This locus harbors two cell cycle regulators CDKN2A/p16/INK4A and INK4A-p14^{ARF}, which are inactivated in various human malignancies. It was therefore hypothesized also plays a role in the development of chondrosarcomas and this locus was investigated at protein, genetic, and epigenetic levels. Loss of p16 protein expression was detected by immunohistochemistry in 12 of 73 central chondrosarcomas and it correlated with increasing histological grade ($p = 0.001$). Loss of p16 protein expression was not found in 51 enchondromas, which are presumed to be potential precursors of conventional central chondrosarcoma. LOH at 9p21 was found in 15 out of 39 chondrosarcomas (38%) but it did not correlate with loss of p16 protein expression. SSCP analysis of p16 did not reveal any mutations in 47 cases. Also, *p14* was not the target of LOH, since it gave no aberrant bands in SSCP. To investigate whether an epigenetic mechanism was operating, methylation-specific PCR was used to look at promotor methylation of *CDKN2A/p16*, which was identified in 5 of 30 tumors. However, this did not correlate with protein expression, or with LOH at 9p21. Cytogenetic data were available in a subset of cases. All tumors that showed chromosome 9 alterations also showed LOH and loss of INK4A/p16 protein expression. It is concluded that although some alterations were found at the DNA level and at the promoter expression level, the lack of correlation between LOH, promotor methylation and protein expression indicates that a locus other than the *CDKN2A/p16* must be the target of LOH at 9p21. The correlation between INK4A/p16 protein expression and tumor grade and the retention of expression in enchondromas, indicates that loss of INK4A/p16 protein expression may be an important event during tumor progression from enchondroma to conventional central chondrosarcoma, and in the progression in grade after recurrence of chondrosarcoma.

Chondrosarcomas are malignant cartilage-forming bone tumors, with an occurrence of about 1 in 100.000 in the general population [1,2]. There are three grades of malignancy that correlate with prognosis [3]. Based upon their location in the bone, there are two major subtypes of conventional chondrosarcomas. The majority of the tumors are localized in the medullary cavity of long bones, so-called conventional central chondrosarcomas. Enchondroma may be its benign precursor in rare cases. Malignant transformation of enchondroma is, however, a rare event, especially in enchondroma of the phalanx, a site where chondrosarcomas are extremely rare, despite the fact that hands and feet harbor 35% of enchondromas [4]. A hereditary form of conventional central chondrosarcomas is not known. Enchondromas arising in the context of Ollier disease and Maffucci syndrome, two non-hereditary conditions displaying multiple enchondromas, have a much higher risk to become malignant, i.e. 30-35% (OMIM #166000) [5]. A minority of chondrosarcomas arise secondary to a pre-existing osteochondroma, and are called secondary peripheral chondrosarcomas [1,2,6]. We have shown that secondary peripheral chondrosarcomas are characterized by gross chromosomal instability reflected by a high percentage of LOH with almost all chromosomes involved, and a broad ploidy range [7-9]. In contrast, genetic aberrations in conventional central chondrosarcomas are sparse and the tumors are often (peri)diploid. This suggests that only limited genetic alterations are sufficient for tumorigenesis in conventional central chondrosarcomas. Previously, we demonstrated LOH at 9p21 in 3 of 12 (25%) conventional central chondrosarcomas. Moreover, a cytogenetic study of 7 of 16 conventional central chondrosarcomas showed cytogenetic aberrations, of which five involved chromosome 9p12-22 [7]. These data suggest that the 9p21 region may be important in its tumorigenesis. At chromosome 9p an important region is the INK4A/ARF locus (9p21), encoding the proteins INK4A/p16 and INK4A/p14^{ARF}. The activation of INK4A/p16 and INK4A/p14ARF results in blockage of cell-cycle progression and inhibition of cellular proliferation. Mutational or transcriptional inactivation of the *CDKN2A/p16* and *p14* genes can lead to uncontrolled growth. *CDKN2A/p16* is inactivated in several tumor types, including bone sarcomas [10-13], either by homozygous deletion, mutation or extensive *de novo* methylation-inhibiting gene transcription [14]. To investigate if this locus is the target of the LOH at chromosome 9p in conventional central chondrosarcomas, we present the results of immunohistochemistry, LOH analysis, SSCP analysis (INK4A/p16 and INK4A/ARF), and promoter methylation status of the INK4A locus on a large well-characterized patient series.

MATERIALS AND METHODS

Patient material

All tissue samples were handled in a coded fashion. The specimen codes indicated patient data were only available via physicians involved in the diagnosis and treatment of these patients. All procedures were performed according to local ethical guidelines. Table 3.1 shows the clinico-pathological data of the patients in this study. In total, 73 tumor specimens from patients treated between 1986 and 2001 with a histological diagnosis of conventional

Table 3.1: Clinico-pathological features

		Enchondroma (n = 51)	Conventional central chondrosarcoma (n = 73)
Male vs female		28 vs 23	40 vs 33
Median age at diagnosis			
years (range)		36.1 (7 – 74)	50.8 (18 – 85)
Histology	grade I	na	28
	grade II	na	33
	grade III	na	12
Ollier disease		4 of 51	4 of 73
Phalanx vs not phalanx		14 vs 37	0 vs 73
Median follow up			
months (range)		75 (2 – 178)	56.5 (1 – 187)
Mean disease-free survival			
months (range)		68.8 (2 – 178)	49.8 (1 – 169)

central chondrosarcoma were retrieved from our files. Peripheral, dedifferentiated, mesenchymal, juxtacortical and clear-cell chondrosarcomas were excluded. Conventional central and secondary peripheral chondrosarcomas were distinguished on the basis of accepted clinico-pathological and radiological criteria [1]. Histological grading was performed according to Evans *et al* [3].

In addition, tumor material from 51 enchondromas was included: 14 enchondromas of the phalanx and 37 enchondromas from other locations of the long bones. According to the 2002 WHO definition, all enchondromas were located centrally in the bone, as documented by X-ray. Phalangeal and non-phalangeal localization were analyzed separately. An expert bone-tumor pathologist reviewed all cases to confirm the diagnosis. Formalin-fixed paraffin wax-embedded tissue was available from all patients; for a subset of chondrosarcomas (n=47), fresh frozen tissue was also available. Follow-up data were retrieved from clinical charts and from files of The Netherlands Committee on Bone Tumors.

Immunohistochemistry

Immunohistochemical staining was performed with the monoclonal INK4A/MTS1 antibody (Neomarkers, Fremont, CA, USA) according to standard laboratory methods [15]. In short, antigen retrieval was performed using 0.01 M Citrate solution. Sections of tonsil were used as a positive control.

The staining intensity (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%, 4 = 75-100%) were evaluated by two observers independently [8]. In of discrepant cases, the sections were re-analyzed to obtain consensus. Haematopoietic cells in bone marrow or endothelial cells served as an internal positive control to evaluate whether negative tumor cells are truly negative or whether prolonged decalcification might have altered the conformation of the

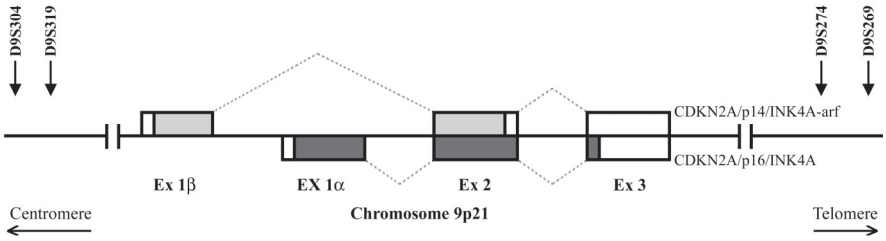

Figure 3.1: *Schematic representation of the CDKN2A/p16 and p14^{ARF} genes, flanked by the markers on chromosome 9p21 that were used for LOH-analysis*

antigen, resulting in a false-negative result. INK4A/p16-negative tumors without a positive internal control were excluded.

Loss of heterozygosity and CDKN2A/p16 and p14^{ARF} SSCP analysis

DNA-isolation

Paraffin wax-embedded cartilaginous tissue is not suitable for DNA analysis because the decalcification process with formic acid results in DNA degradation. From 40 patients with chondrosarcomas, DNA from fresh-frozen tissue and matched normal DNA were available. Only tumor tissue was available from seven patients. Normal DNA was prepared from peripheral blood drawn from patients after informed consent, or from normal muscle or skin obtained from resected specimens. Tumors with tumor cell percentages over 60% were included for genetic analysis [8]. No frozen material was available from enchondromas. DNA isolation from frozen sections was performed using proteinase K treatment and subsequent purification with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. DNA from blood samples was isolated using a salting- out procedure [16].

Loss of heterozygosity analysis

No matching normal DNA was available from seven samples and therefore those seven samples were not suitable for LOH analysis, leaving 40 tumors. For the study of LOH on chromosome 9p, the following polymorphic microsatellite markers were used: D9S269, D9S274, D9S319 and D9S304 (http://gdbwww.gdb.org). The location of the markers in relation to CDKN2A/p16/INK4A is shown in Figure 3.1.

LOH analysis and scoring have been described previously [17]. LOH results had to be reproducible in a consecutive analysis.

CDKN2A SSCP analysis

Genomic DNA from 47 tumors was used for SSCP analysis. Both genes encoded by the CDKN2A locus, p16 and the p14/ARF, were investigated. The CDKN2A/p16 gene was screened as described previously and covers the entire open reading frame [18]. Additional primers were designed for the P14/ARF exon 1β. The exon 1β was divided into two

overlapping fragments. Primers for the two fragments of exon 1β are: F1: 5'-CACCTCTGGTGCCAAAGG-3' and R1: 5'-GCCTCCTCAGTAGCATCAGC-3' (A fragment 219 bp) and F2: 5'-GCCGCGAGTGAGGGTTTT-3'and R2:5'-CACCGCGGTTATCTCCTC-3' (B fragment 257 bp).

Methylation-Specific PCR (MSP) analysis

Promoter methylation was determined according to Herman *et al* [19], based on bisulphite modification and subsequent PCR specific for methylated versus unmethylated *CDKN2A/p16* promoter sequence.

One microgram of genomic DNA from 30 fresh-frozen tumors was used for MSP analysis and modified using the CpGenome DNA Modification Kit (Intergen, New York, USA). Subsequent CDKN2A/p16-promoter methylation status was analyzed using the CpG WIZ Amplification Kit (Intergen). After amplification, samples, methylated, unmethylated, and wildtype controls were analyzed on a 2% agarose gel.

Statistical analysis

The SPSS package was used for all statistical analyses. Histological parameters were analyzed using the chi-square test for trend. Kaplan-Meier curves and the log-rank test were used to study the effect of INK4A/p16 protein expression and LOH on disease free survival.

RESULTS

Immunohistochemistry

In total, 14 of 69 (20 %) chondrosarcomas demonstrated complete loss of INK4A/p16 protein expression (Figure 3.2c), which was restricted to higher-grade lesions: 0 (0%) of 24 grade I chondrosarcomas demonstrated loss of expression, compared to 9 (27%) of 33 grade II and 5 (42 %) of 12 grade III chondrosarcomas (*p = 0.001*, chi-square test for trend). In contrast,

Table 3.2: Results of p16 immunohistochemistry stratified for diagnosis and tumor grade.

	p16-negative
Enchondroma	
Non-phalanx	0/25 (0%)
Phalanx	0/14 (0%)
Total Enchondroma	0/39 (0%)
Conventional central chondrosarcomas	
Grade I	0/24 (0%)
Grade II	9/33 (27%)[a]
Grade III	5/12 (42%)
Total Conventional central chondrosarcomas	14/69 (20%)

[a] One INK4A/p16 negative grade II tumor originated from an Ollier disease patient

none of the enchondroma cases showed loss of INK4A/p16 expression. One of eight patients with Ollier's disease demonstrated loss of INK4A/p16 protein expression in a grade II conventional central chondrosarcoma. The results of the immunohistochemical analysis of INK4A/p16 stainings are shown in Table 3.2. Immunohistochemical INK4A/p16 staining can result in positive nuclear or cytoplasmic staining. In the tumors tested we found both nuclear and cytoplasmic staining (Figure 3.2a). Five of 73 samples showed only cytoplasmic staining. There were also some cases showing focal positive INK4A/p16 staining as shown in Figure 3.2b. Four specimens could not be evaluated due to repeated loss of tissue attachment during microwave procedures.

Loss of heterozygosity and CDKN2A SSCP analysis

SSCP

To investigate if *CDKN2A/p16* and *p14/ARF* are inactivated by a genetic mutation, we carried out SSCP analysis on the four exons encoding these genes and performed LOH analysis with polymorphic microsatellite markers adjacent to the CDKN2A locus. SSCP was chosen as

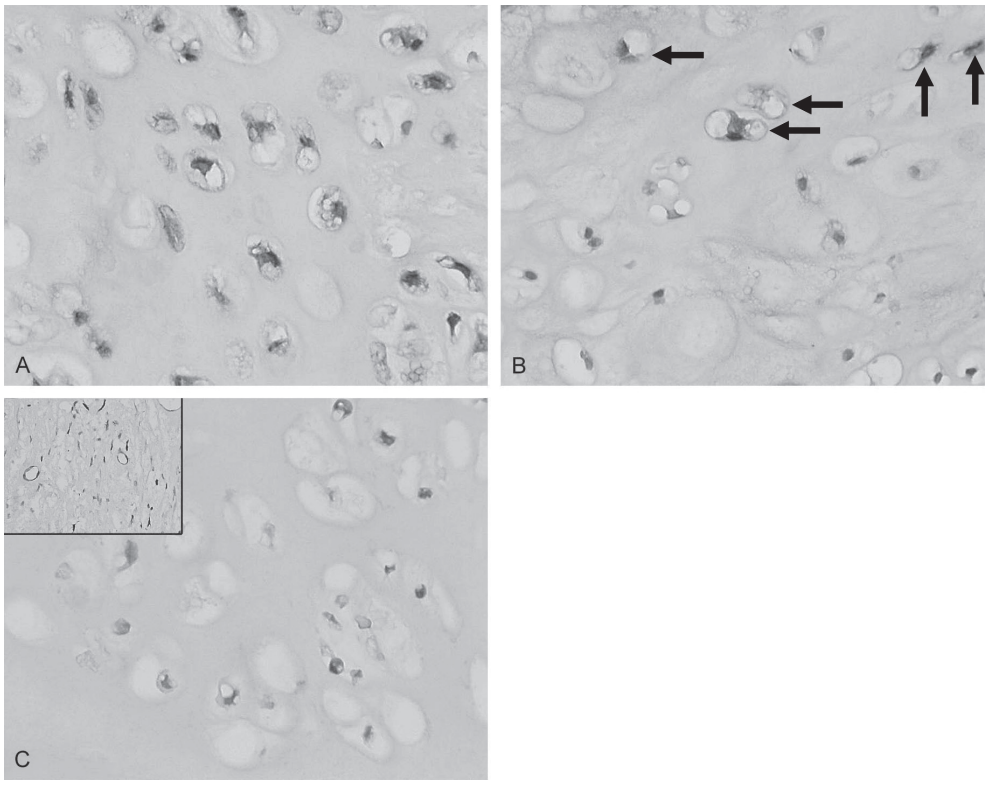

Figure 3.2: *Light micrograph showing the immunohistochemical nuclear and cytoplasmic localization of INK4A/p16. (A) Positive staining of INK4A/p16 in a grade II conventional central chondrosarcoma. (B) Some conventional central chondrosarcomas showed focal positive INK4A/p16 staining. (C) Fifteen per cent of the conventional central chondrosarcomas were entirely negative for INK4A/p16 staining; the inset shows the internal positive control of the tumour (endothelial cells). Color picture can be viewed at page 127*

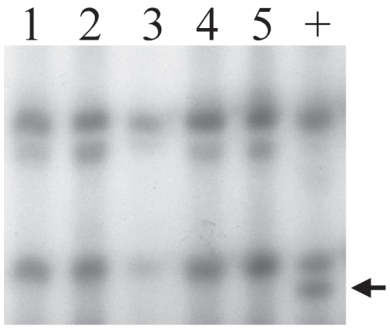

Figure 3.3: *Example of SSCP analysis of CDKN2A/p16 exon 2, c-fragment. Lanes 1-5 represent PCR products from five different conventional central chondrosarcoma samples and + is a melanoma sample containing a known mutation in exon 2. An arrow indicates the band shift in the positive control*

the technique to determine mutations because the method has proven useful for the identification of *INK4A* mutations previously in melanoma [18] and because it is able to detect up to 90% of mutations in a background of non-tumor tissue, which is not possible by sequencing. In two samples from melanoma patients with previously confirmed *CDKN2A/p16* mutation in exon 2 from *CDKN2A/p16* an aberrant SSCP pattern could be detected, validating the SSCP analysis. None of the 47 chondrosarcomas revealed aberrant bands at the *CDKN2A/p16* and *p14/ARF* loci. Figure 3.3 shows an example of an SSCP analysis on 5 chondrosarcomas and a melanoma with a known mutation in *CDKN2A/p16*.

LOH

As shown in Table 3.3, LOH at 9p21 for one or more markers was found in 15 of 40 (37.5%) cases. LOH was mainly found in grade II and III tumors. LOH for one or more markers in the 9p21 region was found in 2 of 10 (22%) grade I, 11 of 21 (52%) grade II and 4 of 6 (67%) grade III chondrosarcomas ($p = 0.054$, chi square test for trend).

For 12 cases, all informative markers at 9p showed LOH. There was also one tumor that showed loss of one marker and retention of an adjacent one, but the region involving LOH included the INK4A locus in all cases.

Table 3.3: Correlation between loss of p16 protein expression and LOH at 9p21.

	Tumors with LOH		Tumors with no LOH		
	LOH 9p21 (n)	p16 negative immunohistochemistry	No LOH 9p21 (n)	p16 negative immunohistochemistry	Total
Grade I	2	0/2	9	0/6[a]	11
Grade II	9	1/7 (14%)[a]	14	1/11 (9%)[a]	23
Grade III	4	2/4 (50%)	2	1/2 (50%)	6
Total	15	3/13 (23%)	25	2/25 (8%)	40

[a] no corresponding p16 protein expression data were available from eight tumors with LOH data.

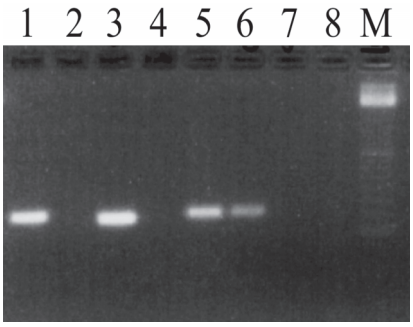

Figure 3.4: *Example of MSP analysis. All DNAs were modified with bisulphite. The samples in lanes 1-4 are amplified with primers for the methylated state and those in lanes 5-8 samples amplified with primers for the unmethylated state. Lanes 1 and 5 are of a conventional central chondrosarcoma; lanes 2 and 6 are unmethylated control DNAs; lanes 3 and 7 are methylated control DNAs; and lanes 4 and 8 are samples amplified without DNA to serve as a negative control. Lane M is a 100 bp ladder*

Remarkably, for all three patients with Ollier disease, LOH for one or more markers in the 9p21 region was found.
As shown in Table 3.3, there is no clear correlation between LOH at 9p and the loss of protein expression.

Promoter Methylation
Table 3.4 shows the results of the promoter methylation analysis by MSP. An example of MSP is shown in Figure 3.4. DNA was available to study promoter methylation from six of ten patients with absent INK4A/p16 protein expression. Twenty-four additional patients were selected with varying INK4A/p16 staining intensities with INK4A/MTS1 antibody.
Methylation of the CDKN2A/p16 promoter was detected in 5 of 30 (13%) cases. Detectable promoter methylation was not clearly associated with loss of INK4A/p16 protein expression (Table 3.4).

Table 3.4: Correlation between *CDKN2A/p16* methylation status, tumor grade, and INK4A/p16 immunohistochemistry.

Conventional central chondrosarcoma	**p16 methylation**		
Grade I	0/7 (0%)		
Grade II	4/17 (24 %)		
Grade III	1/6 (17 %)		
Conventional central chondrosarcoma		**p16 intensity**	**p16% positive cells**
p16 expression 0	1/6 (17%)	0	0
p16 expression 2	2/6 (33%)	1	1
p16 expression 3-4	1/10 (10%)	1	2
p16 expression 5-6	1/5 (20%)	2	3
p16 expression 7	0/3 (0%)		

Statistical analysis

There is a significant relation between loss of INK4A/p16 protein expression and increasing tumor grade ($p = 0.004$, chi-square test for trend). Both LOH for one or more markers in the 9p21 region and complete loss of INK4A/p16 expression also seemed to correlate with a poor prognosis; this was, however, not statistically significant ($p=0.0828$ and $p=0.1271$, respectively, log-rank test) and was not independent of histological grade, which was a strong prognostic parameter ($p = 0.000$, log-rank test).

Ten of 27 chondrosarcomas that showed INK4A/p16 expression showed also LOH. The staining intensity and the percentage of cells demonstrating INK4A/p16 expression seemed slightly lower in INK4A/p16 positive tumors with LOH than in INK4A/p16 positive tumors without LOH although the difference was not statistically significant (mean score for intensity 1.38 versus 1.74, mean score for percentage of positive cells 1.54 versus 2.05, mean total score 2.92 versus 3.79).

DISCUSSION

Few genetic aberrations have been identified in conventional central chondrosarcomas, suggesting that limited genetic alterations are required for tumorigenesis. LOH and other cytogenetic alterations at 9p21 are, however, repeatedly found, suggesting an important role in tumorigenesis for genes located in this region. The *CDKN2A/p16* locus at 9p21 is an excellent candidate, since it was shown to be inactivated in multiple tumor types including bone sarcomas [14]. The *CDKN2A/p16* gene has been investigated previously in chondrosarcoma, but a heterogeneous group of chondrosarcomas was used and only one genetic alteration was found [20]. These authors identified promoter methylation in 5 of 22 cases, but loss of expression was not confirmed by immunohistochemistry.

Here we present a carefully selected group of conventional central chondrosarcomas only, stratified according to differentiation, grade and etiology, and their potential precursors, namely enchondromas. A study for *CDKN2A/p16* was performed on the level of protein, genetic alterations, promoter methylation, and mutational analysis of *p14/ARF*. Furthermore, we investigated whether INK4A/p16 protein expression level can be used as a predictive marker for tumor progression in conventional central chondrosarcomas.

Two separate subgroups were discerned within our tumors:

1. Tumors located in the phalanx versus those located elsewhere, since phalangeal chondrosarcomas are extremely rare and have a far better prognosis compared to chondrosarcomas located elsewhere [4]. Both enchondromas of the phalanx as well as enchondromas located elsewhere failed to demonstrate loss of INK4A/p16 protein expression.
2. Conventional central chondrosarcomas that occur in the context of Ollier disease [5,21] versus sporadic cases. Chondrosarcomas in the context of Ollier disease may have a worse prognosis than normal chondrosarcomas [22,23]. Four chondrosarcomas of Ollier disease patients were available for this study and one showed loss of INK4A/p16 protein expression. Remarkably, however, all three patients with Ollier disease used for the LOH analysis demonstrated LOH for one or more markers in the 9p21 region. This is consistent with

previous data [24] and suggests that INK4A/p16 is not more often involved in Ollier disease than in solitary chondrosarcomas. Rather, another gene located in the 9p21 region may be important in chondrosarcoma in Ollier disease.

This study shows that conventional central chondrosarcomas demonstrate loss of INK4A/p16 expression by immunohistochemistry in 20% of cases tested and this loss increases with histological grade. Enchondromas show no loss of INK4A/p16 expression. This is concordant with the low proliferative activity in enchondromas and the role of INK4A/p16 in inhibition of cell cycle progression. In dysplastic nevi, LOH, mutations, and loss of protein expression of INK4A/p16 have been reported but only in a small fraction, whereas melanomas often show INK4A/p16 alterations, suggesting a role for this gene in tumor progression in this situation too [25-28] . Five tumors showed only cytoplasmic and no nuclear staining. It is previously described that breast tumors with only cytoplasmic INK4A/p16 staining have a more aggressive behavior [29], but this cannot be concluded for chondrosarcomas, because the tumors that showed only cytoplasmic staining were low grade.

In many other tumors, especially melanoma, the *CDKN2A/p16* locus is the target for LOH at 9p21 [18,30,31]. Our LOH analysis shows that *CDKN2A/p16* is included in the region in all cases with LOH of at least one marker at 9p21. LOH at 9p21 was relatively frequent (37%) and comparable with previous results [8,32,33].

LOH at 9p increased with histological grade, which is most probably a reflection of overall increase of genetic instability in higher-grade tumors [34]. Whether *CDKN2A/p16* is the target of LOH at chromosome 9p21 could not be assessed because SSCP analysis did not identify mutational inactivation of the retained copy of *CDKN2A/p16* and *p14/ARF* in any of the tumors tested. Furthermore, there was no association between LOH at 9p21 and immunohistochemical loss of INK4A/p16 protein expression. The 9p12-22 region was involved in five of seven conventional central chondrosarcomas with an aberrant karyotype [7]. Two of the five cases demonstrating –9, t(9;10)(p22;q22) and add(9)(p21) could be included in the present analysis and both showed loss of INK4A/p16 protein expression and LOH at 9p21.

Homozygous deletions of the *CDKN2A* region have been described occasionally in cell lines and primary tumors of different origin [35,36]. We cannot exclude that such deletions have been missed in our study. However, because there is no obvious relation between LOH and INK4A/p16 protein expression in our study, homozygous deletions of *CDKN2A/p16* in conventional central chondrosarcomas are unlikely.

Another mechanism for tumor suppressor gene inactivation is epigenetic promoter methylation, which has been described for the *CDKN2A/p16* gene [37]. Indeed, we found methylation in 5 of 30 patients. However, there was no association between LOH at 9p21 and methylation, since only two of these cases showed LOH. There was no significant relation between protein expression and *CDKN2A/p16* promoter methylation, since we identified four tumors that have promoter methylation without loss of INK4A/p16 protein expression. With increasing age, methylation of several target genes can be detected in normal tissue [38-40]. The average age of the patients that showed *CDKN2A/p16* promoter methylation was 41.4 years. This is even less than the average age of all patients with conventional

central chondrosarcomas, namely 50.8 years, thereby indicating that age-dependent promoter methylation is not likely. An alternative explanation for the discordance between *CDKN2A/p16* promoter methylation and protein expression is the focal INK4A/p16 positive stained areas next to areas with no INK4A/p16 expression (Figure 2b), indicating tumor heterogeneity with regard to INK4A/p16 promoter methylation. Interestingly, we did not find promoter methylation in low-grade chondrosarcomas.

CDKN2A/p16 alterations have also been identified in other bone sarcomas as well, such as osteosarcomas [10,11], Ewing sarcomas [10,12] and malignant fibrous histiocytomas of bone (MFH-b) [13]. About 30% of Ewing sarcoma and osteosarcoma show abnormalities (mutations, deletions, and promoter methylation) of the INK4A locus. For MFH-b only 1 of 19 tumors showed a *CDKN2A/p16* mutation. Ewing sarcomas show a negative correlation between mutations and deletions of the INK4A locus and prognosis.

In conclusion, loss of INK4A/p16 protein expression occurs in one-third of conventional central chondrosarcomas by an as yet unknown mechanism and is associated with higher tumor grade. Although we find LOH at 9p21 in 38% of cases, there was no association between LOH and loss of INK4A/p16 protein expression. Thus, neither the *CDKN2A/p16* nor *p14/ARF* locus appears to be the target and therefore another gene is most likely involved by this recurrent genetic aberration. Also, *CDKN2A/p16* promoter methylation does not explain this phenomenon, because we did not find an association with loss of protein expression. It is of importance that *CDKN2A/p16* loss is more frequent in high-grade tumors and is absent in enchondroma. It has been reported that recurrences of chondrosarcoma sometimes demonstrate an increase in the degree of malignancy [1,2]. This suggests that loss of INK4A/p16 protein expression is an important event during tumor progression in enchondroma, as well as the progression in grade in recurrent chondrosarcoma.

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Chapter 4

Enchondromatosis (Ollier disease, Maffucci syndrome) is not caused by the PTHR1 mutation p.R150C

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Enchondromatosis (Ollier disease, Maffucci syndrome) is a rare developmental disorder characterized by multiple enchondromas. Not much is known about the molecular genetic background. Recently an activating mutation in the parathyroid hormone receptor type 1 (*PTHR1*) gene, c.448C>T (p.R150C), was reported in two of six patients with enchondromatosis. The mutation is thought to result in upregulation of the IHH/PTHLH pathway. This is in contrast to previous studies, showing downregulation of this pathway in other cartilaginous tumors. Therefore, we investigated PTHR1 in enchondromas and chondrosarcomas from 31 enchondromatosis patients from three different European countries, thereby excluding a population bias. PTHR1 protein expression was studied using immunohistochemistry, revealing normal expression. The presence of the described PTHR1 mutation was analyzed, using allele specific oligonucleotide oligonucleotide hybridization confirmed by sequence analysis, in tumors from 26 patients. In addition, 11 patients were screened for other mutations in the *PTHR1* gene by sequence analysis. Using both allele-specific oligonucleotide hybridization and sequencing we could neither confirm the previously found mutation nor find any other mutations in the *PTHR1* gene. These results indicate that the *PTHR1* gene is not, in contrast to previous suggestions, the culprit for enchondromatosis.

Ollier disease (enchondromatosis, MIM #166000) is a rare developmental disorder that seems to distribute in a non-hereditary manner. The syndrome is characterized by skeletal deformities and multiple enchondromas, often with unilateral predominance [1]. Enchondromas are benign cartilaginous lesions, located in the metaphyseal medulla of bone. The solitary form of enchondroma is far more common than the rare occurrence within the context of enchondromatosis.

Maffucci syndrome is a rare variant of enchondromatosis characterized by both multiple enchondromas and benign hemangiomas [2]. Apart from these two enchondromatosis syndromes, recognized by the World Health Organization (WHO) [3], there are other extremely rare distinct variants described, such as spondyloenchondromatosis (MIM #271550) [4-7].

Malignant transformation of enchondromas to chondrosarcomas occurs in <1% of solitary cases [8], and 25 to 30% of enchondromatosis cases [8,9]. Chondrosarcomas can be either conventional central (83%) or secondary peripheral (17%), with central chondrosarcomas arising de novo, or as a result of malignant transformation of an enchondroma [10].

Secondary peripheral chondrosarcomas arise, by its definition, secondarily within the cartilaginous cap of its benign precursor, osteochondroma, which is solitary or occurs in the context of Multiple Osteochondromas (MO) [11]. In contrast to enchondromatosis, MO demonstrates a clear autosomal dominant inheritance pattern, caused by *EXT1* or *EXT2* mutations. EXT mutations are postulated to disturb, via heparan sulphate proteoglycans, the Indian hedgehog (IHH)/parathyroid hormone like hormone (PTHLH) negative feedback loop within the normal human growth plate [11,12], and absence of these signaling molecules has been demonstrated in osteochondromas [13].

Three genetic studies have been reported on enchondromatosis. Cytogenetic analysis showed a interstitial deletion at chromosomal region 1p in one case [14], loss of heterozygosity (LOH) at 13q14 and 9p21 and p53 overexpression in chondrosarcoma in another case [15], and recently, Hopyan *et al.*[16] described a mutation in the parathyroid hormone receptor 1 (*PTHR1*, MIM #168468), PTHR1 c.448C>T (p.R150C PTHR1), which was found in two of six patients with Ollier disease, one a germline and the other probably a somatic mutation. This mutation was absent in 50 solitary chondrosarcomas and 100 unaffected individuals [16]. The single nucleotide change in *PTHR1* codes for an amino acid change, which results in a constitutively active receptor, with reduced translocation to the membrane (as shown by western blot), reduced PTHLH binding (by cell transfection) and a decrease in chondrocyte differentiation (PTHR1 p.R150C mouse model). The authors argued that, by upregulation of the IHH/PTHLH signaling, this leads to the formation of enchondromas [16].

It is difficult to perceive that upregulation of IHH/PTHLH signaling would lead to enchondroma formation, knowing that downregulation of the IHH/PTHLH signaling, as a result of EXT mutation [11-13,17], plays a role in osteochondroma formation. We therefore investigated the role of PTHR1 in enchondromatosis, by studying the expression of the PTHR1 protein and screening for the specified p.R150C *PTHR1* mutation. In addition, in 11 patients all exons of the *PTHR1* gene were screened for mutations.

MATERIAL AND METHODS

Patient data

In total, 23 enchondromas and 18 chondrosarcomas from 31 patients with enchondromatosis were obtained. The samples were collected from the files of The Netherlands Committee for Bone tumors (three patients, six samples), the Leiden University Medical Center (nine patients, 14 samples), Rizzoli Orthopedic Institute, Bologna, Italy (15 patients, 15 samples) and Institute of Pathology, University of Bern, Switzerland (four patients, six samples).
Patient data were obtained by review of clinical charts and radiographs. Grading was performed according to Evans *et al*.[18]. Patients were included if at least two different sites were affected by enchondromas and or chondrosarcomas [3]. For patient descriptions, see Table 4.1. All procedures were performed according to the local ethical guidelines.

PTHR1 immunohistochemistry

Of 24 of the 31 patients paraffin blocks were available for immunohistochemistry. From each patient one tumor was used. Sections (4 μm) were stained with the polyclonal PTHR1 antibody from Babco (Eurogentec, San Diego, CA) in a 1 in 75 dilution, using a citrate antigen retrieval as previously described [19]. As a positive control skin was used and vessel walls and osteoblasts served as an internal positive control. Two independent observers scored the sections.

DNA isolation

Tumor DNA was isolated from paraffin-embedded (n = 24), as well as fresh frozen material (n = 12). From 13 patients normal DNA was also obtained (10 from paraffin, two from frozen tissue and one from blood). The tumor percentage as determined by hematoxylin and eosin stained slides was at least 70%. DNA from paraffin-embedded material was isolated as described earlier [20]. Some samples were microdissected to enrich for tumor percentage or to obtain normal DNA. Samples isolated from paraffin-embedded material with a low DNA concentration were concentrated using the DNA Clean and Concentrator kit (Zymo research, Orange, CA). DNA from fresh frozen material was isolated using a wizard genomic DNA purification kit (Promega, Madison, WI), and DNA of blood was isolated using a salting out procedure according to Miller and Polesky [21].

R150C PTHR1 PCR

Genomic DNA of the *PTHR1* gene (NM_000316.2) containing the position of the p.R150C PTHR1 mutation was amplified using the PCR primers 5'-TGACACACTCGCTGTAGTTGG-3' (PTHR1-F) and 5'-TTGGAGCTAGGGGTTCAGTG-3' (PTHR1-R) generating a 154 bp product. As a positive control, DNA isolated from a normal placenta was used; both to serve as a control for the PCR and as a wild type control for the allele specific oligonucleotide oligonucleotide hybridization.

Table 4.1: Patient descriptions and PTHR1 protein expression data.

Pat	M/F	Syndrome	Family history	Sample	Age at operation (yrs)	Diagnosis	Location	Abnormal spine	Other affected sides	PTHR1 protein expression
1	F	Ollier	no	1	25	EC	Hand	no	Hand L+R, humerus R, ribs	
2*	F	Ollier	no	2.1	30	CS-I	Distal femur R	no	Prox tibia R, prox femur R, prox humerus R	
3*	M	Ollier	no	3.1	24	EC	Hand R	no	Hand L	
4*	F	Maffucci	yes[a]	4.1	41	CS-dediff	Prox humerus L	no	Hand R+L	
5*	F	Ollier	no	5.1	38	EC	Prox humerus L	no	Hand R+L, pubis, prox femur, prox humerus L	
6	M	Ollier	no	6	5	EC	Prox humerus L	no	Femur L	+
7*	M	Maffucci	yes[b]	7.1	36	CS-III	Pelvis	no	Hand R+L, prox femur R	
8	F	Maffucci	no	8	38	CS-II	Pelvis	no	Humerus R+L, femur R+L	+
9	F	Ollier	no	9	15	EC	Hand	no	Hand L+R, pelvis	-
10	M	Ollier	no	10	30	EC	Hand R	no	Femur L, tibia L, hand L	
11*	M	Ollier	no	11.1	23	CS-dediff	Spinal column	tumor		-
12	F	Ollier	no	12.1	6	EC	Femur L	no	Os ilium L, 3 locations in tibia L	-
				12.2	6	EC	Tibia L			
13	M	Ollier	no	13.1	12	EC	Hand phalanx L	no	Hand phalanges dig 1-4 left	++
				13.2	12	EC	Hand R			
14	M	Ollier	no	14	8	EC	Distal fibula R	no		-
15*	F	Ollier	no	15.1	27	EC	Pelvis L	no	Femur L	
				15.2	27	CS-II	Prox fibula R			
				15.3	28	EC	Hand phalanx R			
16*	F	Ollier	no	16.1	62	CS-I	Distal femur L	no	Tibia L, pelvis L	
				16.2	62	CS-I	Distal femur L			
17*	M	Ollier	no	17.1	42	CS-I	Hand metacarpal L	no	Foot L, hand L+R, femur L+R, humerus L, rib	++
				17.2	47	CS-I	Foot metatarsal L		L+R,acetabelum R, ulna L	
18	F	Ollier	no	18	15	CS-I	Humerus R	no	Femur R, scapula R,	
19*	M	Ollier	no	19.1	40	CS-I	prox tibia R	no	Femur R, fibula L, scapula L+R	
				19.2	40	CS-II	Scapula R			
20*	F	Ollier	no	20.1	23	CS-II	Distal femur	no	Femur L+R, tibia L+R, ankle L, lung metastases	++
21*	M	Ollier	no	21.1	26	CS-I	Left tibia L	no	Skull, rib, tibia L+R, fibula L+R, femur L, hand L,	++
				21.2	26	EC	Femur		foot L	
22	F	Ollier	no	22.1	25	EC	Hand phalanges R	no	Humerus L+R, rib R, os pubis R, hand L+R,	++
				22.2	16	EC	Hand		femur R, tibia R	
23	M	Ollier	no	23.1	68	CS-II	Humerus	no		++
				23.2		CS-III	Humerus			
24	M	Ollier	no	24	18	CS-I	Femur R	no	Tibia R	
25*	M	Ollier	no	25.1	15	EC	Hand phalanx L		Hand L+R	++
26	M	Ollier	no	26	14	EC	Distal femur L	yes[c]	Femur L	++
27	F	Ollier	no	27	26	EC	Hand R	no	Humerus R, ribs	
28	M	Ollier	no	28	31	EC	Prox humerus R	no	Prox tibia R, prox femur R	
29	F	Ollier	no	29	38	EC	Prox tibia L	no	Hand L+R	
30	F	Ollier	no	30	41	EC	Prox femur R	no	Hand L+R, humerus L, pubis	
31	M	Ollier	no	31	26	EC	Hand R	no	Hand L	

[a] One of the parents affected by Ollier disease; [b] One of the parents affected by Maffucci syndrome; [c] Radiographical evidence of spinal deformation, no platyspondyly; * of these patients normal DNA was available.
Abbreviations: pat: patient; M: male; F: female; EC: enchondromas; CS: chondrosarcoma; dediff: dedifferentiated; prox: proximal; R: right; L: left;I, II, III are grade I, grade II, grade III; -: no PTHR1 protein staining; +: weak PTHR1 protein staining; ++: positive PTHR1 protein staining

Construction of the mutant sequence

A plasmid containing the PTHR1 fragment with the c to t substitution (c.448C>T) was constructed using mutation specific PCR [22]. Detailed description of the construction can be obtained on request.

ASO hybridization

For detection of the p.R150C PTHR1 mutation PCR fragments of tumor and control DNA were electrophoresed on a 2% agarose gel and blotted to nylon membranes (Hybond™-N+, Amersham bioscience, Piscataway, NJ) as described earlier [23]. A separate blot was made with a dilution series of a mix of wild-type and constructed mutant PCR products in different concentrations. The blots were hybridized with α-^{32}P oligonucleotides specific for wild type (5'-ACGCTGTGAC<u>C</u>GCAATGGCA-3') or mutant (5'-ACGCTGTGAC<u>T</u>GCAATGGCA-3'). Oligonucleotides were labeled in 6 ml containing 20 pmol oligo, 1ml [α-^{32}P] ATP (10 µCi), 1x kinase buffer (70 mM Tris, pH 7.6; 10 mM $MgCl_2$), 9 units T4 PNK kinase (USB; US Biochemicals, Cleveland, OH) for 1 hour at 37°C and 5 min at 65°C. The blot was hybridized overnight with a hybridization mix containing 0.5M $NaHPO_4/NaH_2PO_4$ (pH 7.0), 7% SDS at 65°C (wild-type) and 68°C (mutant). After washing the filters with 1 x SSC/0.5 % SDS, they were exposed to a Phosphor Imager screen (Amersham bioscience).

The blot containing the samples was first hybridized with the mutant probe, then stripped at 68°C with 0.1 x SSC/0.5% SDS and subsequently hybridized with wild-type probe. Complete stripping of the blot was checked by phosphor imaging.

Signal intensities were scored as peak heights as detected by ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Sequencing for the p.R150C PTHR1 mutation

For 19 cases the absence of the mutation, as detected by ASO hybridization, was confirmed by sequence analysis of the PCR fragment, using the forward and/or reverse PCR primer. Among the samples chosen were those tumor samples with a relative signal comparable and lower to the wild type sample. The PCR products of these samples were purified using QIAGEN QIAquick PCR Purification Kit (QIAGEN, Germantown, MD) prior to sequencing. Sequencing was performed using the ABI PRISM® Big Dye Terminators v. 2.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Samples were run on an ABI 3700 Genetic Analyzer (Applied Biosystems).

Sequencing of the entire PTHR1 gene

Fresh frozen tissue to obtain high molecular weight DNA was available for 11 patients affected by Ollier disease, five of which where not analyzed by the ASO hybridization. Genomic DNA was subjected to direct sequencing for the *PTHR1* gene (NM_000316.2). Patient data are resumed in Table 4.1 (patients 17, 19, 21, 22, 24, 25 and 27-31). All coding exons, including exon-intron boundaries, were amplified according to primers described by Schipani et al. [24] and with additional primers described in Table 4.2, based on the sequences U22401-U22409.

Table 4.2: Sequence of the additional designed nucleotide primers of each exon of *PTHR1*

PCR product	Size (bp)	Primer sequence
S	226	5'-AGCTCTGCACCCCCTACC-3'
		5'-GCGTGCCTTAGACCTACTCC-3'
E1	232	5'-AAAGTCCTGCCTGTGGTCTG-3'
		5'-AGCCTTCACCTGGCTCTGTA-3'
E2	167	5'-AGGGAAGCCCAGGAAAGATA-3'
		5'-TCACATCAGAGGGACAGTGC-3'
E3	216	5'-TCCCCTACCCTGTCTGTCTC-3'
		5'-GAGGTCTCGAGGCACTGAAC-3'
G	237	5'-TTGGAGCTAGGGGTTCAGTG-3'
		5'-GTGTGGGTGGGAGTGAATTT-3'
M1	153	5'-GCCTCTTGCTCTTACCCTGA-3'
		5'-GATGAGCACAGCTACGGTGA-3'
M2	175	5'-TGTTCCTGTCCTTCATGCTG-3'
		5'-GGCAGAGGGGTACTCACGTA-3'
M3	176	5'-CCCTGCCCTCTGACTAACAC-3'
		5'-TGTGAAGCCCCACAGGTACT-3'
M4	173	5'-GCTGTGTGGGTCAGTGTCAG-3'
		5'-GGCTGGACTGAGAACTCCTG-3'
EL2	153	5'-ACTTCCAAAGAGGCCTGTGA-3'
		5'-TTGAGGCATTAGCTCCCATC-3'
M5	194	5'-CACTCCCCACAGCTCAACTT-3'
		5'-ATGGGCCACTGTCTTTCACT-3'
M6-7	243	5'-CATTGTCTTCATGGCCACAC-3'
		5'-GCCCTATGCCAACACTGTCT-3'
T1	162	5'-TTGGGAGACACACCTGACTG-3'
		5'-TCACACTTGTGTGGGACACC-3'
T2	150	5'-CTGCCCTGCTACAGGAAGAG-3'
		5'-TTCCCTGTTTTTCCTCTTGG-3'

RESULTS

Patient data

In total 41 tumors from 31 patients with enchondromatosis were collected. Clinical details of all patients are shown in Table 4.1.

PTHR1 immunohistochemistry

Staining results are shown in Table 4.1. Due to loss of attachment of the tissue section from the slide, as a result of the antigen retrieval procedure, 10 of the 24 samples could not be evaluated. Eight out of 14 samples were scored as positive for the presence of PTHR1 protein, five demonstrated only weak positivity, while one tumor was completely negative with a positive internal control. The staining was mainly found in the nucleus, but cytoplasmic

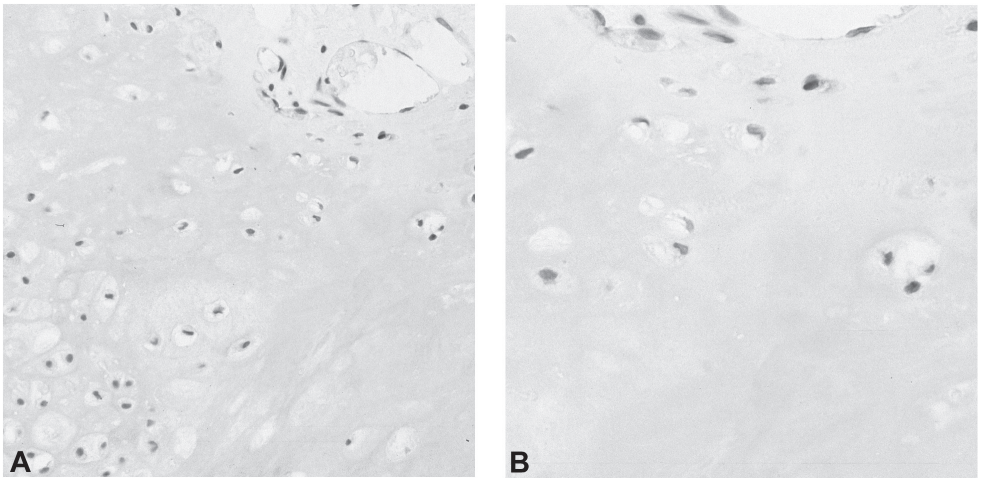

Figure 4.1: *Immunohistochemical staining for the PTHR1 protein. Sample 22.1, an enchondroma of the phalanx, showing positive PTHR1 expression in both nucleus and cytoplasm of tumor cells. The endothelial cells, serving as an internal control, also show PTHR1 expression.* ***A****: 50x,* ***B****: 100x. Color picture can be viewed at page 128*

staining was also found (Figure 4.1). The results were similar to those obtained for 20 sporadic chondrosarcoma cases [13].

ASO hybridization: p.R150C PTHR1 mutation

Of the 36 samples from 26 patients (patients 1-26) 33 samples resulted in a PCR product of the expected size, though the amount of final PCR product varied considerably. The three samples failing were DNA samples isolated from paraffin embedded tissues, one failing normal and tumor sample, the third only failing the tumor sample.

Blots containing PCR products from the *PTHR1* fragment were hybridized for the mutant sequence, resulting in strong hybridization signals with intensities that decreased linear according to a dilution series of PCR products containing the constructed mutant as shown in Figure 4.2a. However, we did see crosshybridization of the mutant probe with wild type PCR products that could not be overcome by more stringent hybridization (Figure 4.2a). The wild-type probe showed strong signals with the wild-type control (placenta DNA from a healthy donor) and samples, but no crosshybridization with the constructed mutant (Figure 4.2b).

In the dilution experiment the mutant sequence could be detected in a background of wild-type sequence containing 90% wild-type PCR product and 10% constructed mutant PCR product. Using this experiment a cut-off value for the probes was determined (0.20). The signal threshold of the mutant oligonucleotide was defined as the strongest signal obtained by phosphor imager analysis, for the wild-type control fragment with the mutant oligonucleotide divided by the signal of the wild-type oligonucleotide for that sample. All patient samples showed a signal for the mutant oligonucleotide/wild-type oligonucleotide

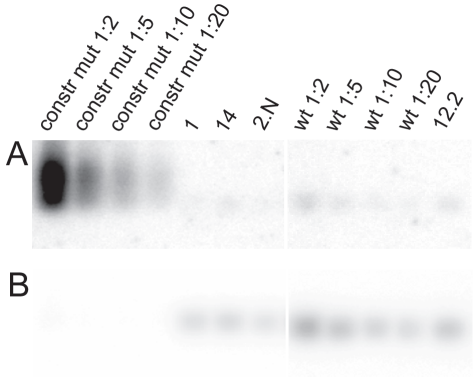

Figure 4.2: *ASO hybridization of the samples with labelled α-^{32}P mutant and wild type oligonucleotides.* ***A****: Blot of ASO hybridization with mutant oligonucleotide, b) Blot of ASO hybridization with wild-type oligonucleotide, showing the same samples as seen in A. constr mut: constructed mutant; wt: wild-type*

ratio below this threshold, ranging from 0.03 to 0.19. In contrast, the signal of constructed mutant, even in a 1 in 10 dilution, was above this threshold (value 0.26).

Sequencing for the p.R150C PTHR1 mutation

A total of 19 samples were selected for sequencing to confirm the results found in the ASO hybridization. All 19 samples showed the wild-type sequence (reviewed but not shown).

Sequencing of the entire *PTHR1* gene

From the 11 patients of whom the whole *PTHR1* gene was sequenced, all PCR reactions resulted in sequencing products. No mutations and/or polymorphisms were detected in the coding exons of the gene.

DISCUSSION

The PTHR1 protein is important in chondrogenesis and skeletogenesis and is involved in the IHH/PTHLH feedback loop present in the growth plate [12,25-27]. IHH binds to its receptor Patched (PTCH) after diffusion, presumably under mediation of heparan sulphate proteoglycans (HSPG) of which the biosynthesis is mediated by EXT. The binding results in PTHLH expression which then binds to PTHR1 in the late proliferating zone [28], resulting in upregulation of BCL2 [27,29]. This signaling regulates the pace of chondrocyte differentiation by delaying the progression of chondrocytes towards the hyperthrophic zone, allowing longitudinal bone growth [27].

Deregulation of this feedback loop can result in many different syndromes. For instance, in patients with Blomstrand Chondrodysplasia (MIM# 215045), inactivating mutations have been identified in *PTHR1* [30], resulting in accelerated chondrocyte differentiation and premature ossification [31]. In contrast, constitutive active *PTHR1* mutations have been

identified in patients with Jansen metaphyseal chondrodysplasia (MIM# 156400) [30]. These patients have a delay in chondrocyte differentiation, in vascular invasion, and a reduction or absence of mineralization of bone elements that are formed through the endochondral process [32]. This syndrome shares some radiographical and histological features with enchondromatosis, like the presence of radiolucent areas containing non calcified cartilage [33].

In patients with MO mutations in the *EXT* genes are found, postulated to lead to a downregulation of the IHH/PTHLH pathway [11,17]. Indeed, in osteochondromas, that are histologically comparable to the human growth plate, absence of IHH/PTHLH signaling was demonstrated [13]. Upon malignant transformation of osteochondromas, upregulation of PTHLH and BCL2 was detected and this was also found during progression of low- towards high-grade conventional central chondrosarcomas.

Surprisingly, the p.R150C PTHR1 mutation is described to lead to an upregulation of IHH [16]. It is difficult to understand that both upregulation (in enchondromatosis) and downregulation (in MO) of the same IHH/PTHLH pathway would cause benign cartilaginous tumors. We therefore wanted to further investigate PTHR1 in a large series of enchondromatosis patients. We looked at the PTHR1 protein expression in 14 patients with enchondromatosis, revealing normal expression in 13 cases. Three of five weakly staining samples originated from young patients (ages 6, 15 and 23). This suggests that there may be an age related expression of PTHR1.

The mutation described by Hopyan *et al* [16] in two of six (33%) enchondromatosis patients was not found in the 31 enchondromatosis patients that were the subject of this paper. Sequencing of all exons, including exon-intron boundaries, of the *PTHR1* gene in 11 of these patients also did not reveal any other mutations. The possible presence of an intronic splice mutation, located outside the sequenced products, is not likely since this would most probably lead to inactivation of the PTHR1 protein, as seen in Blomstrand's Chondrodysplasia [34,35], and not to a receptor with increased signaling, as was described by Hopyan *et al* [16]. This indicates that the *PTHR1* gene is not involved in Ollier disease and Maffucci syndrome in our large multinational series. A possible explanation for this discrepancy could be that the p.R150C PTHR1 mutation described by Hopyan *et al.* is specific for the Canadian population, i.e. a founder mutation.

Technically one could argue that the level of contamination with normal tissue in our tumor samples is just too high for detection of somatic mutations by sequence analysis (in our samples the tumor percentage was at least 70%). However, the detection level for the p.R150C PTHR1 mutation using ASO hybridization is high enough to detect even those cases where the mutated sequence is present in only 10% of the total sample as shown by the dilution experiment. Thus, with the mutation described by Hopyan *et al* [16] as being heterozygous, we should have been able to detect the mutation if it was present.

Another explanation for the discrepancy between the results of Hopyan *et al*. [16] and our results may be found in the exact definition of the clinical syndrome, of which the classification is confusing. Enchondromatosis can be divided using several subclassifications [4-6]. The two most important ones are Ollier disease and Maffucci syndrome, both accepted by the

WHO [3]. Extremely rare is spondyloenchondromatosis, which was described to be autosomal recessive. Its radiographic features include irregular distributed, mostly discrete enchondromas of long tubular bones and generalized severe platyspondyly with mild or no involvement of hands and feet [4,36]. Generalized enchondromatosis, with patients having platyspondyly and metaphyseal manifestations of enchondromatosis with severe involvement of the hands and feet, has also been described [4,37].

If the patient carrying the germline p.R150C PTHR1 mutation [16], belongs to one of these rare subclasses of enchondromatosis instead of having Ollier disease, this mutation may be specific for this rare variant of enchondromatosis. Hopyan *et al*. [16] describe that one of the two patients having the p.R150C PTHR1 mutation inherited the mutation from his father, who had short statue, similar to a patient described by Halal and Azouz [4] diagnosed with "generalized enchondromatosis" and following an autosomal recessive inheritance pattern. Our population consisted strictly of patients with Ollier disease or Maffucci syndrome, lacking platyspondyly after reviewing of clinical charts and radiographs.

In conclusion, in our large, well-characterized, multinational group of enchondromatosis patients we cannot confirm the involvement of mutations in the *PTHR1* gene, indicating that PTHR1 is not causative for enchondromatosis in contrast to previous reports.

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Chapter 5

Absence of IHH and retention of PTHrP signaling in enchondromas and central chondrosarcomas

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Enchondromas and conventional central chondrosarcomas are, respectively, benign and malignant hyaline cartilage-forming tumors 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 tumors 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, PTHLH) signaling, an important pathway in chondrocyte proliferation and differentiation within the normal growth plate, was studied by immunohistochemical analysis of the expression of PTHLH, PTHR1, BCL2, p21, cyclin D1 and cyclin E. Quantitative real-time PCR for *IHH*, *PTCH*, *SMOH* and *GLI2* was performed on a subset of tumors. The data show that IHH signaling is absent in enchondromas and central chondrosarcomas, while PTHLH signaling is active. There was no difference in expression of any of the molecules between 35 enchondromas and 26 grade I central chondrosarcomas, indicating that PTHLH signaling is not important in malignant transformation of enchondroma. Higher expression of PTHR1 and BCL2 was associated with increasing histological grade in chondrosarcoma, suggesting involvement in tumor progression. No difference was found between samples from enchondromatosis patients and solitary cases, suggesting no difference in PTHLH signaling. A small subset of phalangeal chondrosarcomas demonstrated downregulation of PTHLH, which may be related to its more indolent clinical behavior. Thus, in both enchondromas and central chondrosarcomas PTHLH signaling is active and independent of IHH signaling, irrespective of the presence or absence of enchondromatosis.

Chondrosarcoma of bone is a malignant tumor 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 tumor, 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 hands and feet, while chondrosarcomas are rare at this location [2] and, in contrast to elsewhere, only rarely metastasize [4]. This raises the question whether cartilaginous tumors 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 malignant transformation of enchondroma and, secondary, to investigate possible biological differences between conventional central cartilaginous tumors 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 5.1). We investigated molecules involved in PTHLH signaling by comparing the protein expression of these signaling molecules between the different subsets of tumors and assessed whether putative differences in this signaling pathway are associated with different clinical behavior. Since commercially available antibodies for IHH signaling molecules have been shown previously not to work reliably in our hands [13], expression was studied with quantitative RT-PCR in a subset of tumors to evaluate their role in tumorigenesis and tumor progression.

MATERIALS AND METHODS

Pathological material

Formalin-fixed, paraffin wax-embedded material was used from 153 tumors (Table 5.1). Cases were collected from (consultation) files of the Leiden University Medical Center.

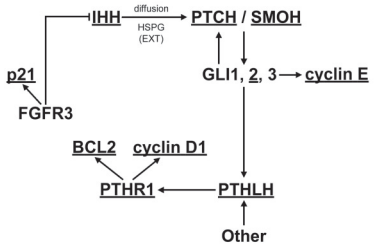

***Figure 5.1**: Signaling within the normal human growth plate. Indian Hedgehog (IHH), produced by pre-hypertrophic chondrocytes, binds after diffusion mediated by heparan sulphate proteoglycans (HSPG), to its receptor Patched (PTCH). This results in the release of Smoothened (SMOH) from PTCH, which 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 peptide (PTHrP, PTHLH)[9]. PTHLH binds to its receptor (PTHR1) in the late proliferating zone, resulting in up-regulation of the anti-apoptotic protein BCL2 [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/PTHLH signaling, and to up-regulation of the cell cycle inhibitor p21 [12]. The molecules investigated in this study are underlined*

Patient data (Table 5.1) 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).

Table 5.1: Patient and tumor 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 (range)	25.4 (12.3-74.3)	37.8 (4.3-68.8)	61.6 (8-83.4)	50 (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 (range)	87.5 (2-226)[a]	83.5 (1-170)[b]	96 (15-249)[c]	59.5 (2-212)[d]

* histological grading in chondrosarcoma of the phalanx proved not useful [4]. Data available from [a]14, [b]26, [c]15 and [d]64 patients

Table 5.2: Details of antibodies used and immunohistochemical protocols employed

Antigen	Producer	Mono-/ poly-clonal	Staining	Positive control	Internal positive control	Dilution	Antigen Retrieval[e]
PTHLH	Oncogene	Poly-clonal (IgG[c])	Cyto-plasm	Normal skin	Occasionally osteoclasts	1:25	Trypsin
PTHR1	Upstate	Mono-clonal (IgG1[d])	Nucleus	Normal skin	Osteoblasts, vessel walls	1:100	Citrate
BCL2[a]	Roche	Mono-clonal (IgG1[d])	Cyto-plasm	Normal tonsil	Osteoclasts, lymphocytes	1:500	Citrate
Cyclin D1	Neomarkers	Mono-clonal (IgG1[d])	Nucleus	Normal tonsil	Occasionally endothelial cells	1:500	Citrate
Cyclin E	Neomarkers	Mono-clonal (IgG2A[d])	Nucleus	Placenta	None	1:100	Citrate
p21[b]	Calbiochem	Mono-clonal (IgG1[d])	Nucleus	Normal colon	Occasionally vessels, osteoclasts	1:400	Citrate

[a]: clone 124; [b]: $p21^{WAF/CYP1}$; [c]: rabbit antibody; [d]: mouse antibody; [e]: Trypsin (30 min, 37 °C), Citrate (120 min, 95 °C)

Immunohistochemistry

Antibodies, controls, and antigen retrieval are described in Table 5.2. 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 5.2) to exclude absence of staining due to decalcification. Negative controls were performed using isotype controls. Immunohistochemical staining was performed as described previously [13].

Evaluation and Scoring

Three observers (LBR, PCWH and JVMGB) evaluated the slides independently and discrepancies were discussed. All observers were blinded towards clinico-pathological data. Scoring was performed as described [13]. In short, staining intensities (0= negative, 1= weak, 2= moderate and 3= strong intensity) and 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 staining intensity and percentage of positive cells were greater than 3 for PTHLH, PTHR1 and BCL2 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 tumors [2 phalangeal enchondromas (enchondromatosis patients), 1 solitary grade I chondrosarcoma, 5 grade II chondrosarcomas (3 from enchondromatosis patients), and 2 solitary grade III chondrosarcomas] and 7 normal samples (4 growth plates and 3 normal resting cartilage samples) as described previously [17].

One micorgram of total RNA was converted to complementary DNA (cDNA) by using AMV Reverse Transcriptase (Roche Applied Science). qPCR was performed for *IHH, PTCH, SMOH, GLI2,* and four normalization genes (*CPSF6, GPR108, HNRPH1* and *SRPR*; primers and qPCR conditions 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 manufacturer's protocol. For each gene a standardcurve, 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), representative for the amount of mRNA in each sample. Expression levels in the tumors were related to those of four normal growth plates, where IHH signaling is known to be active [9].

Statistical analysis

Comparison of immunohistochemical data between two different groups was analyzed using Pearson chi-Square/Fisher's exact test, two-sided. Correlation between staining and histological grade was tested with chi-square, linear by linear. Correlation with outcome was analyzed using log-rank test. To correct for multiple testing, p values ≤ 0.01 were considered significant. Relative RNA expression levels from the different tumor groups were compared to growth plates by one-way ANOVA with Bonferroni correction, after log transformation. A p value ≤ 0.05 was considered significant.

RESULTS

Immunohistochemistry

PTHLH signaling molecules are present in enchondromas and central chondrosarcomas. p21 and PTHLH were positive in almost all samples, whereas PTHR1, BCL2 and cyclin D1 showed more variation (Table 5.3). 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 (PTHLH) to 29 (Cyclin D1), could not be evaluated due to detachment to the glass slides.

Table 5.3: Scoring results of the immunohistochemical staining in enchondromas and chondrosarcomas

		PTHLH		PTHR1		BCL2		Cyclin D1		Cyclin E		p21	
		pos[a]	% pos[b]	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

[a] 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

Statistical analysis of immunohistochemistry

Enchondroma vs chondrosarcoma grade I

No significant difference was found for any of the investigated molecules 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 [$p=0.035$ (PTHLH) - 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 PTHLH differed significantly, being expressed in 12 of 16 (75%) phalangeal chondrosarcomas compared with 100% (n = 25) of chondrosarcomas grade II located elsewhere ($p = 0.010$) (Figure 5.2).

In addition, expression of BCL2 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

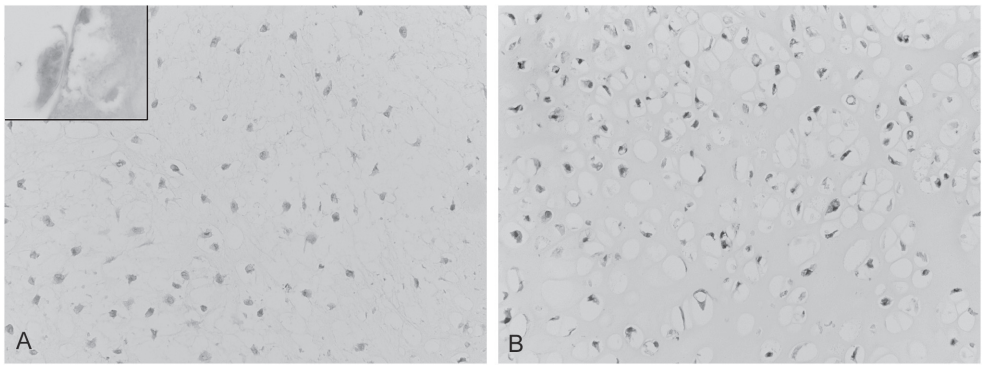

***Figure 5.2**: Lack of PTHLH expression in a subset of phalangeal chondrosarcomas. (A) PTHLH expression is absent in a phalangeal chondrosarcoma, with a positive internal control (inset), while (B) strong expression of PTHLH is found in grade II chondrosarcoma. Color picture can be viewed at page 129*

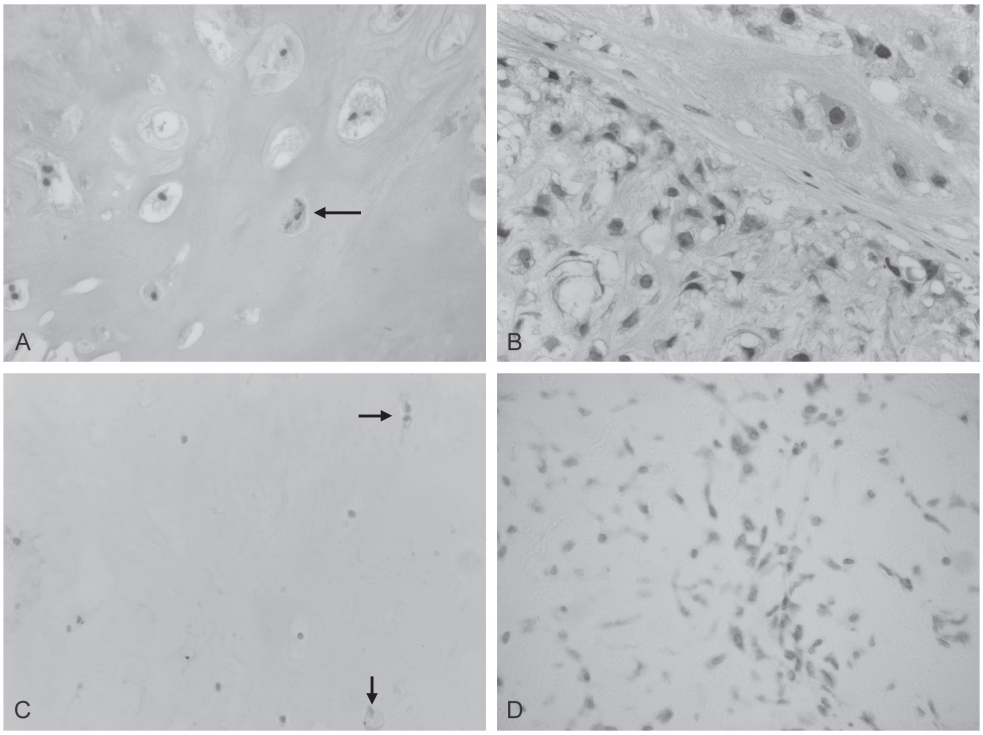

***Figure 5.3**: Immunohistochemical staining for BCL2 and PTHR1 in low- and high-grade conventional central chondrosarcoma. PTHR1 (A, B) and BCL2 (C, D) staining shows low intensity and a low percentage of positive cells in grade I conventional central chondrosarcomas (A and C, arrows), while increased staining is found in high-grade conventional central chondrosarcomas (B and D, grade III). Color picture can be viewed at page 129*

was found towards higher expression for PTHR1 in all phalangeal enchondromas [13 of 18 (72%) positive] compared to all enchondromas elsewhere [9 of 23 (39%) positive] was found (*p=0.072*).

Enchondromatosis vs solitary cases

Expression was compared both for all tumors 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

Analyzing all samples, increased PTHR1 and BCL2 expression correlated with increasing histological grade (*p=0.002* and *p=0.000*, respectively; Figure 5.3). For BCL2, 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 was 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 BCL2 and PTHR1 (*p=0.000*).

qPCR

Expression of *IHH*, *PTCH*, *GLI2* and *SMOH* 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 tumors as compared with the normal growth plate (Table 5.4). Although the sample size is small, *PTCH* expression was lower in normal cartilage (*p=0.019*),

Table 5.4: Average mRNA expression of *IHH*, *PTCH*, *SMOH* and *GLI2* per subgroup, relative to the average expression in the human growth plate

Sub types	IHH	PTCH	SMOH	GLI2
Resting cartilage (n = 3)	0.28	0.10[b]	1.51	0.70
Human growth plate (n = 4)	1.00	1.00	1.00	1.00
Phalangeal enchondroma (n = 2)	0.48	0.48	0.54	0.22
Grade I chondrosarcoma (n = 1)[a]	0.20	0.01	-	0.21
Grade II chondrosarcoma (n = 5)	0.49	0.06[b]	0.73	0.23[b]
Grade III chondrosarcoma (n = 2)	0.51	0.06[b]	0.63	0.15[b]

[a]: significant difference as compared to growth plate
[b]: statistical test could not be performed on this group since it contains only one sample

chondrosarcoma grade II (*p=0.000*) and III (*p=0.000*) and *GLI2* expression 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 PTHLH signaling in both enchondroma and central chondrosarcoma by protein expression of PTHLH, PTHR1, BCL2 and Cyclin D1. Expression of *PTCH* RNA was very low or absent. Activation of IHH signaling leads to the activation of target genes, including *PTCH* and *GLI* [6,7]. Therefore, transcriptional activation of PTCH is used as reporter for Hedgehog signaling [6]. The absence of PTCH thus indicates that IHH signaling 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 signaling. Thus, PTHLH signaling 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 PTHLH [19] and is expressed in chondrosarcoma [20].

Our findings confirm the importance of PTHLH signaling 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 PTHLH signaling 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, PTHLH signaling is absent [13], most likely due to mutational inactivation and/or loss of *EXT* genes [22]. Upon malignant transformation PTHLH and BCL2 expression is upregulated [13]. This makes absence of PTHLH signaling specific for osteochondromas since PTHLH signaling is active in enchondromas and chondroblastomas [15].

We have confirmed that higher BCL2 expression is associated with progression towards high-grade central chondrosarcoma, as suggested by our previous results [13]. BCL2 negatively controls programmed cell death both in growth plate chondrocytes *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, PTHLH 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 tumors was made, expression of PTHLH and PTHR1 has also been examined by others, showing up-regulation with grade [23,24]. Thus, in enchondroma and central chondrosarcoma PTHLH signaling 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 tumors are biologically different from other sites. Phalangeal chondrosarcomas display locally aggressive behavior with very low metastatic potential (<2%)[4]. Remarkably, only 4 of 142 tumors were PTHLH negative and all four were chondrosarcomas of the phalanx. This down-regulation of PTHLH

signaling 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 behavior. 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 BCL2 and PTHR1 was higher in solitary phalangeal enchondromas than in solitary enchondromas elsewhere. Since PTHR1 and BCL2 correlates with increasing histological grade in non-phalangeal chondrosarcomas, these results probably are 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 tumors. An activating mutation has been reported in *PTHR1* in two enchondromatosis patients [25]. The mutation would lead to up-regulation of IHH signaling [25]. Here we demonstrate absence of IHH signaling in two enchondromas and three chondrosarcomas of 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 tumors. 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 downregulation of IHH signaling that we observed.

In conclusion, we demonstrate that IHH signaling 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, PTHLH signaling 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 signaling.

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Chapter 6

cDNA expression profiling of chondrosarcomas: Ollier disease resembles solitary tumors and alteration in genes coding for components of energy metabolism occurs with increasing grade

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Conventional central chondrosarcomas are malignant cartilaginous tumors, occasionally arising secondary to either solitary or multiple (Ollier disease) enchondromas. Recurrences may have progressed in grade. The aims of the present study were to identify putative differences in gene expression between solitary and Ollier disease-related tumors, and to elucidate signaling pathways involved in tumor progression by genome-wide cDNA expression analysis. Arrays enriched for cartilage-specific cDNAs and genes involved in general tumorigenesis were used to analyse enchondromas (n = 3, two with Ollier disease), chondrosarcomas of different grades (n = 19, three with Ollier disease), normal resting-zone cartilage (n = 2) and chondrosarcoma cells in culture (n = 7). The arrays were analyzed by unsupervised hierarchical clustering, significant analysis of microarray and *T*-tests. Confirmation of data was performed by immunohistochemistry and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Ollier disease cases and solitary tumors revealed similar expression profiles, suggesting that the same signaling pathways are involved in tumorigenesis. Interestingly, JUNB protein expression was significantly higher in grade I chondrosarcomas than enchondromas ($p = 0.009$), which could be of diagnostic relevance. Upon chondrosarcoma progression, matrix-associated genes are down-regulated, reflecting the histology of high-grade tumors. An increase of glycolysis-associated, and a decrease of oxidative phosphorylation-related, genes was found in high-grade tumors. These findings suggest an adaptation in energy supply upon progression towards higher grade.

Chondrosarcoma of bone is a malignant hyaline cartilage-forming tumor that affects males and females equally, with a peak incidence around the fifth decade [1]. Most chondrosarcomas (~ 83%) arise centrally within the medulla and can develop from a pre-existing enchondroma [1]. A minority (~17%) are located at the surface of bone (secondary peripheral chondrosarcomas) [1,2].

While most enchondromas are solitary, patients with Ollier disease (enchondromatosis) have multiple enchondromas, scattered throughout the skeleton, often with a unilateral predominance [3]. Enchondromas of Ollier disease patients display a more indolent clinical behavior. These enchondromas can contain more worrisome histological features (higher cellularity, pleomorphism and binucleated cells), compared to solitary enchondromas [3,4]. The overall risk of malignant transformation in patients with Ollier disease is higher and has been estimated at 25-30%, compared to less than 1% for solitary enchondromas [3].

Three histological grades of malignancy are distinguished [5]. Increasing grade correlates with worse prognosis [5,6]. The fact that recurrences of chondrosarcomas can exhibit a higher grade of malignancy compared to previous lesions suggests that these tumors may progress [5,7].

Little is known about the pathways involved in the development and progression of central chondrosarcomas. Loss of heterozygosity (LOH) analysis shows limited changes, with some recurrent LOH at 9p21 [8]. Increased PTHLH (parathyroid hormone like hormone) signaling is present in high-grade tumors [9,10]. p53 overexpression and mutations [8,11], as well as absence of p16/INK4A [12,13], are also found to correlate with high histological grade.

For Ollier disease, case reports describe an interstitial deletion at chromosomal region 1p [14], LOH at 13q14 and 9p21, and p53 overexpression [15]. Hopyan *et al.*[16] reported a mutation, p.R150C, in the *PTHR1* (parathyroid hormone receptor 1) gene in two of six patients with Ollier disease: this mutation was absent in 50 solitary chondrosarcomas and 100 unaffected individuals. However, in a larger, multi-institutional series of 31 Ollier disease patients, this specific mutation was not identified, nor did sequencing the entire gene in 11 patients reveal any other mutations [17]. Moreover, in contrast to the up-regulation of IHH (indian hedgehog) signaling due to *PTHR1* mutations claimed by Hopyan *et al.*[16], we demonstrated that IHH signaling was very low or absent, while PTHLH signaling was active in both Ollier disease and solitary tumors [10].

We wanted to investigate whether Ollier disease tumors have a distinct gene expression profile when compared with solitary tumors by cDNA microarray analysis, to find an explanation for the clinical differences. To elucidate the pathways involved in progression of chondrosarcomas, we compared gene expression levels between low- and high-grade chondrosarcomas.

MATERIAL AND METHODS

Clinical Samples

Table 6.1: Clinico-pathological and clinical follow-up data of the solid (tumor) samples used in cDNA microarray analysis

Sample	Diagnosis	Location	M/F	Age at diagnosis (years)	Follow-up Months	Outcome
L867	Normal Cartilage	Foot	M	1	-	-
L1163	Normal Cartilage	-	-	16 wks GA	-	-
L892	EC	Phalanx	M	17	2	Rem
L206	EC (OD)	Phalanx	F	25	44	Rem
L1251	EC (OD)	Phalanx	M	14	8	Rec
L43	C-CS I	Humerus	M	31	128	Rem
L45	C-CS I	Femur	F	62	129	Rem
L327	C-CS I	Femur	M	19	84	Rem
L738	C-CS I	Humerus	F	34	59	Rem
L761	C-CS I	Femur	M	60	36	Rem
L803	C-CS I	Femur	F	39	28	Rem
L875	C-CS I	Femur	M	17	19	Rec
L172	C-CS II (OD)	Scapula	M	40	7	Rem, lfu
L286	C-CS II (OD)	Femur	F	22	41	Meta, dod
L813	C-CS II (OD)	Humerus	M	68	2	Rem, lfu
L654	C-CS II	Fibula	M	50	17	Rec
L861	C-CS II	SI-joint	M	6	1	Meta, dod
L869	C-CS II	Tibia	M	52	87	Rem
L908	C-CS II	Humerus	M	35	36	Rem
L171	C-CS III	Humerus	M	78	4	Rec, dod
L622	C-CS III	Humerus	F	73	9	Meta, dod
L795	C-CS III	Scapula	M	42	8	Rec
L835	C-CS III	Humerus	M	55	1	Meta, dod
L1066	C-CS III	Humerus	M	51	16	Meta

EC: Enchondroma, C-CS: conventional central chondrosarcoma; I: grade I; II: grade II; III: grade III; OD: Ollier disease; M/F: Male/Female; GA: Gestation age; Rem: Remission; Rec: Recurrence; Meta: Metastasis; lfu: lost to follow-up; dod: died of disease

Fresh frozen enchondroma and conventional central chondrosarcoma samples were collected from the archives of the Department of Pathology of the Leiden University Medical Center. Twenty-two tumor samples (3 enchondromas, 7 grade I, 7 grade II, and 5 grade III chondrosarcomas), five of which Ollier disease-related (two enchondromas, and three grade II chondrosarcomas), and two normal resting-zone cartilage samples were used (Table 6.1). In addition, two chondrosarcoma cell lines and five primary chondrosarcoma cell cultures were used (1 grade I, 3 grade II, and 3 grade III). All enchondroma specimens were of phalangeal localization, which should be regarded a separate subgroup because of their more worrisome histological features compared with enchondromas elsewhere [18]. All specimens were handled according to the ethical guidelines as described in “Code for Proper Secondary Use of Human Tissue in The Netherlands”, Dutch Federation of Medical Scientific Societies.

Reference Panel

As a common reference panel, used to compare cDNA array experiments, 15 highly diverse cancer cell lines were used, of which eight cell lines were selected from Scherf *et al.*[19] [HL-60 (acute myeloid leukemia); NCI-H226 (non-small-cell-lung); COLO 205 (colon); SB-19 (central nervous system); CAKI-1 (renal); OVCAR-3 (ovarian); PC-3 (prostate), MCF7 (breast)]. In addition, SAOS-2 and MG-63 (osteosarcoma), OUMS-27 and SW1353 (chondrosarcoma), and three additional primary central chondrosarcoma cell cultures were

added as samples expressing bone and cartilage-specific genes. RNA from all cell lines was mixed in equal amounts.

RNA isolation

For RNA isolation, we used samples containing at least 70% of tumor cells, as estimated on haematoxylin and eosin-stained frozen sections. RNA was isolated as previously described [20]. The amount of RNA was determined with a spectrophotometer and the integrity of the RNA was assessed by gel electrophoresis of 0.5-1.0 μg RNA on a 1% agarose/formalin gel.

cDNA microarray analysis

A cDNA array was designed containing 8696 cDNA clones, representing common genes and a selection of genes (~500) known to be expressed in cartilage, growth plate or involved in carcinogenesis in general (genelist available on request). Most of these genes were selected from the sequence-verified human ResGen 40K library (Research Genetics, Invitrogen, Huntsville, AL, USA); others were ordered from DKFZ (RZPD, Berlin, Germany) or custom made. For these clones, cDNA primers were derived from the reference sequence from NCBI. RT-PCR reactions were performed on RNA from tissue samples reported to express these genes (http://cgap.nci.nih.gov/Genes/GeneFinder). PCR-products were cloned in a vector using TOPO TA Cloning (Invitrogen) and verified by sequencing. All clones were PCR-amplified [21] and verified for size on a 1% agarose gel. After precipitation, PCR products were dissolved in 40 μl 3x SSC.
The arrays were printed on Micromax SuperChip glass slides (Perkin Elmer, Wellesley, MA, USA) using an OmniGrid 100 (GeneMachines, San Carlos, CA, USA) in a 2 x 8 printer tip configuration according to manufacturer's protocol. All clones were printed in duplicate.

Hybridization

One μg of total RNA from the samples was labeled with biotin and 1 μg of reference panel total RNA with fluorescein, generating biotin and fluorescein-labeled cDNAs with the Micromax TSA Labeling Kit (Perkin Elmer) according to the supplied protocol. Slides were hybridized with labeled cDNAs in Corning hybridization chambers (Corning, NY, USA). TSA detection was done using Shandon cover plates (Thermo Shandon, Thermo Electron Corporation, Waltham, MA, USA).

Scanning and spot analysis

Following hybridization, arrays were scanned using a GeneTac LSV scanner (Genomic Solutions, Ann Arbor, MI, USA). To collect as many data as possible from both weakly and strongly expressed genes, two images were made with a different scanning intensity (gain) for each dye.
The intensity of the fluorescent signals (Cy3 and Cy5) was collected in four different images (high and low gain) and quantified with Genepix Pro 4.1 software (Axon Instruments, Union City, CA, USA). A Microsoft Excel macro was applied that systematically selects bona fide spotted clones from Genepix data. In this macro, the spot diameter (>70 μm), signal above

background (>500 counts), saturation (<10%), and the ratio between low and high gain scans were criteria to select valid spotted clones. Signals were normalized by dividing through the median signal per array. Next, the log10 transformed Cy3/Cy5 ratio for both high and low gain scans was calculated for the valid spotted clones. Finally, the data from high and low gain scans were averaged, resulting in a single log10 ratio for each valid spot.
The PCR products of spotted clones that were identified as significantly different were sequence-verified.

Statistical analysis of microarray data
The arrays were analyzed by unsupervised hierarchical clustering and comparison of groups using the Spotfire Decisionsite™ for functional genomics (Somerville, MA, USA). Data from samples that were hybridized twice, either as duplicate or dye swap, were averaged. Normalized data were used for further analysis. Additional analysis of differential gene expression between two groups was performed with significance analysis of microarray (SAM) [22]. The global testing method was used to find differentially expressed categories of genes between two groups [23]. To correct for multiple testing, a false discovery rate (FDR) of 10% was accepted, using a step-up procedure [24].

Quantitative reverse transcriptase PCR (Q-PCR)
Nineteen samples (3 normal cartilage, 2 phalangeal enchondromas, 1 grade I, 8 grade II and 5 grade III chondrosarcomas) were used for verification of array data. Five of these (1 normal cartilage, 2 grade II and 2 grade III chondrosarcomas) had not been used previously for hybridization. One μg of total RNA was reverse transcribed with AMV Reverse Transcriptase (Roche). Primers were designed for the selected genes (Supplementary table 6.1). For each gene, a calibration curve, consisting of a mixture of eight samples, was included to allow calculation of relative starting quantities of each gene. These were used in the normalization and statistical analysis.
Candidate genes for normalization were selected from array results demonstrating the least variation between all samples (Supplementary table 6.1). Normalization was based on geometric averaging of the candidate normalization genes, as previously described [25], to acquire a reliable normalization of the Q-PCR experiments. This method provides a normalization factor (NF), representative of the amount of mRNA in each sample.

Immunohistochemistry
An intron-exon boundary is absent from *JUNB*, making the distinction between RNA and DNA difficult, if not impossible. JUNB protein expression (sc-8051, Santa Cruz Biotechnology, Inc., dilution 1:200) was tested in a large group of paraffin-embedded enchondromas and chondrosarcomas described previously [10]. 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 [9]. Osteoblasts and osteoclasts were used as a positive internal control. A sum score of more than 3 was considered positive, excluding samples with only sporadic positive cells.

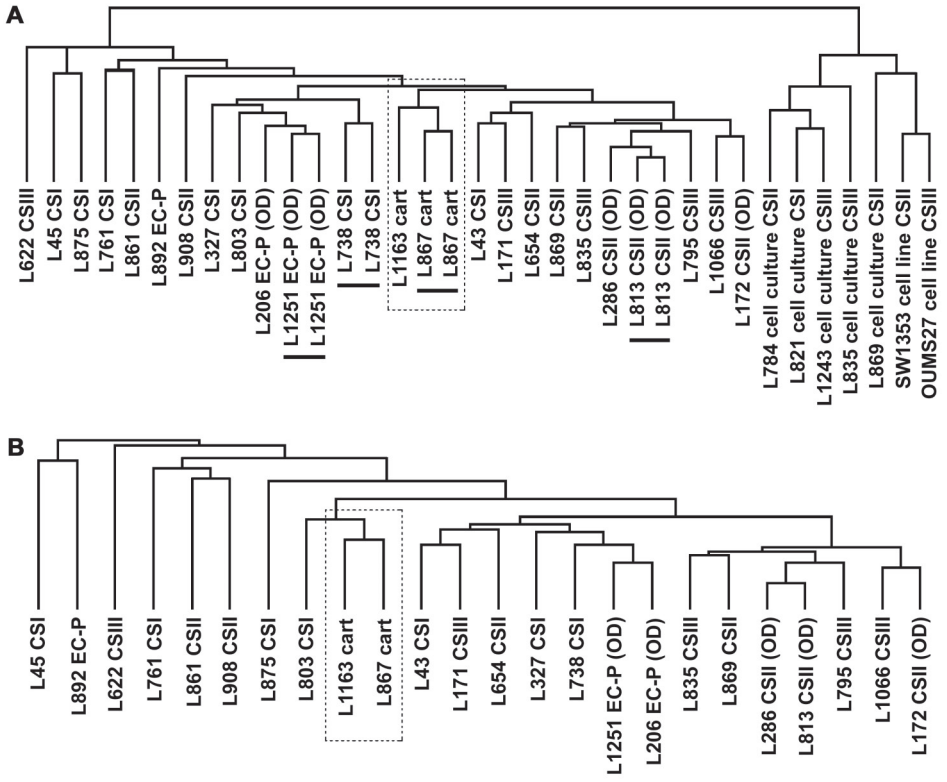

Figure 6.1*: Unsupervised hierarchical clustering of the samples. (**A**) Hierarchical clustering on the array including the samples that were hybridized in duplicate, which cluster together (black horizontal bars). The cell lines/ cultures cluster separately from the primary samples. The samples from normal cartilage also cluster together and form a separate cluster (dotted box). Note that the hybridizations of the paired solid tumor and primary cell cultures from the two samples for which these were available (L869 and L835) do not cluster together. (**B**) Hierarchical clustering of the arrays, excluding the cell lines, after averaging of the duplicate hybridizations. CS: chondrosarcoma; I: grade I; II: grade II; III: grade III; EC-P: phalangeal enchondroma; OD: Ollier disease*

RESULTS

cDNA microarray

Verification of array quality

All samples were first analyzed by unsupervised hierarchical clustering. All duplicate experiments (including dye swaps) clustered together (Figure 6.1a), indicating technical reproducibility and absence of a dye effect. Duplicate samples were averaged in further analysis. The two normal cartilage samples clustered together (Figures 6.1a and b). No distinct clusters of the tumor samples were found, indicating that more subtle changes in gene expression characterize the different histological grades. Chondrosarcoma cell lines and cultures clustered separately from primary tissues, reflecting, for instance, the decrease of

extracellular matrix and increased proliferation. The cells were, however, still of cartilaginous origin, demonstrated by low expression of cartilage-specific genes (aggrecan, collagen II and SOX9). This probably reflects the "dedifferentiation" described in cultivated articular chondrocytes [26]. Duplicate spotted clones correlated significantly with each other. These data suggest that this home-made array is potentially able to distinguish biological samples on the basis of their expression profile.
Sequencing of selected clones (~140) revealed that ~90% of the clones were correct. The other products contained the wrong sequence or PCR analysis revealed multiple bands, indicating contamination.

Ollier disease versus sporadic central chondrosarcomas
The analysis of Ollier disease-associated versus solitary tumors was initially performed using the grade II chondrosarcoma samples (n = 7, three with Ollier disease). Identified genes were subsequently analyzed in solitary and Ollier disease-related phalangeal enchondromas. SAM analysis revealed no differentially expressed genes. Group comparison (option "treatment comparison" from the Spotfire Decisionsite™ for functional genomics) with cDNAs present in at least 80% of all samples demonstrated a difference. In total, 68 genes (81 spotted clones) demonstrated a $p < 0.05$ (T-test, two-sided); 26 genes (37 spotted clones) were expressed more highly; and 42 genes (43 spotted clones) were expressed at a lower level, in Ollier disease samples (Supplementary table 6.2). For 28 of these genes (38 spotted clones, 47.5%) three phalangeal enchondromas (1 solitary and 2 Ollier disease-related) showed similar results. Amongst these were genes as *JUNB*, *MYC* and *CYR61*. For five genes (6%), this information was not available because the spotted clones did not give a bona fide signal on the arrays of phalangeal tumors. However, the differences in expression between grade II Ollier disease and solitary chondrosarcomas were not statistically significant after correction by FDR (false discovery rate).
To find pathways, we performed global testing, using gene ontology terms, revealing seven groups of genes differentially expressed (p values ranging between 0.005-0.031, Supplementary table 6.3). None was significant after correction by FDR. Thus, by microarray analysis the Ollier disease samples used here did not reveal an expression pattern that was significantly different from solitary tumors.

Low-grade versus. high-grade central chondrosarcomas
To study tumor progression, seven grade I and five grade III chondrosarcomas were compared. The most optimal SAM analysis resulted in 14 differentially expressed genes corresponding to 21 spotted clones with 1 false positive. Eleven genes (17 spotted clones) were more highly expressed and three (four spotted clones) were expressed to a lower level in grade III samples (Table 6.2). In general, grade II tumors showed an expression level for these genes intermediate to grade I and III samples. Remarkably, the phalangeal enchondromas had expression levels that were generally ranging between those of grade I and grade II chondrosarcomas. As expected, genes involved in the extracellular matrix, such as *COL9A2* (collagen, type IX,

Table 6.2: Significant differentially expressed genes found by comparison of the grade I central chondrosarcomas and the grade III central chondrosarcomas as identified by SAM analysis

				Average expression ratio				
Gene symbol	Description	Accession no/ref seq	Location	EC-P	Grade I	Grade II	Grade III	Main Gene Ontology term[a]
a) Genes more highly expressed in grade III than grade I chondrosarcoma								
ALDOA (3x)	Aldolase A	NM_000034	16q22-q24	1.04	0.39	1.42	1.62	Glycolysis
FN1	Fibronection 1	N26285	2q34	1.41	0.36	8.37	10.61	Extracellular matrix structural constituent
GAPD (4x)	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	12p13	1.49	0.57	2.03	3.54	Glycolysis
METTL1	Methyltransferase-like 1	AA422058	12q13	0.35	0.27	0.36	0.76	Methyltransferase activity
P4HB	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	R27004	17q25	4.33	1.27	2.51	5.38	Electron transport
PLAU	Plasminogen activator, urokinase	AA284668	10q24	0.45	0.05	0.33	0.45	Extracellular space
PLOD3 (2x)	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	AA459305	7q22	0.77	0.37	2.14	2.67	Protein modification
PRKCSH	Protein kinase C substrate 80K-H	AA496810	9p13.2	0.96	0.41	0.76	2.26	Protein kinase cascade
SCARB1	Scavenger receptor class B, member 1	AA443899	12q23.31	0.00	0.49	0.63	5.74	Cell adhesion
VEGF	Vascular endothelial growth factor	NM_003376	6p12	0.86	0.73	6.75	6.35	Angiogenesis
ZNF205	Zinc finger protein 205	T69522	16p13.3	0.00	0.04	0.23	2.80	Regulation of transcription, DNA-dependent
b) Genes lower expressed in chondrosarcoma grade III compared to grade I								
MTCYB (2x)	Cytochrome b	AI289238	Mitochondrium	2.59	1.91	0.86	0.34	Mitochondrial electron transport
HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	W02101	7p15	1.50	0.49	0.47	0.17	Nucleic acid binding
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	AA670438	4p14	0.05	0.23	0.06	0.01	Ubiquitin-dependent protein catabolism

ref seq: reference sequence; EC-P: enchondroma of the phalanx; [a]: further Gene Ontology terms can be found on the website: http://www.godatabase.org/cgi-bin/amigo/go.cgi

alpha 2) and *COL2A1* (collagen, type II, alpha 1) showed a trend towards down-regulation in grade III chondrosarcomas (*p = 1.5E-04* and *3.7E-10*, respectively).

MTCYB (mitochondrial cytochrome B), involved in oxidative phosphorylation (OXPHOS), was expressed to a lower level in grade III chondrosarcomas (*p = 5.9E-09*). Multiple clones from *ALDOA* (aldolase A, *n* =3) and *GAPD* (glyceraldehyde-3-phosphate dehydrogenase, *n =4*), both involved in glycolysis, were expressed more highly in grade III samples (*p = 2.6E-07* and *p = 1.2E-07*, respectively). Multiple clones of *PLOD3* (procollagen-lysine, 2-

Table 6.3: Gene Ontology (GO) terms found to differ in expression between grade I and grade III samples, as found by global testing

Gene ontology-term	No of spotted clones (genes)	*p*-value	comp *p* value	Up-regulated in
Glycolysis	70 (16)	0.0021	0.000	Grade III
Extracellular space	460 (164)	0.0034	0.016	Grade III
Nucleotide-sugar transporter activity	8 (4)	0.0038	0.004	Grade III
Regulation of cell growth	106 (48)	0.0049	0.000	Grade III
Collagen type IX	16 (3)	0.0063	0.002	Grade I
Skeletal development	284 (130)	0.0085	0.001	Grade I
Plasminogen activator activity	8 (4)	0.0090	0.004	Grade III
Mitochondrial electron transport, NADH to ubiquinone	18 (8)	0.0092	0.049	Grade III
Heparin binding	104	0.0168	0.003	Grade III
Procollagen-lysine 5-dioxigenase activity	18 (3)	0.0183	0.003	Grade III

No spotted clones: number of spotted clones present in this GO term; comp p-value: comparative p value (this calculates the chance that significance is reached when taking randomly the same number of spotted clones)

oxoglutarate 5-dioxygenase 3, $n = 2$), a matrix associated gene, showed up-regulation in grade III tumors ($p = 6.0E\text{-}06$).

Global testing, using gene ontology terms, identified 10 functional groups of genes with a *p* value ranging from 0.002 to 0.018, including " collagen type IX ", "glycolysis" and "mitochondrial electron transport, NADH to ubiquinone", a subclassification of OXPHOS (Table 6.3).

No significantly differentially expressed genes were found by SAM analysis (censored survival data) to be related with the prognosis (good [minimal 1-year follow-up, remission or recurrence] versus poor prognosis [metastasis and/or died of disease]).

Q-PCR

ALDOA, *PLOD3*, and *NDUFB8* and *NDUFS3* (involved in OXPHOS) were analyzed by Q-PCR. Five genes whose RNA were most stably expressed were selected for normalization. Using GeNorm, we showed that these genes were indeed stably expressed when analyzed with Q-PCR. Of these five genes, four (*CAPNS1*, *CPSF6*, *GRP108* and *HNRPH1*) were found to be the most stable and were therefore used for normalization. The expression as detected by Q-PCR showed the same trend as seen in the micro array (Figure 6.2). In case of *PLOD3*, a trend of up-regulation with grade was seen, corresponding to the array results ($p = 0.000$, Spearman correlation) and for *ALDOA*, higher expression in the grade II and III tumors compared to the one grade I was present, although the correlation with the array was not significant ($p = 0.6$). *NDUFB8* and *NDUFS3* demonstrated a trend for down-regulation with increasing grade, as was also seen with the array data ($p = 0.025$ and 0.1, respectively, Spearman correlation; Figure 6.2).

Immunohistochemistry

JUNB immunohistochemistry was performed since a trend for higher expression in Ollier disease compared with solitary tumors was found. In total, 89 samples were evaluable, 36 of

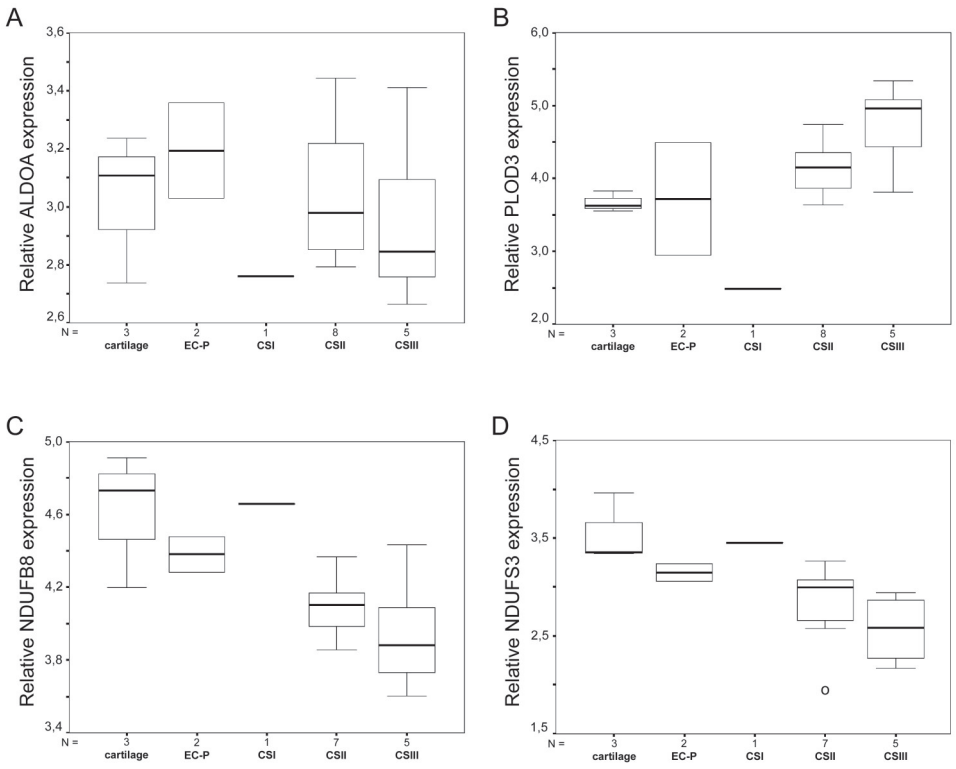

***Figure 6.2**: Box plots of the relative RNA expression measured by Q-PCR. Relative log10 expression of ALDOA (**A**), PLOD3 (**B**), up-regulated in high-grade tumors and, NDUFB8 (**C**) and NDUFS3 (**D**) both downregulated in high-grade tumors, per subgroup of cartilage, enchondroma of the phalanx (EC-P), chondrosarcoma grade I (CS I), chondrosarcoma grade II (CS II), and chondrosarcoma grade III (CS III)*

which were positive (Ollier disease–related enchondromas 2 of 6, chondrosarcomas 3 of 7; solitary enchondromas 3 of 21, chondrosarcomas 28 of 55).

cDNA array results were be confirmed by immunohistochemistry evaluating only those samples used for cDNA array ($p = 0.010$, Spearman correlation and $p = 0.014$, *T*-test, Ollier disease-related and solitary grade II chondrosarcomas). However, further analysis of the whole group revealed no difference in expression ($p = 1.0$, Fisher's exact test, two-sided).

Interestingly, JUNB expression was significantly lower in non-phalangeal enchondromas [1 of 12 (8%) positive) as compared to non-phalangeal chondrosarcomas (27 of 53 (51%) positive] (all three grades, $p = 0.009$, Fisher's Exact test, two-sided) and also as compared to chondrosarcomas grade I (10 of 20 (50%) positive) ($p = 0.023$, Fisher's Exact test, two-sided) (Figure 6.3). No significant difference in expression was detected between the different grades of chondrosarcoma ($p = 0.45$, chi square, linear by linear).

DISCUSSION

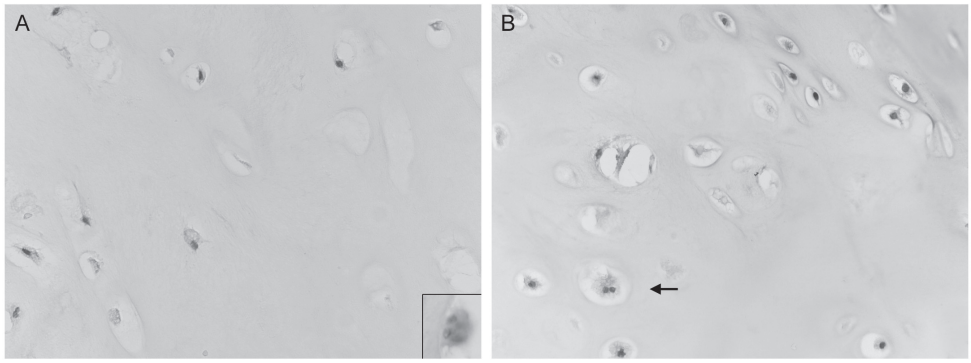

***Figure 6.3**: JUNB protein expression in enchondromas and central chondrosarcomas. JUNB immunohistochemical (nuclear) staining demonstrates JunB expression to be absent or very low expressed in enchondromas (**A**), with positive internal controls (inset). Chondrosarcomas were positive in about 50% of the cases (**B**, grade I chondrosarcoma, with arrow pointing to double nucleus). Original magnification 40x. Color picture can be viewed at page 130*

So far, few data are available on the gene expression patterns of conventional central chondrosarcomas. Expression array analyses have been described [20,27] using arrays containing genes from libraries other than cartilaginous tissue. We have designed an array containing 8696 cDNA clones of which ~500 genes are known to be expressed in cartilage. We previously developed a robust RNA isolation method that enables us to obtain adequate amounts of RNA from sometimes pauci-cellular cartilaginous tissue to perform cDNA microarray analysis [20]. Because only limited amounts of RNA can be extracted from benign and low-grade tumors, due to low cellularity and high amounts of matrix, the hybridization signal was amplified with Tyramide signal amplification, enabling the use of 1 ìg of total RNA. Despite these adaptations, sufficient RNA could not be obtained from non-phalangeal enchondromas, excluding the possibility to investigate gene expression differences related to malignant transformation of enchondroma to chondrosarcoma.

In a previous cDNA microarray study on commercially available arrays, we compared central and peripheral chondrosarcoma, revealing, amongst other things, overexpression of ribosomal genes in central chondrosarcomas and overexpression of immunoglobulins in peripheral chondrosarcomas [20]. Others, studying a larger group of conventional chondrosarcomas, showed that these tumors were heterogeneous [27]. Comparing tumors to normal fetal cartilage, they observed up-regulation of genes related to cell adhesion and motility, both up- and down-regulation of growth factors, and down-regulation of DNA damage repair and developmental regulators [27]. To our knowledge, this is the first study relating expression profiles of central chondrosarcomas to histological grade and Ollier disease.

Comparison of our array data with immunohistochemistry data from this and previous studies confirmed the quality of our array. JUNB protein expression significantly correlated with the array results. Cyclin D1 and CDKN1A (p21) immunohistochemistry previously revealed no difference between the different groups [10], and similar results were obtained using the arrays. cDNA levels of *p53*, for which protein expression (and the presence of mutations)

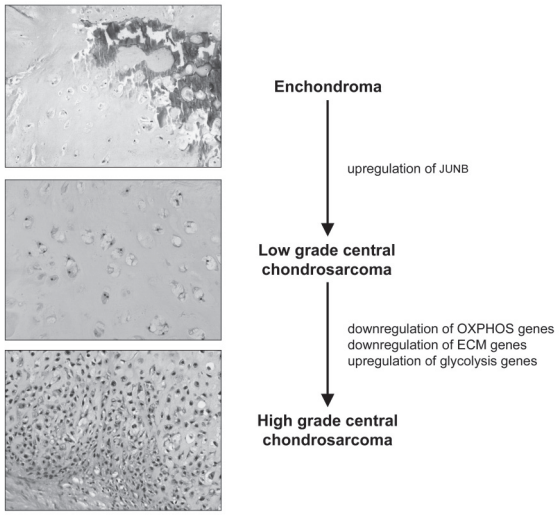

Figure 6.4: *Summary of the most important results. Enchondromas are characterized by the production of hyaline cartilage. Upon malignant transformation, the lesion becomes slightly more cellular, with binucleate cells and sometimes muco-myxoid matrix changes and host bone entrapment. The tumors have little vascularisation. The present study demonstrates up-regulation of the expression of JUNB in low-grade chondrosarcoma, compared with enchondroma. The progression of low- to high-grade (grade II and III) chondrosarcoma is marked by increased cellularity, increased nuclear atypia, decreased amounts of extracellular matrix, and increased vascularisation. Gene expression profiles show changes not only in extracellular matrix, but also in the metabolism of tumor cells. Original magnification 20x*

was increased in high-grade tumors [11,28], were similar in all grades. This discrepancy can be explained by the fact that p53 is regulated on protein level, by MDM2 [29,30], and therefore differential expression on RNA level is not expected. Unfortunately, for genes involved in the IHH and PTHLH signaling pathways, as well as CDKN2A (p16), for which immunohistochemical results were reported previously [10,13], no data were available, because the RNA levels of these signalling molecules are very low in our assay. The quantitative RT PCR of the present study also confirmed the array data.

We investigated if the same pathways were affected in solitary and Ollier disease-related tumors. Within the limitations of the small sample size available for this extremely rare syndrome, the biology of Ollier disease-related tumors could not be distinguished from solitary tumors, indicating only subtle differences underlie the more indolent clinical behavior [3,4] and overall higher risk of malignant transformation of Ollier disease-related enchondromas [1]. To unravel any pathway pointing to the etiology of this rare non-hereditary syndrome, larger cohorts of patients and more specific knowledge of chondrosarcomagenesis seem necessary. One of the genes that showed a trend, although not significant, towards higher expression in Ollier disease was *JUNB*, a subunit of the AP-1 transcription factor family, which is implicated in chondrogenic differentiation [31]. JUNB is expressed in normal growth plates [32] and *JunB* knockout mice show reduced proliferation of growth plate chondrocytes and osteoblasts [32]. Up-regulation of JUNB could have explained the more worrisome histological features in Ollier disease. However, no difference was found between 13 Ollier

disease-related and 76 solitary samples, suggesting that JUNB was identified as differentially expressed by chance because of multiple testing.
Interestingly, the expression of JUNB protein was significantly lower in 12 enchondromas compared with 20 grade I central chondrosarcomas, which is in line with its stimulatory effect on chondrocyte proliferation [32,33]. This suggests that JUNB is important in malignant transformation of enchondroma, making it an excellent candidate as a diagnostic marker for malignancy (Figure 6.4).
Cartilaginous tumors of the phalanx are considered a separate subgroup since worrisome histological features are tolerated in phalangeal enchondroma, and phalangeal chondrosarcoma, being extremely rare at this localization, has a favorable prognosis [1,18]. We have shown the gene expression levels of phalangeal enchondroma to range between those of grade I and grade II chondrosarcomas located elsewhere. Unfortunately, frozen material from phalangeal chondrosarcomas was not available for comparison.
To elucidate pathways involved in tumor progression, we compared grade I and grade III chondrosarcomas. Remarkably, no cell cycle genes were identified in this comparison, as would be expected based upon the higher mitotic rate of grade III chondrosarcomas compared with grade I chondrosarcomas. However, we previously found no significant difference in protein expression between grade I and grade III chondrosarcomas for some of the proteins involved in the cell cycle, such as cyclin E and cyclin D1 [10], comparable to what is seen in the array experiment. As could be expected, several matrix-associated genes were down-regulated in grade III chondrosarcomas. This reflects their histology since high-grade chondrosarcomas are more cellular, with less matrix with mucoid and/or myxoid appearance [1,34] (Figure 6.4). In contrast, enchondromas and grade I chondrosarcomas histologically resemble normal hyaline cartilage, a tissue that lacks blood vessels and is highly hypoxic. In high-grade chondrosarcomas increased micro-vascularisation is seen [35,36] (Figure 6.4). In the present study, we have shown up-regulation of glycolysis and down-regulation of oxidative phosphorylation (OXPHOS) in high-grade chondrosarcomas. Increased glycolysis is described in several cancer types [37,38], and even suggested to be a hallmark of invasive tumors [39]. The up-regulation of anaerobic glycolysis in the hypoxic (ie low-grade) situation is hypothesized to give a growth advantage [39]. With increased vascularisation (ie high-grade), more oxygen is available to the cells and the use of the glycolytic pathway is not down-regulated, but most likely changed from anaerobic to aerobic [39]. The down-regulation of the OXPHOS may result from the increased glycolysis and can be reversed in some cases [40]. Other explanations for the up-regulation of glycolysis and down-regulation of the OXPHOS could be the need for products produced in the glycolysis, the inability to store glucose in the cancer cells, failure of the OXPHOS complex, or to decrease the amount of reactive oxygen species that are can induce DNA damage. Hif-1α (hypoxia-inducible factor 1α) could be important in chondrosarcoma progression since it can induce both up-regulation of glycolysis [37] and vascular in-growth. However, conflicting results are reported regarding the up- and down-regulation of this protein in chondrosarcomas [41,42] and no data could be retrieved from our array. As a consequence of glycolysis, lactate is formed, resulting in microenvironmental acidosis being toxic to cells, resulting in necrosis or apoptosis through

p53- and caspase-3-dependent mechanisms [43,44]. In chondrosarcomas, the anti-apoptotic protein BCL2 is up-regulated [9,10], which may be required to escape the acidosis-induced apoptosis.

In conclusion, we have shown that chondrosarcomas from patients with Ollier disease and solitary chondrosarcomas display similar gene expression profiles, suggesting that the same pathways are involved in histogenesis. JUNB was expressed significantly more highly in chondrosarcomas than in enchondromas, suggesting it to be a good diagnostic marker for malignancy. Progression of chondrosarcomas towards a higher grade is accompanied by up-regulation of glycolysis and down-regulation of oxidative phosphorylation genes suggesting that adaptation to the energy supply of tumor cells is related to chondrosarcoma progression.

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Supplementary table 1: Primers and conditions for the genes used for Q-PCR

Symbol	Accession nr	Forward	Reverse	Product length (bp)	$MgCl_2$
SRPR[a]	NM_003139	CATTGCTTTTGCAC GTAACCAA	ATTGTCTTGCATGC GGCC	70	3 mM
CPSF6[a]	NM_007007	AAGATTGCCTTCAT GGAATTGAG	TCGTGATCTACTAT GGTCCCTCTCT	89	5 mM
GPR108[a]	XM_290854	AGATGCCCCTTTTC AAGCTCTAC	GCCATGAGCCAGTG GATCTTG	122	2 mM
CAPNS1[a]	NM_001749	ATGGTTTTGGCATT GACACATG	GCTTGCCTGTGGTG TCGC	66	2 mM
HNRPH1[a]	NM_005520	GATGTAGCAAGGA AGAAATTGTTCAG	CACCGGCAATGTTA TCCCAT	74	2 mM
ALDOA	NM_000034	GTATGTCAAGCGAG CCCTGG	TAGAGACGAAGAG GGACTCGC	101	3 mM
PLOD3	NM_001084	CCCGAGTGTGAGTT CTACTTCAG	GGGCGATCACCTTC CTGTTC	100	3 mM
NDUFB8	NM_005004	CGGTTTCCTGGCTT TCATGATATT	TAGTGAACCACCCG CTCTGG	150	4 mM
NDUFS3	NM_004551	GGCTTCGAGGGACA TCCTTTC	CGGAACTCTTGGGC CAACTC	119	4 mM

[a]: genes that were used as candidate normalization genes

Supplementary table 2: List of the differently, but not significant, expressed genes found in the comparison of solitary to Ollier disease-related grade II central chondrosarcomas

Gene symbol	Description	Accession no/ref seq	Location	T-test	Main Gene Ontology term[a]
a) Genes lower expressed in Ollier disease-related grade II chondrosarcoma compared to solitary grade II chondrosarcomas					
[1]Unknown		AK022464		0.0141	
[1]APTX	Aprataxin	R43471	9p13.3	0.0144	
[1]CDC27	Cell division cycle 27	AA489324	17q12-17q23.2	0.0204	Cell proliferation
[2]CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	AA421819	5p14-15.1	0.0083	Cell adhesion
[2]COPS7A	COP9 constitutive photomorphogenic homolog subunit 7A (Arabidopsis)	AA455119	12p13.31	0.0383	
[1]COPZ1	Coatomer protein complex, subunit zeta 1	AA486228	12q13.2-q13.3	0.0307	Intracellular protein transport;
[1]COX6C	Cytochrome c oxidase subunit VIc	NM_004374	8q22-q23	0.0355	Cytochrome-c oxidase activity
[3]CRYZ	Crystallin, zeta (quinone reductase)	R13434	1p22-p31	0.0112	NADPH:quinone reductase activity
[1]CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	NM_003798	9q31.2	0.0011	Apoptosis
[2]DCC1	Defective in sister chromatid cohesion homolog 1 (S. cerevisiae)	AA843451	8q24.12	0.0362	
[3]DPEP2	Dipeptidase 2	AA451861	16q22.1	0.0453	Proteolysis and peptidolysis
[3]EWSR1	Ewing sarcoma breakpoint region 1	R32756	22q12.2	0.0117	cell growth and/or maintenance
[3]FGA	Fibrinogen, A alpha polypeptide	AA011414	4q28	0.0345	Fibrinogen complex
[1]FKBP11	FK506 binding protein 11, 19 kDa	N72137	12q13.12	0.0253	Protein folding
[3]GA17	Dendritic cell protein	AA101348	11p13	0.0090	
[3]GP9	Glycoprotein IX (platelet)	AA701315	3q21	0.0221	Protein binding
[3]HBXIP	Hepatitis B virus x interacting protein	AI218994	1p13.3	0.0034	
[3]IL13RA1	Interleukin 13 receptor, alpha	AA411324	1 Xq24	0.0475	Cell surface receptor linked signal transduction
[3]KIAA0683	KIAA0683 gene product	NM_016111	16p13.3	0.0376	
[3]KIAA1049	KIAA1049 protein	NM_014972	16q24.3	0.0160	
[2]MAML1	Mastermind-like 1 (Drosophila)	R37148	5q35	0.0077	
[1]MAPRE1	Microtubule-associated protein, RP/EB family, member 1	AA922700	20q11.1-11.23	0.0003	Regulation of cell cycle
[3]MGC20262	Hypothetical protein MGC20262	W74293	9q34.3	0.0144	
[3]MGC2198	Hypothetical protein MGC2198	H81199	5q35.2	0.0282	
[1]MRPL4	Mitochondrial ribosomal protein L4	AA490981	19	0.0128	Protein biosynthesis
[1]NASP	Nuclear autoantigenic sperm protein (histone-binding)	AA644128	1p34.1	0.0040	DNA packaging
[3]NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	AI096694	10q23.2-q23.33	0.0121	Mitochondrial electron transport, NADH to ubiquinone
[1]NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	AA629687	2q31	0.0289	Regulation of transcription, DNA-dependent
[3]NQO1	NAD(P)H dehydrogenase, quinone 1	NM_000903	16q22.1	0.0305	NAD(P)H dehydrogenase (quinone) activity

Supplementary table 2, continued

Gene symbol	Description	Accession no/ref seq	Location	T-test	Main Gene Ontology term[a]
a) Genes lower expressed in Ollier disease-related grade II chondrosarcoma compared to solitary grade II chondrosarcomas, continued					
[3]OS-9	Amplified in osteosarcoma	AA013336	12q13	0.0422	
[1]PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	NM_002576	11q13-q14	0.0023	Apoptosis
[3]PFKM	Phosphofructokinase, muscle	AA099169	12q13.3	0.0480	
[3]PTOP	POT1 and TIN2 organizing protein	NM_022914	16q22.1	0.0006	
[2]PUS1	Pseudouridylate synthase 1	AA496794	12q24.33	0.0260	Lyase activity
[3]RPS6	Ribosomal protein S6	N91584	9p21	0.0054	Protein biosynthesis
[3]SH2BP1 (2x)	SH2 domain binding protein 1 (tetratricopeptide repeat containing)	AA133684	11p15.3	0.0083	
[3]SRPR	Signal recognition particle receptor (‘docking protein’)	AA598621	11q24.3	0.0418	Signal recognition particle receptor complex
[3]STAT1	Signal transducer and activator of transcription 1, 91kDa	AA486367	2q32.2	0.0332	Caspase activation
[3]TFPI2	Tissue factor pathway inhibitor 2	AA399473	7q22	0.0294	Extracellular matrix
[1]UNG	Uracil-DNA glycosylase	H15111	12q23-q24.1	0.0290	Base-excision repair
[3]VEGFC	Vascular endothelial growth factor C	H07991	4q34.1-q34.3	0.0237	Angiogenesis
[3]ZNF43	Zinc finger protein 43 (HTF6)	AA773894	19p12-p13.1	0.0432	Regulation of transcription, DNA-dependent
b) Genes more highly expressed in Ollier disease-related grade II chondrosarcoma than solitary grade II chondrosarcomas					
[1]unknown		AA496863		0.0084	
[3]ADAMTS6	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 6	AA400393	5	0.0151	Extracellular matrix
[1]RHOD	Ras homolog gene family, member D	AA143436	11q14.3	0.0382	GTP binding
[3]BMP4	Bone morphogenetic protein 4 1	AA463225	4q22-q23	0.0181	Skeletal development
[3]CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	6p21.2	0.0382	Cell cycle arrest
[3]CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	AA284072	14q22	0.0465	Cell cycle arrest
[1]CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	AA442092	3p21	0.0064	Wnt receptor signaling pathway
[1]CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	W42723	4q21	0.0065	Cell proliferation
[1]CYR61 (4x)	Cysteine-rich, angiogenic inducer, 61	AA777187	1p31-p22	0.0242	Cell proliferation
[3]GSTM2	Glutathione S-transferase M2 (muscle)	AA290737	1p13.3	0.0492	
[3]GTF2B	General transcription factor IIB	H23978	1p22-p21	0.0165	Regulation of transcription, DNA-dependent
[1]JUNB (4x)	Jun B proto-oncogene	N94468	19p13.2	0.0143	Transcription factor activity
[1]KAL1 (2x)	Kallmann syndrome 1 sequence	H17882	Xp22.32	0.0236	Extracellular matrix structural constituent

Supplementary table 2, continued

Gene symbol	Description	Accession no/ref seq	Location	T-test	Main Gene Ontology term[a]
b) Genes more highly expressed in Ollier disease-related grade II chondrosarcoma than solitary grade II chondrosarcomas, continued					
[4]MYC (3x)	v-myc myelocytomatosis viral oncogene homolog (avian)	AA464600	8q24.12-q24.13	0.0237	cell proliferation
[1]PDPR (2x)	Pyruvate dehydrogenase phosphatase regulatory subunit	AL117440	16q22.1	0.0385	Amino-methyl-transferase activity
[1]PHB	Prohibitin	AA055656	17q21	0.0345	cell growth and/or maintenance
[3]PTMA	Prothymosin, alpha (gene sequence 28)	H48420	2q35-q36	0.0338	Regulation of cell cycle
[1]RAB2 (2x)	RAB2, member RAS oncogene family	T82414	8q12.1	0.0091	Intracellular protein transport
[3]RFC4	Replication factor C (activator 1) 4, 37kDa	H54752	3q27	0.0245	DNA replication
[3]SAH	SA hypertension-associated homolog (rat)	W01011	16p13.11	0.0204	Metabolism
[2]SLC39A6	Solute carrier family 39 (zinc transporter), member 6	H29407	18q12.2	0.0368	
[1]SNAPC3	Small nuclear RNA activating complex, polypeptide 3, 50kDa	AA043334	9p22.3	0.0443	Regulation of transcription, DNA-dependent
[1]TINP1	TGF beta-inducible nuclear protein 1	AA172048	5q13.3	0.0172	Protein biosynthesis
[1]TRAF4	TNF receptor-associated factor 4	AA598826	17q11-q12	0.0322	Apoptosis
[1]THRAP1	Thyroid hormone receptor associated protein 1	AA457462	17q22-q23	0.0469	Thyroid hormone receptor binding
[3]TUBB4	Tubulin, beta, 4	NM_006086	16q24.3	0.0076	Structural constituent of cytoskeleton

ref seq: reference sequence
[a] further GO terms can be found on the website: http://www.godatabase.org/cgi-bin/amigo/go.cgi
[1] phalangeal enchondromas showed same trend of lower or higher expression in Ollier disease tumors
[2] no data available for the solitary phalangeal
[3] enchondroma phalangeal enchondromas did not show the same trend of lower or higher expression in Ollier disease tumors
[4] phalangeal enchondromas showed higher expression in Ollier disease tumors for 2 spotted clones, and lower expression for 1 spot

Supplementary table 3: Gene Ontology (GO) terms found not to differ significantly in expression between Ollier disease and solitary grade II samples, as found by global testing. None of the groups of differentially expressed genes was significant after correction by FDR (see text of main paper)

Gene Ontology term	No of spotted clones (genes)	*p*-value	Comp *p* value	Up-regulated in
Translation factor activity, nucleic acid binding	98 (38)	0.0052	0.034	Ollier disease
Cell cycle arrest	116 (35)	0.0108	0.000	Ollier disease
Iron ion homeostasis	38 (9)	0.0117	0.002	Ollier disease
Axon guidance	22 (11)	0.0179	0.024	Ollier disease
Chemokine activity	62 (23)	0.0221	0.018	Ollier disease
Hemostasis	104 (42)	0.0191	0.007	Ollier disease
Regulation of CDK activity	76 (22)	0.0308	0.018	Ollier disease

No spotted clones: number of spotted clones present in this GO term; comp *p* value: comparative *p* value (this calculates the chance that significance is reached when taking randomly the same number of spotted clones)

Chapter 7

Array-CGH of central chondrosarcoma: identification of RPS6 and CDK4 as candidate target genes for genomic aberrations

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Submitted

Enchondromas are benign lesions, which can occur solitary or multiple (Ollier disease) and could be precursors of central chondrosarcomas. Recurrences of chondrosarcoma can be of higher grade compared to the primary tumor, suggesting possible progression. Tumor specific genomic abnormalities are unknown so far. Genome-wide array-CGH (comparative genomic hybridization) was used to investigate copy number changes in enchondromas and central chondrosarcomas to elucidate both primary genetic events, and those related to tumor progression. ANOVA, T-test and hierarchical clustering were used for analysis. Array-CGH data were compared to cDNA expression array data. No genomic imbalances specific for Ollier disease were found. Genomic imbalances were rare in enchondromas and grade I chondrosarcomas, while frequently in high-grade tumors. We identified 22 chromosome regions, imbalanced in ≥ 25% of tumors, three of which located on chromosome 12 (12p13, 12p11.21-p11.23 and 12q13, containing amongst other the gene *PPFIBP1* [PTPRF interacting protein, binding protein 1]). Loss of chromosome 6 and gain of 12q12 were associated with higher grade. Loss of 4q13, 4q34, 10 and gain of 9q34 correlated with adverse prognosis, and higher grade. Comparison of array-CGH with cDNA expression showed correlation for *RPS6* (ribosomal protein S6) and *CDK4* (cyclin-dependent kinase 4). We identified genomic regions and new candidate genes (*RPS6*, *CDK4* and *PPFIBP1*) associated with tumor progression and prognosis in high-grade chondrosarcomas.

Chondrosarcoma of bone is a slowly growing malignant tumor characterized by the formation of cartilage. The tumors have an equal sex incidence and principally occur in adults of 30–60 years. Chondrosarcomas are found most frequently in the long bones (33%), pelvic bones (27%), and in ribs and scapula (10%). The majority of chondrosarcomas (~83%) arise centrally within the medullary cavity of bone and are called primary conventional central chondrosarcomas, or secondary central chondrosarcomas if they develop from a pre-existing enchondroma [1]. The latter can be solitary or multiple in the context of Ollier disease (enchondromatosis). A minority (~17%) of chondrosarcomas are subclassified as secondary peripheral, developing at the surface of bone within the cartilaginous cap of a documented pre-existing osteochondroma [2,3]. Conventional central and secondary peripheral chondrosarcoma share similar cytonuclear features and three grades of malignancy are discerned [4], correlating with the occurrence of metastases and survival [1]. However, there is clear evidence for genetic differences between central and peripheral chondrosarcoma [5,6].

In few publications only the distinction between central and secondary peripheral chondrosarcomas is made. As shown by DNA flow cytometry central chondrosarcomas are predominantly near-diploid [6], whereas peripheral chondrosarcomas are aneuploid [5-8]. Loss of heterozygosity (LOH), classic CGH and karyotyping show a broad range of presumably mostly random genomic alterations in high grade cases with some indications that chromosome 9 is more often affected [6,8-10].

Cytogenetic data of studies in which no distinction was made between central and peripheral chondrosarcomas revealed several aberrations in recurrent chromosomal regions (reviewed by Sandberg and Bridge [10]) of which some, e.g. the loss of 13q, were reported to have a prognostic impact [11]. Enchondromas show mainly a normal karyotype [10,12].

Several genes, such as *TP53* and *CDKN2A/p16*, have been tested in central chondrosarcomas for the presence of mutations. The *TP53* gene on chromosome 17p13 was found to be deleted or mutated in some chondrosarcomas, mainly in high-grade tumors [13-17]. *CDKN2A*, located on chromosome 9p21, has been studied extensively in central chondrosarcomas. Although cytogenetics, CGH and LOH point to the 9p21 region as an important candidate locus for central chondrosarcoma development [6,8,9], mutations and methylation (a mechanism of gene downregulation) of the *CDKN2A* gene combined with absent p16 protein expression is found in only a subset of mainly high grade central chondrosarcomas [18-20].

For enchondromas in the context of Ollier disease the overall percentage of malignant transformation is much higher. It has been estimated at 25-30% per patient with Ollier disease compared to less than 1% for patients with solitary enchondromas [1,21]. Genetic data on patients with Ollier disease are sparse. LOH was identified at 13q14 and 9p21 [22], whereas cytogenetic studies revealed no [8] or only one alteration per tumor, i.e., either deletion of 1p [23] or an inversion on chromosome 9 [8]. A mutation in 2 out of 6 patients with Ollier disease was reported in the *PTHR1* gene [24], although another study on 31 patients with Ollier disease could not confirm this [25].

In order to further identify genomic alterations we performed a genome wide screen by high-resolution array-CGH on solitary and Ollier disease-related enchondromas and conventional

central chondrosarcomas. We looked for genomic alterations, specific for central chondrosarcoma and Ollier disease, and those related with tumor progression and prognosis. The results were compared to expression array data. Tumors from patients with Ollier disease were included to identify putative genetic changes specific for this syndrome.

MATERIAL AND METHODS

Samples

Fresh frozen enchondroma and conventional central chondrosarcoma samples were collected from the archives of the department of Pathology of Leiden University Medical Center. All samples were either primary conventional chondrosarcoma, or secondary to a radiological longstanding documented enchondroma. No samples originating from recurrent tumors were used. In total, 21 tumor samples (three enchondromas, seven grade I, seven grade II and four grade III chondrosarcomas) were used (Table 7.1). One of these patients was previously diagnosed with a breast carcinoma [26]. Patient data were obtained by review of clinical charts and radiographs. All but one patients were operated by one othopaedic oncologist (A.H.M.T.). Grading was performed according to Evans *et al.* [4]. All samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines "Code for Proper Secondary Use of Human Tissue in The Netherlands" (Dutch Federation of Medical Scientific Societies).

Table 7.1: Clinico-pathological data of the samples used in genomic array analysis

Sample	M/F	Diagnosis	Ollier disease	Location	Size (cm)	Follow up (months; outcome)
L206	F	EC	Yes	Phalanx	1x0.3x0.3	42; remission
L1251	M	EC	Yes	Phalanx	max diam 1.3	8; recurrence
L892	M	EC	No	Phalanx	?	2; remission, lfu
L185	F	C-CS I	No	Femur	max diam 1	101; remission
L321	M	C-CS I	No	Femur	7x2.6x3.2	54; remission
L738	F	C-CS I	No	Humerus	5.9x2.5x3.4	59; remission
L761	M	C-CS I	No	Femur	max diam 2.5	16; remission
L803	F	C-CS I	No[a]	Femur	4.5x2.5x2	28; remission, doc
L853	F	C-CS I	No	Humerus	3x3x3	31; remission
L1212	F	C-CS I	No	Humerus	6.5x5x6	16; remission
L172	M	C-CS II	Yes	Scapula	max diam 4	7; remission, lfu
L130	M	C-CS II	No	Rib	12x8x3.5	23; recurrence
L646	F	C-CS II	No	Femur	11x2.5	72; remission
L654	M	C-CS II	No	Fibula	2.5x3.5x4.5	17; recurrence
L813	M	C-CS II	Yes	Humerus	?	2; remission, lfu
L861	M	C-CS II	No	SI joint	max diam 1	1; metastasis, dod
L908	M	C-CS II	No	Humerus	max diam 4.8	30; remission
L171	M	C-CS III	No	Humerus	13x10x9	6; recurrence, dod
L795	M	C-CS III	No	Scapula	11x9x6.5	8; recurrence
L903	F	C-CS III	No	Femur	21x5.5x4.2[b]	32; recurrence
L1066	M	C-CS III	No	Humerus	20.3x14	16; metastasis

M/F: male/female; EC: Enchondroma; C-CS: conventional central chondrosarcoma; I: grade I; II: gradeII; III: grade III; [a] patient also had a breast carcinoma [26]; max diam: maximal diameter of tumor; ?: unknown size; [b]: contaminated margin; lfu: lost to follow up; doc: died of other causes; dod: died of disease. Of the underlined samples cDNA expression data are available [32].

DNA isolation

For DNA isolation, we used samples containing at least 70% of tumor cells, as estimated from analyses of haematoxylin and eosin stained frozen sections. Four samples were microdissected to enrich the tumor percentage [5]. The DNA was isolated using a Wizard genomic DNA purification kit (Promega Benelux, Leiden, The Netherlands), according to the manufacturer instructions. The concentration of the DNA was quantified spectrophotometrically and the DNA fragment sizes were determined on a 0.7% agarose gel. Our samples were hybridized against a sex-matched control DNA pool created from >10 normal blood DNAs (Promega).

Array-CGH

The 1 Mb resolution arrays were constructed using a BAC/PAC clone set provided by the Welcome Trust Sanger Institute (UK), and information regarding the full set is available at the Sanger Center mapping database site, Ensembl (http://www.ensembl.org/). The array production, hybridization and image acquisition procedures were performed as previously described by us [27]. In short, all samples were labeled with Cy3-dCTPs and hybridized on a the slides together with Cy5-dCTPs labeled reference DNA. Hybridized slides were scanned with an Agilent DNA microarray scanner.

Data analysis

Spot intensities were measured by GenePix Pro 4.1 software. Those that did not fulfill quality criteria were excluded, namely: spots with low intensity (below five times the average of the background), or non-similar values among replicates [27]. Further analysis and averaging of the triplicate spotted clones was performed using home made Excel algorithms [27] and CGH Analyzer MeV (University of Pennsylvania, Abramson Cancer Research Institute, Weber Lab)[28]. The determination of significant copy number changes detected in array-CGH for tumor samples, where a substantial part of the genome is altered, is not straightforward. The identification of breakpoints and the determination of the true copy number values is problematic due to aneuploidy and admixture of non-tumor cells. To facilitate and standardize the data analysis aCGH-Smooth was used [29]. Clones that showed imbalances also in normal controls were considered polymorphic [30], and were excluded from further analysis.

FISH (Fluorescent In Situ Hybridization)

Interphase FISH was performed to confirm the imbalance in tumor, and exclude its presence in the constitutive line of this patient. Nuclei from paraffin embedded normal and tumor tissue were isolated from sample L803 as described previously [31]. Three probes were selected, two located within the amplified region, and one immediately adjacent to this region. The DNA used as probes in the FISH experiments represented aliquots of the same amplified DNA used to spot the arrays. DNAs were directly labeled by nick translation with fluorochrome conjugated d-UTPs, and hybridized according to standard protocols.

Correlation of genomic alterations and expression levels

Expression array data were available for 13 of the 21 tumors studied [32]. RNA from these tumors was hybridized to a cDNA array containing 8696 cDNA clones, representing common genes and a selection of genes (~500) known to be expressed in cartilage, growth plate or involved in carcinogenesis in general. Most of these genes were selected from the human ResGen 40K bank (Research Genetics, Invitrogen, Huntsville, AL, USA), others were ordered from DKFZ (RZPD, Berlin, Germany) or custom made. The clones were printed in duplicate on Micromax SuperChip glass slides (Perkin Elmer, Wellesley, MA, USA) and 1 mg of total RNA from the samples was labeled with biotin and one mg of reference panel total RNA was labeled with fluorescein generating biotin and fluorescein labeled cDNAs with the Micromax TSA labeling kit (Perkin Elmer, Wellesley, MA, USA) according to the supplied protocol.

Of the SRO's (smallest region of overlap), array GCH and cDNA-array data were compared, to relate gene expression effects to DNA copy number alterations.

RESULTS

Array-CGH

After hybridization to the BAC arrays, 95-99% of the spots fulfill the quality criteria [27] and were used in the analysis. In 10 cases with statistically significant copy number alterations the samples showed with high normal cell contamination, as was reported previously [6], despite the efforts to enrich the tumor percentage by micro dissection in some cases. In these cases extra adjustments (aCGH-Smooth) were applied to be able to input data to MeV software package (samples L761, L1212, L172, L654, L813, L861, L908, L795, L903, and L1066).

Genomic alterations were mainly found in the high-grade samples. No DNA copy number alterations were found in the samples 2-6 and 8 (two enchondromas and four grade I chondrosarcomas). Overall, grade III chondrosarcomas showed the largest number of DNA imbalances (enchondromas average 8 imbalances, range 4-11; grade I chondrosarcomas average 18 imbalances, range 3-26; grade II chondrosarcomas average 28 imbalances, range 9-39; and grade III chondrosarcomas average 43 imbalances, range 26-74) (Figure 7.1). DNA copy number alterations that show an overlapping region present in at least five different samples (smallest region of overlap [SRO]) and comprising at least 3 adjacent clones, are given in Table 7.2.

Analysis of genomic alterations in Ollier disease-related tumors

In order to identify possible Ollier disease specific alterations, we looked at the alterations found in the four samples with Ollier disease (2 phalangeal enchondromas and 2 grade II chondrosarcomas). Of the 2 phalangeal enchondromas one revealed no alterations, whereas the other sample showed loss of complete chromosome 6. Two grade II chondrosarcomas showed more alterations, of which one showed gain of almost the entire chromosomes 2, 5, 8, 15, 19, 20, 21 and 22 and gain of parts of the chromosomes 1, 5, 7, 9, 16, 17 and 18. The

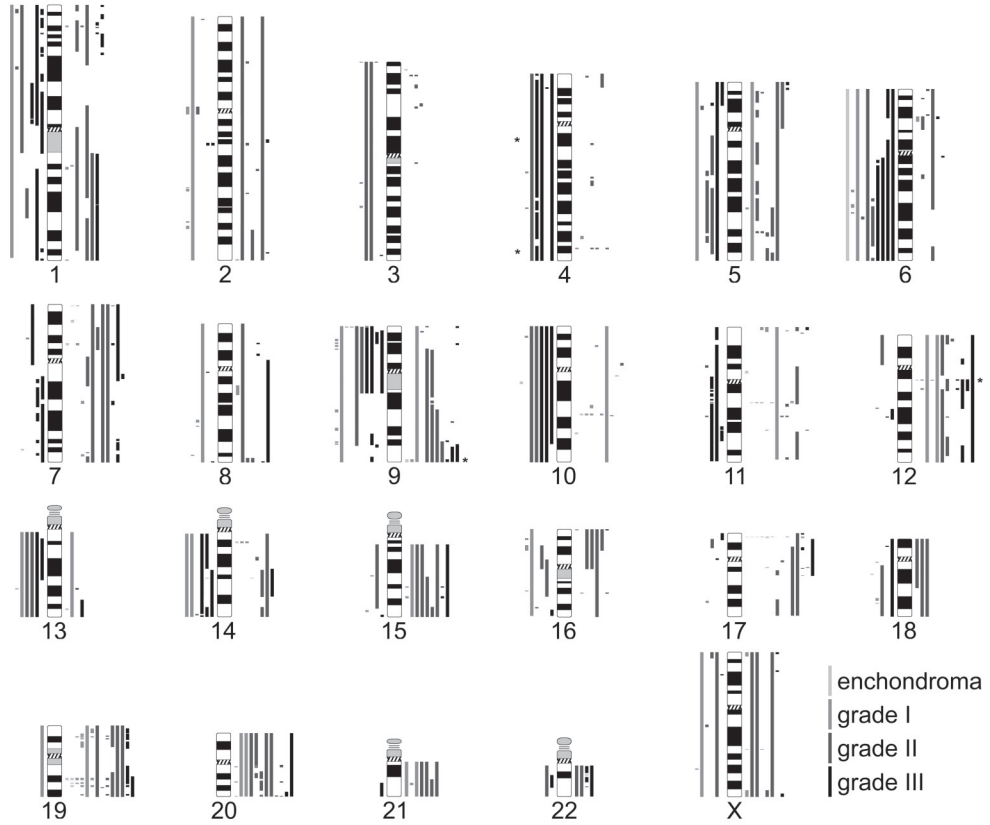

Figure 7.1: *Genomic alterations found in enchondromas and conventional central chondrosarcomas. The ideogram shows the distribution of the numerical aberrations subdivided in enchondroma and the three different grades of chondrosarcoma. Gains are shown on the right, losses on the left. Gain of chromosomal region 12q12 is associated with grade III chondrosarcomas. Loss of chromosomes 10, 4q13, 4q34.3 and gain of 9q34 are associated with adverse prognosis. * marks the location of 4q13, 4q34.3, 9q34 and 12q12*

second Ollier disease-related grade II chondrosarcoma contained both losses (on chromosomes 1, 3, 4, 6, 9, 10, 13, 15, 16, 22) and amplifications (on chromosomes 6, 7, 12, 14, 15, 16, 17, 18, 19). None of the alterations recurred in all four, or even three of the four samples, nor were any of the changes specific for Ollier disease (i.e. absent in solitary tumors).

Correlation with grade

Clear differences were found in the number of aberrations between low- and high-grade tumors. To investigate whether some of these alterations specifically correlated with histological grade, ANOVA and T-test analyses were performed. No clear distinctions between the grades were seen, when all grades were taken in account. By comparing only grade II to grade III chondrosarcomas we found that gain of four clones on chromosome 12 ($p \leq 0.001$), of which three are adjacent on chromosomal region 12q12 (~1.3 Mb), was significantly

Table 7.2: Smallest regions of overlap (SRO) that were amplified or deleted in minimally five samples

Chr	Type	Region	SRO start clone	SRO stop clone	SRO size (Mb)	No of tumors affected	Candidate genes
1	del	p36.22-p36.31	RP3-438L4	RP3-438L4	3.2	5	
1	del	p13.2-p22.1	RP5-1033H22	RP4-770C6	21	5	EXTL2
5	amp	q23.3	RP1-241C15	RP1-241C15	0.55	5	P4HA2
6	del	p21.32-p25.3 (tip)	PAC62L11	RP5-1077I5	33	5	histones
6	del	q22-q25	RP1-94G16	RP11-13P5	43	8	ESR1, PERP
7	amp	p12.3-p15.3	RP11-99O17	RP11-21H20	24	5	RALA
7	amp	p11.2-q11.23	RP5-905H7	RP11-107L23	17	5	GUSB
7	amp	q36.1-q36.3 (tip)	RP11-422E4	CTB-3K23	6.4	5	SHH, C7orf2
8	amp	q24.3 (tip)	RP5-1056B24	CTC-489D14	5.5	7	MAFA
9	del	p21.3-p24.1	RP11-527D15	RP11-149I2	15	7	CDKN2A, RPS6
9	amp	q33.3-q34.3	RP11-373J8	GS1-135I17	14	7	ABL1, VAV2
10	del	pter-q25.2		RP11-426E5	113	5	RSU1, PTEN, NDUFB8
12	amp	p13	RP11-277E18	RP11-277E18	2.9	6	
12	amp	p11.21-p11.23	RP11-425D17	RP11-50I19	5.9	5	PTHLH, PPFIBP1
12	amp	q13	RP5-1057I20	RP11-571M6	11	6	ERBB3, SAS, CDK4, GLI
15	amp	q25.3	RP11-133L19	CTB-154P1	21	7	FES
19	amp	p13.11-p13.3	RP11-500M22	CTD-3149D2	17	7	VAV1, JUNB, JUND
19	amp	q13.11-q13.31	CTD-2527I21	RP11-569M1	9.2	7	AKT2
20	amp	q11.21	RP11-410N8	RP11-410N8	0.66	9	
20	amp	q12	RP11-122O1	RP5-892M9	3.2	8	MAFB
20	amp	q13.33 (tip)	RP4-563E14	RP13-152O15	4.0	9	BIRC7
21	amp	q22.11-q22.3	RP11-410P24	CTB-63H24	19	6	ETS2

The size of SRO presented in the table represents the distance between the first and last BAC in a recurrent DNA copy number alteration. Chr: chromosome; del: deletion; amp: amplification

more frequent in grade III chondrosarcomas (Figure 7.2). This region contains amongst others *HDAC7a* (histon deacetylase 7A) and *SENP1* (SUMO1/sentrin specific protease1). Previously it was reported that loss of 6q is associated with impaired metastasis-free survival [11]. Hierarchical clustering of the samples with solely clones from chromosome 6 partly separated the grade III chondrosarcomas from the other tumor grades ($p < 0.05$, Figure 7.3a). Hierarchical clustering of clones from chromosome 10 showed separate clusters for 6 tumors with recurrence, metastasis and/or dead of disease ($p < 0.05$, not shown). Hierarchical clustering on other previously reported chromosomal areas (i.e. of chromosome 11, 13, 22,

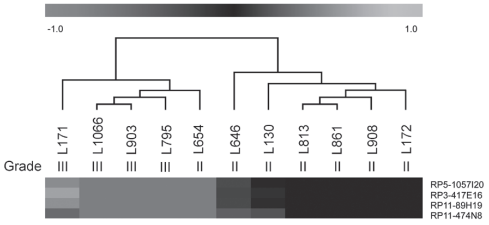

Figure 7.2: *Hierarchical clustering of the four clones of chromosome 12 differentiating between grade II and grade III chondrosarcomas. Two clusters, one containing mainly grade III (amplified) samples and the other one containing grade II chondrosarcomas, can be discerned. Color picture can be viewed at page 130*

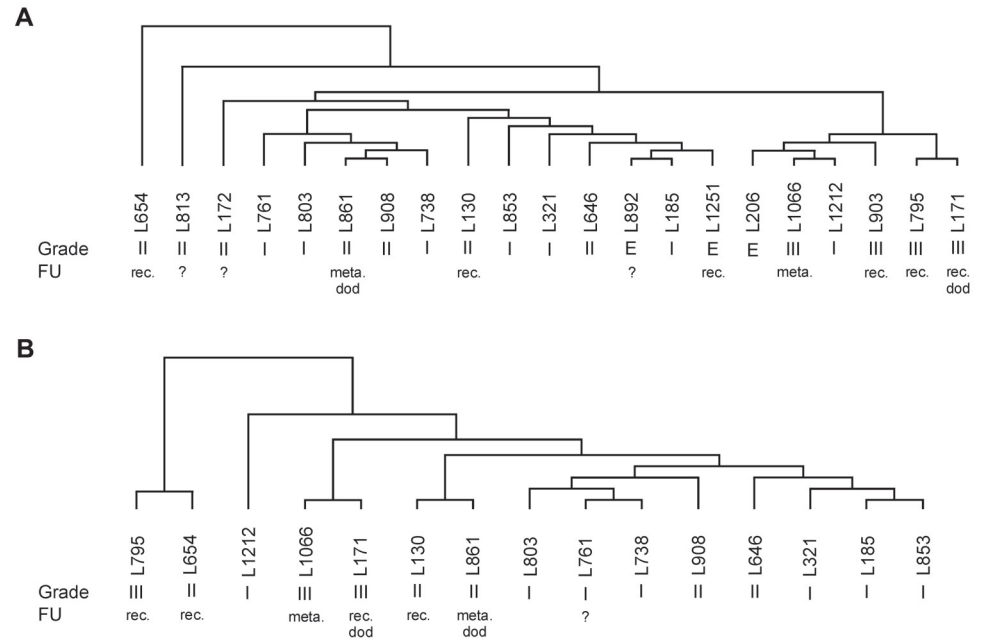

Figure 7.3: *Hierarchical clustering of the tumors. A) hierarchical clustering on the clones of chromosome 6. A distinct cluster containing all grade III chondrosarcomas, one grade I chondrosarcoma and one enchondroma is evident, separating the grade III chondrosarcomas from almost all other grades. B) Hierarchical clustering on the 131 BAC clones, as found by T-test for prognosis shows the clustered distribution of the samples with no evidence of disease as compared to those with recurrences, metastasis or dead of disease. (Abbreviations: III: grade III chondrosarcoma; II: grade II chondrosarcoma; I: grade I chondrosarcoma; E: enchondroma; FU: follow up; rec.: recurrence; ?: lost to follow up; meta.: metastasis; dod: died of disease)*

as reported by Mandahl et al [11] revealed no separate cluster formation of grade and/or outcome.

Correlation with prognosis

To identify possible regions associated with adverse prognosis we compared cases with no evidence of disease to those with recurrence (excluding tumors with a positive surgical margin), metastasis and/or dead of disease, and for which the follow up was minimal 1 year, except if an event had occurred within the first year. T-test identified 165 BAC clones ($p \leq 0.001$) that showed differences between the two groups and hierarchical clustering with these clones identified a near perfect distinction (Figure 7.3b), correlating with histological grade. Of these BACs 95 were located on chromosome 10 (almost all consecutive on 10pter - 10q25.3). Two consecutive regions were present on chromosome 4, ~4.2 Mb on 4q13 and ~4.4 Mb on 4q34.3, lost in 3 grade III chondrosarcomas with recurrence (L171), metastasis and recurrence (L1066) and dead of disease (L795). One continuous region was identified on chromosome 9, ~6.2 Mb on 9q34.

Correlation of genomic alterations and expression levels

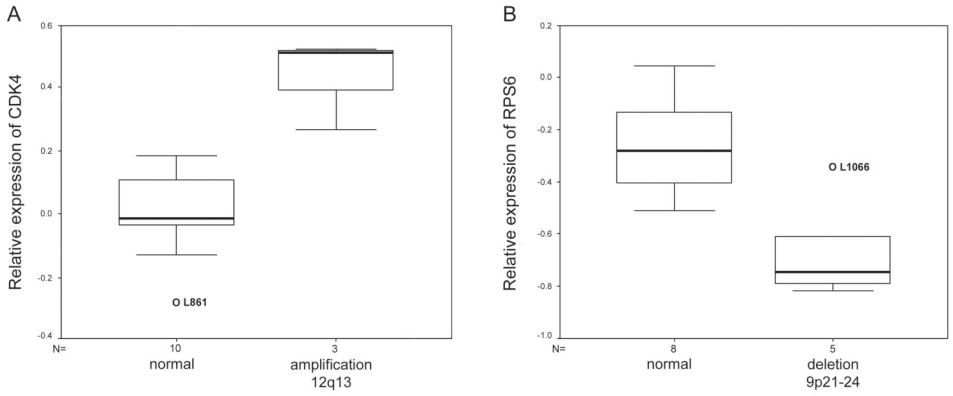

Figure 7.4: *Correlation RNA expression with amplification/deletion found by array-CGH. A) CDK4 [p = 0.000, Pearson's R], B) RPS6 [p = 0.004, Pearson's R]*

We subsequently analyzed gene expression profiles of the SRO's identified by array-CGH (Table 7.2) for 13 tumors (see Table 7.1) of which expression array data were available [32]. Gene expression of tumors with loss/gain in the SRO was compared to gene expression of tumors without the loss/gain (Table 7.2). Overall the genomic alterations did not result in a significant alteration of the gene expression levels. However, expression of two of the aproximately 300 in this experiment tested genes, *CDK4* (12q13) and *RPS6* (9p21-24), appeared to correlate (*p = 0.000* and *0.004* Pearson's R, respectively) with the genomic alteration (Figure 7.4).

FISH

Tumor L803 showed a normal profile, except for a copy number gain of three adjacent clones on chromosome 4q32 (RP11-218F10, RP11-336N6 and RP11-25N12). Interphase FISH analysis was performed to confirm that the amplification of the region is related to the tumor, and to exclude the possibility of a germline amplification, using two of the amplified clones and one adjacent non-amplified clone. The amplification was confirmed in tumor cells and was absent in non-tumor cells of the patient (data not shown).

DISCUSSION

We used array-GCH to examine if DNA imbalances were present in solitary and Ollier disease-related enchondromas and conventional central chondrosarcomas. It has already been demonstrated in osteosarcoma [33] and other tumor types that array-CGH may uncover previously undetected DNA copy number changes [34], and can be used to indicate chromosome regions containing genes involved in tumor origin and progression. This study represents the first application of genome-wide copy number screening by array-GCH in chondrosarcomas.

Our array GCH results are in agreement with genomic changes previously reported at the cytogenetic level (4-10Mb resolution) by G-banding and chromosome CGH [9,10,35], with absent or a limited number of alterations in enchondromas and the presence of several genomic alterations in chondrosarcomas (such as deletion of 9p, gain of parts of chromosome 12 and gain of chromosome 20 and 21; Figure 7.1). The number of changes is increased in tumors with higher grade.

The genetic changes that underlie the origin of Ollier disease, and distinguish its behavior from solitary tumors, are still unclear [1,21]. We compared the tumors from patients with Ollier disease with each other and to the solitary tumors. No genomic alteration was found to be present in all, or exclusively, in Ollier disease samples. Also at expression level, our cDNA array analysis did not reveal differences between Ollier disease-related and solitary enchondromas and chondrosarcomas [32]. However, we cannot exclude that more subtle changes could underlie this syndrome, such as amplifications/deletions smaller than ~1 Mb, or balanced rearrangements, which cannot be identified with array-CGH. However, balanced rearrangements have not been reported in Ollier disease-related tumors or conventional central chondrosarcomas [8,10,12,23]. Another possibility is that Ollier disease is caused by point mutation of a gene. The suggested role of *PTHR1* was excluded by us [25].

Our study revealed that gains and losses of large DNA segments (>1 Mb) are not present in all tumors, and 2 out of 3 enchondromas and 4 out of 7 grade I chondrosarcomas exhibited no detectable alterations in DNA copy numbers. High numbers of alterations mainly including large DNA segments (arms/whole chromosomes) were present predominantly in the high-grade tumors. These alterations can be both recurrent and random, the latter presumably representing chromosomal instability. Applying a cut-off of minimally present in five samples we identified 22 smallest regions of overlap (Table 7.2), ranging from 0.55-113 Mb.

One SRO involved a deletion of 9p21.3-p24.1. This region has been reported to be deleted in chondrosarcomas before [10], and contains the locus INK4A/INK4A-ARF, coding for the tumor suppressor genes *CDKN2A/CDKN2C*. Previously we [20] and others [18] have investigated this locus, and found that loss of this locus or the protein was associated with high histological grade. However, we identified another gene involved in the 9p deletion, *RPS6* (ribosomal protein S6), of which low expression levels significantly correlated with the deletion. This protein belongs to the S6E family of ribosomal proteins, regulated by phosphorylation. The protein may contribute to the control of cell growth and proliferation through the selective translation of particular classes of mRNA [36], and is therefore a candidate tumor suppressor gene in chondrosarcomas.

Chromosome 1 contained two SRO's that were deleted, 1p36.22-p36.31 and 1p13.2-p22.1 [also reported previously [10]]. The region 1p13.2-p22.1 contains amongst others *EXTL2*. In patients with multiple osteochondromas, a hereditary syndrome with multiple exostoses that may transform into secondary peripheral chondrosarcomas, two other members of this gene family are involved [37]. These patients have mutations in the *EXT1* or *EXT2* genes that encode for proteins involved in the heparan sulphate side chain elongation. *EXTL2* is homologous to *EXT1* and *2*, and initiates the heparan sulphate synthesis [38]. Since conventional central chondrosarcomas histologically resemble the secondary peripheral

chondrosarcomas, *EXTL2* could be a target for deletion. However, cDNA microarray analysis revealed no difference in RNA expression of *EXTL2* between the tumors that contained a deletion of this SRO and those without this deletion.

Chromosome 12 contained three SRO's. Chromosomal region 12q13 was found gained in 6 tumors (2 grade I, 1 grade II and 3 grade III chondrosarcomas). This region has been reported previously in chondrosarcomas and other sarcomas [10,39]. Several genes in this region have been indicated to be of importance for tumorigenesis. For instance *SAS* (sarcoma amplified sequence), *CDK4* (cyclin dependent kinase 4), and *GLI* (glioma-associated oncogene homologue) are located in the amplified zone. Two other often implicated genes in sarcomas, *HMGA2* (high mobility group AT-hook 2) and *MDM2*, are located just outside the region we found. cDNA microarray analysis revealed a higher expression of *CDK4* in tumors in which this region was amplified (Figure 7.4), also reported for other tumors [40]. Expression profiles of other genes in this region were not correlating with the genomic gain. However, further studies are required to confirm these findings in other tumor samples.

Chromosomal region 12p11.21-p11.23 was gained in 5 tumors (2 grade I, 1 grade II and 2 grade III chondrosarcomas). One of the genes in this region is *PTHLH* (parathyroid Hormone like hormone), which is an important gene for chondrocyte growth and differentiation. Previously we reported that this protein is expressed in almost all enchondromas and chondrosarcomas [41,42]. This region also contains *PPFIBP1* (PTPRF interacting protein, binding protein 1), which was found to interact with S100A4, a calcium-binding protein related to tumor invasiveness and metastasis [43]. For these genes no data were available in the cDNA microarray experiment.

Genomic gain of the oncogenes *JUNB* and *JUND*, located in the SRO 19p13.11-p13.3 was seen in 7 tumors. We previously found that JUNB protein expression was found to be upregulated in chondrosarcomas compared to enchondromas [32]. Both *JUNB* and *JUND* are proto-oncogenes, overexpressed in several cancer types [44,45] and are transcription factors influencing the cell cycle regulation [46]. cDNA expression analysis revealed expression of both *JUNB* and *JUND* in almost all tumors, at similar levels regardless of amplification of the SRO 19p13.11-p13.3.

In general, most alterations were found only in high-grade tumors. Analysis of the different grades identified 2 regions capable of partly separating the different grades. Copy number gain of region 12q12 separated grade II from grade III chondrosarcomas in hierarchical clustering (Figure 7.2). This region (~1.3 Mb) contains amongst others *HDAC7a* (histone deacetylase 7A) and *SENP1* (SUMO1/sentrin specific protease1). SENP1 is capable of reducing the deacetylase activity of HDAC1 (histone deacetylase 1) [47] and therefore both are involved in histone activity. Also chromosome 6 contains several histone genes (deleted SRO, 6p22-p21.3), and hierarchical clustering of the clones on this chromosome also partly separated the grade III chondrosarcomas from the others (Figure 7.3). Two other regions containing a cluster of histone genes, 1q21 and 1q42, were not affected. Histones are involved in the packaging of the genome, protecting it from damage, and regulation of gene expression by making the gene (in)accessible for transcription [48]. The SRO 6p21 en SRO 12 may therefore affect the genome stability resulting in damage of DNA.

Investigating a potential correlation with prognosis revealed that tumors with loss of chromosome 4 (4q13 and 4q34) and 10 and gain of 9 (9q34) may have a bad prognosis. These aberrations also correlate with increase in histological grade and tumor size. Cluster analysis of all tumors used in this article of clones from chromosome 10 did not result in such a clear separation, as was the case for the cluster analysis of those on the tumors used for association with prognosis. However, the here found prognostic gains and losses should be tested further on a separate group of tumors, to proof their validity. In cluster analysis one tumor performed somewhat unexpected, clustering together with the tumors with adverse prognosis while no recurrence or metastasis has been reported in this patient. However, follow up for this patient has only been available for 16 months, which is relatively short for chondrosarcomas [49], since recurrences may still occur within five years after surgery and metastases after 10.

In conclusion, recurrent alterations (SRO's) were found in chondrosarcomas, as well as non-specific genomic instability, predominantly in high-grade chondrosarcomas. These alterations involve chromosome 12, of which multiple regions are amplified (three SRO's: 12p13, 12p11.21-p11.23, 12q13 and one related with prognosis: 12q12) and chromosome 6. We therefore hypothesize that these parts of the chromosome play an important role in the tumor progression of chondrosarcoma. The expression of *CDK4* correlated with the genomic alteration on 12q13. For the well-known loss of chromosomal region 9p21, we introduce *RPS6* as a possible other gene of interest, in addition to *CDKN2A*.

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Chapter 8

Summary and Conclusion

CONTENTS

I. Expression analysis of central chondrosarcoma

The mechanism behind malignant transformation of enchondromas into chondrosarcomas is not known, and as yet no molecular markers have been identified for the difficult histological differential diagnosis of enchondroma versus low-grade central chondrosarcoma. The histological grading system of the chondrosarcomas is hampered by interobserver variations in evaluation of the different histological features. Chondrosarcomas are one of the rare mesenchymal tumors that upon recurrence may show progression in grade. Thus investigation of the expression patterns characterizing different grades may give an insight in the mechanisms of this process and may also shed light on genetic pathways involved in metastasis and disease-related death.

I.a. Specific (hypothesis driven) expression analysis

RNA and protein expression was investigated in the **chapters 3** (CDKN2A), **chapter 5** (IHH and PTHLH signaling) and **chapter 6** (JUNB).

I.a.i. CDKN2A

The gene coding for the p16 protein is located in a region that is deleted in a subset of central chondrosarcomas. Combined analysis of genetics and protein expression revealed loss of this genomic region and loss of protein expression in mainly high-grade chondrosarcomas (**chapter 3**). However, there was no correlation between loss of protein expression, LOH and or promotor methylation, suggesting an alternative manner of downregulation of gene expression.

I.a.ii. JUNB

JUNB was one of the genes that showed a small, but non-significant, difference comparing three Ollier disease-related grade II chondrosarcomas with four solitary grade II chondrosarcomas using a large scale gene expression analysis (**chapter 6**). This protein is a subunit of the AP-1 transcription factor family, and is implicated in chondrogenic differentiation. *JunB* knock-out mice show reduced proliferation of growth plate chondrocytes and osteoblasts. Comparing the protein expression of this gene in a larger group of Ollier disease-related and solitary enchondromas and central chondrosarcomas revealed that no difference was present between these two groups. However, comparing enchondromas and low-grade chondrosarcomas, a significant increase of protein expression was found in chondrosarcomas, suggesting the possible use of JUNB as a diagnostic marker in the distinction between enchondromas and low-grade chondrosarcomas.

I.a.iii. IHH and PTHLH signaling

In **chapter 1** and **2** it was discussed that the IHH/PTHLH signaling plays an important role in the growth and differentiation of the normal growth plate. In hereditary osteochondromas these signaling pathways are affected by mutations in the *EXT1* or *EXT2* gene. The expression of molecules involved in the IHH/PTHLH signaling in enchondromas and central chondrosarcomas was investigated in **chapter 5.**

Proteins of both signaling pathways were studied by immunohistochemical analysis (PTHLH signaling: PTHLH, PTHR1, BCL2, p21, cyclin D1 and cyclin E) or RNA expression (IHH signaling: *IHH*, *PTCH*, *SMOH* and *GLI2*). The data show that IHH signaling is absent in enchondromas and central chondrosarcomas, while PTHLH signaling is active. There was no difference in expression of any of the molecules between 35 enchondromas and 26 grade I central chondrosarcomas, indicating that PTHLH signaling is not important in malignant transformation of enchondroma. Higher expression of PTHR1 and BCL2 at the immunohistochemical level was associated with increasing histological grade in chondrosarcoma, suggesting involvement in tumor progression.

I.b. Large scale gene expression analysis

In **chapter 6** RNA expression levels of enchondromas and central chondrosarcomas was studied genome wide, by cDNA micro array analysis. Possible changes underlying malignant transformation could not be investigated, due to the fact that RNA isolation of non-phalangeal enchondromas did not yield enough RNA for expression studies. Expression patterns related to progression were investigated by comparing grade I and grade III central chondrosarcomas. Apart from already known/suspected alterations, such as downregulation of extracellular matrix genes, other processes were significantly different between these two groups.

One of these was downregulation of the oxidative phosphorylation, and upregulation of the glycolysis in grade III chondrosarcomas. Both processes are involved in the energy supply of the cell. Increased glycolysis is described in several cancer types, and even suggested to be a hallmark of invasive tumors. The upregulation of anaerobic glycolysis in the hypoxic (i.e. low-grade) situation is hypothesized to give a growth advantage. With increased vascularization (i.e. high-grade), more oxygen is available to the cells and the use of the glycolytic pathway is not downregulated, but most likely changed from anaerobic to aerobic. The downregulation of the OXPHOS may result from the increased glycolysis and can be reversed in some cases. Other explanations for the upregulation of glycolysis and downregulation of the OXPHOS could be the need for products produced in the glycolysis, the inability to store glucose in the cancer cells, failure of the OXPHOS complex, or to decrease the amount of Reactive Oxygen Species, that are capable to induce DNA damage.

II. Genetic aberrations of central chondrosarcoma

So far few specific genomic alterations have been found specific for enchondromas and conventional central chondrosarcomas (9p21, 12q). In previous studies often both conventional central and secondary peripheral chondrosarcomas were analyzed as one group, obscuring possible specific aberrations in either subgroup.

In **chapter 3**, based on previous studies, the location of loss on 9p21, which seems to be specific for central chondrosarcomas was investigated further. In this area one of the genes was the tumor suppressor *CDKN2A*, which is involved in cell cycle regulation and is a target for deletion in many other types of cancers. Loss of heterozygosity (LOH) of four markers, surrounding the CDKN2A/INK4a locus, was found in 15 out of 39 chondrosarcomas (38%). Screening for mutations in the genes *CDKN2A* and *p14ARF*, by SSCP analysis, did not reveal

any mutations in 47 cases in either gene. Our results suggest that a locus other than the CDKN2A/INK4a must be the target of LOH at 9p21.
In **chapter 7** we investigated genome wide, using array-CGH, the genomic aberrations in 21 primary tumor samples, three enchondromas, seven grade I, seven grade II and four grade III chondrosarcomas. We observed an increase in the number and size of the aberrations, correlating with increased histological grade. In case of enchondromas (of the phalanx) and grade I chondrosarcomas generally the number of aberrations was limited and the size of the amplifications/deletions was small, although for instance in one enchondroma loss of complete chromosome 6 was observed. In the high-grade tumors substantially more alterations were observed, often in a seeming random manor, as if genomic instability is taking place.
None of the alterations were present in all tumors, but recurrent alterations were observed. By applying a cut-off of minimally affected in 5 or more tumors in the same manor (all lost, or all gained) and a minimum of 3 adjacent clones, we identified 22 regions, ranging from 0.55 till 113 Mb. Some of these regions were reported previously, while others were not. Interestingly, some chromosomes contained multiple regions, like chromosomes 7 (7p12.3-p15.3, 7p11.2-q11.23, 7q36.1-q36.3), 12 (12p13, 12p11.21-p11.23 and 12q13) and 20 (20q11.21, 20q12, 20q13.33). Analysis of the data revealed that some chromosomes and regions seemed specific for either progression and/or prognosis. Loss of chromosomes 6, 10 and gain of 12q12 was correlated with increasing grade and loss of chromosome 10 (10pter - 10q25.3), 4q13, 4q34.3 and gain of 9q34 was correlated with adverse prognosis.
In general, amplifications of chromosome 12 are reported frequently in tumors. Especially in several sarcoma types, such as osteosarcomas and liposarcomas, amplification of 12q13-15 is a frequent finding. This region contains amongst others the genes *CDK4*, *MDM2*, *SAS* and genes coding for high mobility group (HMG) proteins. Amplification of the short arm of chromosome 12 is seen in other cancer types, such as testicular germ-cell tumors, distal bile duct carcinoma and pancreatic carcinoma. In both testicular germ-cell tumors and pancreatic carcinomas specifically the region 12p11 is also implicated. Gain of 12p is thought to be related to malignant progression.
Combining the results from the cDNA expression array (**chapter 6**) and array-CGH (**chapter 7**), the RNA expression levels of genes located in the recurrent regions of amplification or deletion were analyzed (**chapter 7**). We found that for two genes on our cDNA array the expression matched the changes seen in the BAC array. *CDK4*, located on 12q13, was higher expressed in tumors containing an amplification of this region. Of the second gene, *RPS6* (ribosomal protein S6), located in the deleted region 9p21.3-p24.1, low expression levels significantly correlated with the deletion. These data suggest that the mentioned genes may be the targets for the amplification and deletion found.

III. Multi-step model for tumorigenesis

Combining all data, a multi step model for the development of central cartilaginous tumors can be proposed (Figure 8.1).
With the malignant transformation of enchondroma towards chondrosarcoma upregulation of the protein JUNB is observed (**chapter 6**). The progression of low- to high-grade

chondrosarcomas shows the upregulation of the glycolysis, combined with the downregulation of the oxidative phosphorylation (**chapter 6**). Increased protein expression of PTHR1 and BCL2 is also observed with increasing histological grade (**chapter 5**), whereas the protein expression of p16 decreases (**chapter 3**). On the genomic level, no specific alterations were observed in enchondromas and low-grade central chondrosarcomas. The transition of low- to high-grade central chondrosarcomas is characterized by loss of chromosome 6, 10 and gain of chromosomal region 12q12, although loss of chromosome 6 was also observed in one enchondroma. In addition, the number of losses and gains as well as the size of these aberrations increased in high-grade tumors, possibly as a result of genomic instability (**chapter 7**). Correlating with adverse outcome are the loss of 4q13, 4q34, and 10pter-10q25 and gain of 9q34 (**chapter 7**).

IV. Conventional central chondrosarcoma subtyping

Within the conventional type of central chondrosarcomas, based upon clinical data, different subtypes can be distinguished. Chondrosarcomas located in the phalanx display a more indolent clinical behavior compared to those with similar histological features located in other regions of the skeleton. Also in patients with enchondromatosis, characterized by multiple central cartilaginous lesions, criteria are different, as a consequence of the different clinical features, compared to those of solitary tumors.

IV.a. Phalangeal lesions

In **chapters 5 and 6**, RNA and protein expression levels of tumors located in the phalanx were compared to enchondromas and chondrosarcomas located elsewhere to elucidate whether these tumors are biologically different from those located at other regions, or whether the location is causative for their good prognosis.

For immunohistochemical analysis (**chapter 5**) the chondrosarcomas located in the phalanx were compared to chondrosarcomas grade II located elsewhere, based upon similarities in their histology. In total for 64 enchondromas (21 located in phalanx) and 89 chondrosarcomas

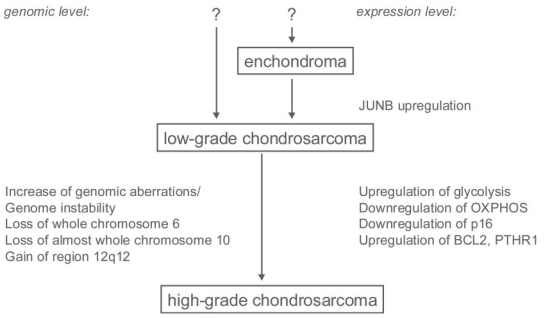

Figure 8.1: *Multi-step model for the development of central cartilaginous tumors. A question mark is placed since the origin of enchondromas and chondrosarcomas is not known, and it has been debated whether they arise from pluripotent mesenchymal stem cells or from cartilage rests from the growth plate that have not undergone calcification*

(17 located in phalanx) the IHH and PTHLH signaling was investigated. Overall statistical analysis revealed no significant differences between chondrosarcomas located in the phalanx and chondrosarcoma grade II located elsewhere in the skeleton. However, a small subset of phalangeal chondrosarcomas demonstrated downregulation of PTHLH.
RNA expression analysis, by cDNA microarray analysis (**chapter 6**), was performed on three phalangeal enchondromas and 19 chondrosarcomas elsewhere of different grades. Overall the expression levels of the phalangeal enchondromas were found to be intermediate to those of grade I and III chondrosarcomas. Moreover, the immunohistochemical comparison of phalangeal enchondromas with enchondromas located elsewhere revealed increased expression of BCL2 and a trend for increased PTHR1 expression in the phalangeal enchondromas.
The number of genomic aberrations (**chapter 7**) in phalangeal enchondromas is small, both in size and number. Unfortunately, phalangeal chondrosarcomas and enchondromas located elsewhere could not be investigated, due to the lack of fresh frozen material and fragmentation of the DNA in parrafin blocks due to decalcification, thus a comparison at the genomic level could not be made.
Combining these results, the better prognosis of phalangeal chondrosarcomas (i.e. low risk of metastasis) could be partly due to the downregulation of the PTHLH signaling. Downregulation of PTHLH and BCL2 is also observed in osteochondromas, the benign counterparts of secondary peripheral chondrosarcomas. It therefore may be a representation of benign behavior. However, most enchondromas display active PTHLH signaling. Also the smaller number of genomic alterations in phalangeal enchondromas, as compared to chondrosarcomas grade I-II with similar histological features, may partly cause the better prognosis. However, studies of genomic aberrations in phalangeal chondrosarcomas would be required to test this hypothesis. In contrast, RNA expression levels of phalangeal enchondromas suggest that these tumors have malignant features, comparable to grade I-II chondrosarcomas, with which these tumors share several histological features. However, no data were available for non-phalangeal enchondromas, and therefore we cannot exclude that these tumors have overall expression levels comparable to the grade I-II chondrosarcomas. Together these results support both the theory that the more indolent clinical behavior has a molecular origin reflecting a different biological make up (lower PTHLH expression, low number of genomic aberrations), as well as the theory that the location is the most important cause for the better prognosis (RNA expression levels of phalangeal enchondromas are in line with of those of chondrosarcomas grade II located elsewhere).

IV.b. Enchondromatosis

Enchondromatosis is defined by the presence of multiple enchondromas at different sites. We investigated whether tumors in this context have different molecular features compared to solitary tumors. These results then may give clues to the underlying molecular defect and/or explain the less malignant behavior of these tumors in patients with enchondromatosis.
In the **chapters 4-7** tumors of patients with enchondromatosis (Ollier disease, Maffucci syndrome) were investigated. In **chapter 4** we looked for the presence of a previously reported

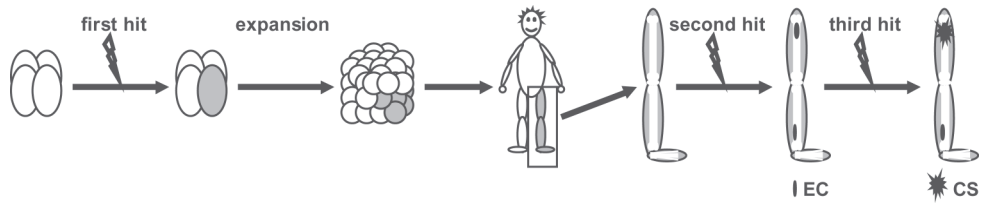

Figure 8.2: *Hypothesis regarding mosaicism and enchondromatosis. In the early embryogenesis a first genetic hit takes place, of which the mechanism is unknown, causing the alteration of one of the cells. The embryo continues to expand with only part of the body affected by this genetic alteration, in this example one of the lower extremities. A second hit, of which the mechanism is also unknown, will subsequently result in the formation of enchondromas/central chondrosarcomas. This second hit would only result in enchondroma formation if it occurs in regions in which the first genetic alteration is present. A third hit will result in malignant transformation, creating a (low-grade) chondrosarcoma. Abbreviations: EC: enchondroma; CS: chondrosarcoma*

mutation in *PTHR1* (c.448C>T, p.R150C). We collected enchondromas and chondrosarcomas from 31 enchondromatosis patients from three different European countries. Screening for this specific mutation in 26 patients did not reveal the presence of it, nor did direct sequencing of all coding regions of *PTHR1* result in any other mutation. In **chapter 7** we investigated the presence of large genomic aberrations (>~1Mb) in four tumors of patients with Ollier disease. Although individual tumors showed aberrations, none of these were present in all of the investigated Ollier disease-related tumors and none were specific for Ollier disease.
With the studies in **chapters 4** and **5** we excluded that differences in the PTHLH signaling, and specifically alterations of the *PTHR1* gene were causative for enchondromatosis. RNA expression studies (**chapter 6**) revealed only small non-significant differences comparing three Ollier disease related grade II chondrosarcomas to four solitary grade II chondrosarcomas, although some genes listed in may become significant, if tested on larger groups
So, in all non of our studies significant differences were found between enchondromatosis-related tumors and solitary tumors, suggesting that in both occasions the same pathways are affected.
The cause of enchondromatosis is still unknown. The syndrome is not hereditary and the fact that in some patients only certain parts of the body are affected (for instance only right side, upper body, etc), suggest that the cause of these syndromes lies in early development. In that case, in the early stages a single cell could be affected, for instance by a mutation. This cell could than potentially grow out to cells populating one part of the body (mosaicism, reported for McCune-Albright syndrome, OMIM #174800). Subsequently these cells could be more vulnerable for a second hit which would be required for the development of enchondromas (Figure 8.2). Further research will be required to test this hypothesis. To identify the cause of enchondromatosis it could be investigated as was done for McCune-Albright syndrome. The gene (*GNAS1*) causing this was identified by searching for an hereditary syndrome, displaying similar biochemical and clinical features. This was the case for Albright hereditary osteodystrophy, for which causative mutations had already been identified. Unfortunately, although in case of Enchondromatosis some hereditary syndromes are known, but these

have an even lower incidence than Ollier disease or Maffucci syndrome. Therefore, clues of what is causing enchondromatosis are most likely to be found by genome wide analysis of RNA or protein expression of a larger group of Ollier disease-related tumors.

V. Concluding remarks and directions for future research

The purpose of this thesis was to investigate the molecular processes involved in development and progression of enchondromas and conventional central chondrosarcomas, including the subgroups of those arising in patients with Ollier disease and in the phalanx.

Within these groups, though clearly clinical differences are observed between the three different subgroups (Ollier disease–related, phalangeal localization and solitary non-phalangeal tumors), no clear molecular differences are observed. Ollier-disease-related tumors showed no significant differences from solitary tumors. Phalangeal lesions showed a decrease of PTHLH expression in only a subset of the phalangeal chondrosarcomas.

Our aim was to further elucidate the multi-step model and identify diagnostic and prognostic markers.

We constructed a multi-step model, showing alterations as described in this thesis regarding malignant transformation, progression and correlation with adverse prognosis. We identified protein expression of JUNB as a candidate diagnostic tool, showing increased expression in (low-grade) chondrosarcomas compared to enchondromas. Related with prognosis we found upregulation of PTHLH signaling in high-grade chondrosarcomas and upregulation of glycolysis-associated genes combined with downregulation of genes involved in the oxidative phosphorylation in high-grade chondrosarcomas.

Our genome wide analysis at DNA and RNA level pointed to the candidate genes *RPS6* and *CDK4*, which are the subject of continuing research, to unravel their role in the pathogenesis of enchondromas and conventional central chondrosarcomas.

NEDERLANDSE SAMENVATTING

Moleculaire opmaak van solitaire en Morbus Ollier gerelateerde centrale chondrosarcomen:
Een onderzoek naar DNA afwijkingen, mRNA en eiwit expressie.

INHOUD

Chondrosarcomen zijn kwaadaardige kraakbeen-producerende tumoren. Deze tumoren zijn zeldzaam en groeien meestal langzaam. Het merendeel behoort - microscopisch gezien - tot de subgroep conventionele chondrosarcomen. Binnen deze groep worden er 3 graden van maligniteit onderscheiden (graad I-III). De tumoren met de hoogste graad hebben een slechtere prognose. Binnen de groep van conventionele chondrosarcomen zijn twee groepen te onderscheiden op basis van waar deze zich bevinden ten opzichte van het bot waarvan ze uitgaan en al of niet bestaan van een goedaardige voorloper. Het perifere chondrosarcoom dat altijd ontstaat aan het oppervlak in de kraakbeenkap van zijn goedaardige tegenhanger het osteochondroom, en het centrale chondrosarcoom dat primair centraal in het bot gelegen is en kan al of niet ontstaan zijn in een goedaardige voorloper, genaamd enchondroom. Deze centrale chondrosarcomen zijn het meest voorkomend (± 80%). Dit proefschrift concentreert zich op deze specifieke groep tumoren.
Enchondromen zijn meestal solitair, dat wil zeggen dat er per patiënt maar één tumor wordt gevonden. Er zijn echter patiënten waarbij meerdere van deze tumoren voorkomen. Deze mensen lijden aan een syndroom genaamd "enchondromatose". Binnen dit syndroom wordt er onderscheid gemaakt tussen verschillende subtypen waarvan Morbus Ollier (M. Ollier) en het Maffucci syndroom het meest voorkomend zijn.
Naast graad is ook de lokalisatie in het skelet van de tumoren van belang voor de prognose. De tumoren gelegen in de botten van de vingers en tenen tonen, in tegenstelling tot wat hun histologie doet denken, een relatief gunstige prognose.

I. Expressie analyse van central chondrosarcomen
Het onderliggend mechanisme van de ontaarding van enchondromen naar laaggradige chondrosarcomen is onbekend. Ook zijn er geen moleculaire merkers bekend die kunnen helpen om het verschil tussen deze benigne en maligne tumoren te verduidelijken. Dit zou goed van pas komen omdat het verschil soms moeilijk vast te stellen is door de patholoog. Daarnaast is het histologische graderingsysteem wat wordt gebruikt voor deze tumoren niet altijd glashelder, doordat de histologische kenmerken die van belang zijn voor de gradering verschillend geïnterpreteerd kunnen worden.
Chondrosarcomen zijn een van de weinige mesenchymale tumoren waarbij progressie in graad kan plaatsvinden. Het bestuderen van expressie niveaus in de verschillende graden kan daarom aanwijzingen geven wat betreft de mechanismen die betrokken zijn bij dit proces van progressie en de daaraan gecorreleerde risico's op metastase en tumor gerelateerd overlijden.

I.a. Specifieke (hypothese gerelateerde) expressie analyse
RNA en eiwit expressie is onderzocht in de **hoofdstukken 3** (CDKN2A), **5** (IHH en PTHLH signaal transductie) en **6** (JUNB).

I.a.1. CDKN2A
Het gen (*CDKN2A*) coderend voor het eiwit genaamd p16 (betrokken bij de regulatie van cel groei en deling en zo genoemd naar aanleiding het molecuul gewicht van dit eiwit) ligt in

een regio van het genoom (9p21; een gebied op de korte arm van chromosoom 9, band p21) dat gedeeltelijk verloren is gegaan in een deel van de centrale chondrosarcomen. Gecombineerde analyse van zowel het mogelijk verlies van de regio 9p21 en de mate van eiwitexpressie van p16 liet zien dat verlies van de regio en p16 eiwitexpressie voornamelijk iets is dat voorkomt in hooggradige chondrosarcomen (**hoofdstuk 3**). Er is echter geen relatie gevonden tussen het verlies van p16 eiwitexpressie, het verlies van het stukje genoom 9p21, of de aanwezigheid van methylering van het gen (een mechanisme dat kan zorgen voor een verminderde eiwitexpressie). Daarom is er waarschijnlijk een ander, nog niet geïdentificeerd, mechanisme actief dat leidt tot verminderde expressie van p16. Verlies van het eiwit p16 speelt een rol in de progressie van chondrosarcomen. Dit is een gegeven dat ook beschreven is bij andere tumoren zoals het rondcel liposarcoom.

I.a.ii. JUNB

JUNB is een van de genen die een klein, maar niet significant, verschil heeft gegeven bij de vergelijking van M. Ollier gerelateerde graad II chondrosarcomen met solitaire graad II chondrosarcomen zoals beschreven in **hoofdstuk 6**. Het eiwit JUNB maakt deel uit van een familie van transcriptie factoren (factoren die op basis van hun kenmerken het afschrijven van genen zowel positief als negatief kunnen beïnvloeden), genaamd AP-1. Deze factor speelt een rol bij de controle van groei en differentiatie van kraakbeen. Muizen waarbij *JunB* experimenteel is uitgeschakeld laten een gereduceerde groei van zowel kraakbeen als botvormende cellen zien.

Bij de analyse van JUNB eiwitexpressie in een grotere groep tumoren - opgesplitst in solitaire en M. Ollier gerelateerde tumoren - is er echter geen verschil gezien tussen deze twee groepen. Er is wel een verschil gevonden in eiwitexpressie tussen enchondromen en laaggradige chondrosarcomen. Hierbij is een toename van expressie geconstateerd in de chondrosarcomen. Dit suggereert dat JUNB expressie mogelijk kan worden gebruikt om het onderscheid tussen enchondromen en laaggradige chondrosarcomen te maken.

I.a.iii. IHH en PTHLH signaal transductie

In de **hoofdstukken 1** en **2** wordt de signaaltransductie regelkring zoals die beschreven is voor Indian Hedgehog (IHH) en parathyroid hormoon-gelijkend hormoon (PTHLH) geïntroduceerd. IHH komt aan zijn naam omdat wanneer een eiwit dat er veel op lijkt, in de fruitvlieg word verwijderd dit leidt tot een larve met allemaal stekeltjes (engels: “hedgehog”), de naamgeving Indian komt van het feit dat dit gen als eerste werd ontdekt in India. De IHH/ PTHLH regelkring speelt een belangrijke rol in de normale groei en ontwikkeling van de groeischijf. In erfelijke osteochondromen, de goedaardige tegenhangers van perifere chondrosarcomen, is de signaaltransductie van deze regelkring beïnvloed door mutaties in de genen *EXT1* en *EXT2*. Daarom zijn de expressie niveaus van moleculen betrokken bij deze regelkring in enchondromen en centrale chondrosarcomen bestudeerd in **hoofdstuk 5**. De moleculen zijn bestudeerd op eiwit niveau (immunohistochemie) voor PTHLH, PTHR1 (de receptor voor PTHLH), BCL2 (een eiwit betrokken bij het geprogrammeerde sterfproces van de cel), p21, cycline D1 en cycline E (drie eiwitten betrokken bij de regulatie van de cel

deling). Op RNA niveau is er gekeken naar *IHH*, *PTCH* (patched, de receptor van IHH), *SMOH* (smoothened, een eiwit dat vast kan zitten aan PTCH en een rol speelt bij het doorgeven van het signaal) en *GLI2* (een eiwit dat codeert voor een transcriptie factor en eveneens een rol speelt bij het doorgeven van het signaal)(Figuur 5.1). De data laten zien dat de IHH signaal transductie regelkring afwezig is in enchondromen en centrale chondrosarcomen, terwijl de PTHLH regelkring wel actief is. Er is geen verschil in expressie niveaus gevonden tussen enchondromen en graad I chondrosarcomen. Het lijkt er dus op dat de PTHLH regelkring geen belangrijke rol speelt in de overgang van enchondroom naar chondrosarcoom. Een hoger expressie niveau correlerend met toenemende hogere histologische graad is gevonden voor de eiwitten PTHR1 en BCL2. Dit suggereert dat deze eiwitten mogelijk een rol spelen in de tumor progressie.

I.b. Algemene gen expressie analyse

In hoofdstuk 6 zijn de RNA expressie niveaus van duizenden genen tegelijkertijd in enchondromen en centrale chondrosarcomen gemeten, met cDNA microarray analyse. De progressie is onderzocht door het vergelijken van de expressie patronen per gen in graad I en graad III chondrosarcomen. Naast de veranderingen die al bekend zijn, zoals de afname van extracellulaire matrix genen (graad III chondrosarcomen zijn celrijker en hebben minder substantie liggen tussen de tumor cellen) werden er ook andere processen die significant verschillend zijn tussen de graad I en graad III chondrosarcomen gevonden.

Een van deze processen is de energie huishouding. In graad III chondrosarcomen is er een lagere expressie van genen gevonden betrokken bij de oxidatieve fosforylering. Dit is een proces dat zorgt dat suikers samen met zuurstof (aëroob) worden omgezet in energie. Daarnaast is er in hooggradige tumoren een hogere expressie gevonden van genen betrokken bij de glycolyse. Glycolyse is het proces waarbij suiker wordt omgezet in energie, zonder dat er zuurstof wordt gebuikt (anaëroob). De toename van glycolyse is beschreven bij verschillende kanker soorten, en er wordt zelfs gesuggereerd dat het een kenmerk is van invasieve tumoren. De stimulering van het proces van glycolyse leidt mogelijk in de laaggradige tumoren tot een groeivoordeel, aangezien er in deze tumoren weinig bloedvaten (en dus zuurstof) aanwezig zijn. Bij toename in graad neemt ook de vaatingroei toe, waardoor er meer zuurstof beschikbaar is voor de cellen. De glycolyse die netto resulteert in minder energie per suiker molecuul, gaat echter niet omlaag. Waarschijnlijk zijn de cellen dus omgeschakeld van aërobe naar anaërobe energie voorziening tijdens progressie. Mogelijk leidt de verhoogde activiteit van de glycolyse tot een verlaging van de activiteit van de oxidatieve fosforylering. Andere verklaringen zijn dat tumorcellen de producten die vrijkomen bij de glycolyse nodig hebben, dat het proces van oxidatieve fosforylering niet goed functioneert (bijvoorbeeld door mutaties), of dat cellen de hoeveelheid vrije radicalen (een bijproduct van oxidatieve fosforylering dat het DNA aan kan tasten) willen beperken.

II. Genetische veranderingen in centrale chondrosarcomen

Tot nu toe waren er weinig specifieke veranderingen van het genoom bekend in het geval van enchondromen en conventionele centrale chondrosarcomen (9p21, 12q). Vorige studies

combineerden vaak de conventionele centrale chondrosarcomen en de perifere chondrosarcomen, waardoor mogelijke verschillen specifiek voor een van deze twee groepen moeilijk te detecteren zijn.

In **hoofdstuk 3** is, gebaseerd op eerdere studies, het verlies van de regio 9p21 verder bestudeerd. Verlies hiervan lijkt specifiek te zijn voor conventionele centrale chondrosarcomen. De regio bevat onder andere het gen *CDKN2A*, wat codeert voor het p16 eiwit (zie boven). Doordat het eiwit in sommige gevallen de ontwikkeling en groei van tumoren kan belemmeren, is dit gen vaak het doel van het verlies. Met behulp van 4 merkers, gelokaliseerd op chromosoom 9 rondom de regio waar het *CDKN2A* gene ligt, is aangetoond dat een kopie van deze regio is verloren in 15 van 39 chondrosarcomen. Er zijn geen mutaties in de genen *CDKN2A* en *p14ARF* (een gen dat gedeeltelijk overlap vertoont met *CDKN2A*) gevonden in alle 47 geteste tumoren. Dit alles te samen suggereert dat dit gebied, met de genen *CDKN2A* en *p14ARF*, niet het doel van het verlies is, maar dat er in deze regio (9p21) een ander gen van belang is.

In **hoofdstuk 7** wordt beschreven hoe over het gehele genoom onderzoek is gedaan met array-CGH (een experiment waarmee de mate van winst of verlies van het genetisch materiaal in een keer kan worden meten). Er is gekeken naar genomische afwijkingen in primaire tumoren: enchondromen, graad I, graad II en graad III chondrosarcomen. Hierbij is een toename in zowel het aantal als de uitgebreidheid van de veranderingen in tumoren van hogere graad gevonden. In het algemeen waren de afwijkingen van de enchondromen (alle drie gelokaliseerd in de botten van vingers en tenen) en de graad I chondrosarcomen beperkt en indien aanwezig klein, hoewel één van de enchondromen verlies vertoonde van geheel chromosoom 6. In de hooggradige chondrosarcomen zijn beduidend meer, schijnbaar willekeurige, veranderingen waargenomen, alsof het genoom van deze tumoren instabiel is geworden.

Geen van de veranderingen was aanwezig in alle geteste tumoren, maar er zijn wel veranderingen die werden gevonden in meerder tumoren. Door het toepassen van een scheidslijn waarbij minimaal 5 tumoren dezelfde veranderingen moeten bevatten zijn er 22 regio's geïdentificeerd. De grootte van deze regio's varieerde van 0.55 Mb tot 113 Mb. Enkele van deze regio's zijn eerder genoemd in andere studies, terwijl andere nog niet eerder waren gerapporteerd. In het geval van de chromosomen 7, 12 en 20 zijn meerdere stukjes met verlies of winst gevonden. Op chromosoom 7 zijn dat de regio's 7p12.3-p15.3, 7p11.2-q11.23, 7q36.1-q36.3, op chromosoom 12: 12p13, 12p11.21-p11.23 and 12q13 en op chromosoom 20: 20q11.21, 20q12, 20q13.33. Verdere analyse toonde aan dat winst of verlies van sommige chromosomen of chromosoom regio's specifiek lijken te zijn voor progressie of prognose. Verlies van chromosoom 6 of 10 en winst van de regio 12q12 correleert met een hogere graad. Verlies van chromosoom 10 (10pter-10q25.3), 4q13, 4q34.3 en toename van 9q34 correleert met een slechtere prognose.

Winst van genetisch materiaal op chromosoom 12 wordt waargenomen in meerdere tumorsoorten. Vooral in sarcomen, zoals osteosarcomen en liposarcomen, worden amplificaties van de regio 12q13-15 vaker gezien. In dit gebied liggen onder andere de genen *CDK4*, *MDM2*, *SAS* en genen van de familie van "high mobility group" eiwitten. Winst van de korte

arm van chromosoom 12 (12p) wordt ook gezien in andere typen kanker, zoals bepaalde types kiembaan tumoren van de testis en pancreas carcinomen. In beide type tumoren wordt, net als in chondrosarcomen, amplificatie van 12p11 gevonden. Winst van 12p wordt gezien als een mogelijke stap naar verdere kwaadaardige progressie.
Uiteindelijk zijn de resultaten van de cDNA expressie array (**hoofdstuk 6**) en die van de array-CGH (**hoofdstuk 7**) gecombineerd om te zien of het niveau van de gen expressie mee verandert met het verlies of winst van gedeeltes van het genoom. Er zijn twee genen geïdentificeerd, *CDK4* (12q13) kwam hoger tot expressie in tumoren waarvan deze regio amplificatie vertoonde en *RPS6* (9p21.3-p24.1) had lagere expressie niveaus in tumoren met verlies van deze regio. Deze data suggereren dat de hiergenoemde genen kandidaat zijn als doelwit voor de gevonden winst en respectievelijk verlies.

III. Model voor tumorvorming

Met behulp van de hierboven staande data is een model voor tumor vorming van conventionele central chondrosarcomen opgesteld (Figuur 8.1).
Bij de transformatie van enchondroom naar laaggradig chondrosarcoom wordt een toename van expressie van het eiwit JUNB gevonden (**hoofdstuk 6**). De progressie van laag- naar hooggradig chondrosarcoom wordt gekenmerkt door de verhoogde expressie van genen betrokken bij de glycolyse, gecombineerd met de afname in expressie van genen betrokken bij de oxidatieve fosforylering (**hoofdstuk 6**). Daarnaast wordt een toename van PTHR1 en BCL2 eiwitexpressie gezien met toename in histologische graad (**hoofdstuk 5**), terwijl de eiwitexpressie van p16 afneemt (**hoofdstuk 3**). Op genomisch niveau worden er geen verschillen waargenomen tussen enchondromen en laaggradige chondrosarcomen. Bij de overgang van laag- naar hooggradige tumoren wordt verlies van chromosoom 6, 10 en amplificatie van de regio 12q12 gevonden, hoewel verlies van chromosoom 6 ook is gevonden in één van de drie enchondromen. Tevens nam het aantal en de grootte van de genomische veranderingen toe in de hooggradige tumoren, mogelijk door genomische instabiliteit (**hoofdstuk 7**). Het verlies van 4q13, 4q34, en 10pter-10q25 en de amplificatie van 9q34 bleken te correleren met een slechtere prognose (**hoofdstuk 7**).

IV. Conventionele centrale chondrosarcoma subtypes

Binnen de groep van conventionele centrale chondrosarcomen zijn er, gelet op de klinische data, verschillende subtypes te onderscheiden. Patiënten met chondrosarcomen gelokaliseerd in de botten van de vingers en tenen hebben een lagere kans op uitzaaiingen vergeleken met patiënten waarvan de tumoren soortgelijke histologische kernmerken vertonen, maar die elders gelokaliseerd zijn. Ook voor patiënten met enchondromatose, gekarakteriseerd door meerdere centraal in het bot gelegen kraakbeen-vormende tumoren, worden er als gevolg van verschillend klinisch gedrag andere histologische criteria gebruikt, vergeleken met solitaire tumoren.

IV.a. Tumoren gelokaliseerd in vingers en tenen

In de **hoofdstukken 5** en **6** zijn de RNA- en eiwit-expressieniveaus van tumoren gelokaliseerd in vingers en tenen (hieronder groep I genoemd) in zichzelf vergeleken met de tumoren die zich elders in het skelet bevinden (hieronder groep II genoemd). Dit is gedaan om te onderzoeken of de tumoren biologisch verschillend zijn, of dat de locatie van deze tumoren in vinger en tenen de oorzaak is van hun goede prognose.
Wat betreft eiwitexpressie (**hoofdstuk 5**) zijn vanwege de overeenkomsten in de histologie de chondrosarcomen in groep I vergeleken met graad II chondrosarcomas uit groep II. In totaal zijn 64 enchondromen (waarvan 21 behoren tot groep I) en 89 chondrosarcomen (waarvan 17 behoren tot groep I) onderzocht op verschillen in de IHH en PTHLH signaal transductie regelkring. Er zijn geen significante verschillen gevonden tussen de chondrosarcomen van groep I en de graad II chondrosarcomen uit groep II. Er was echter wel een kleine groep van chondrosarcomen gelokaliseerd in de botten van vinger en tenen (groep I) die verminderde expressie toonden van het eiwit PTHLH. De BCL2 en PTHR1 eiwitexpressie van enchondromen uit groep I waren hoger wanneer deze werden vergeleken met enchondromen uit groep II (**hoofdstuk 5**).
RNA expressie analyse is met behulp van cDNA microarray analyse (**hoofdstuk 6**) uitgevoerd op 3 enchondromen van groep I en 19 chondrosarcomen uit groep II. Over het algemeen bevonden de expressie niveaus van de enchondromen uit groep I zich tussen die van de graad I en graad III chondrosarcomen.
In **hoofdstuk 7** is aangetoond dat het aantal genomische veranderingen in enchondromen uit groep I beperkt is, zowel in grootte als in aantal.
De verminderde expressie van de PTHLH die enkele van de tumoren uit groep I vertonen zou dus een oorzaak kunnen zijn van hun relatief gunstige prognose. Een aanwijzing dat er mogelijk een biologisch verschil ten grondslag aan het verschillend klinisch gedrag zou kunnen liggen is de geringe hoeveelheid genomische veranderingen.

IV.b. Tumoren in de context van enchondromatose
Enchondromatose wordt gekenmerkt door de aanwezigheid van meerdere enchondromen op verschillende locaties in één patiënt. Gedurende dit onderzoek is in **hoofdstuk 4-7** onderzocht of de tumoren van patiënten met enchondromatose verschillende moleculaire kenmerken hebben ten opzichte van solitaire tumoren. Deze resultaten zouden mogelijk aanwijzingen kunnen geven die leiden tot het vinden van de oorzaak van dit syndroom. Daarnaast zou er mogelijk een verklaring gevonden kunnen worden voor feit dat de histologie een slechte voorspeller is voor gedrag in patiënten met enchondromatose.
In **hoofdstuk 4** is gekeken naar de aanwezigheid van een eerder in de literatuur beschreven mutatie in het gen *PTHR1* (c.448C>T, p.R150C). Hiervoor zijn enchondromen en chondrosarcomen van 31 patiënten uit drie verschillende Europese landen verzameld die gediagnosticeerd waren met enchondromatose. De hierboven genoemde mutatie is niet gevonden in 31 (100%) van de geteste patiënten, noch is er enige aanwijzing gevonden voor aanwezigheid van een andere mutatie in dit gen. In **hoofdstuk 7** is de aanwezigheid van grote genomische veranderingen (>~1 Mb) onderzocht in 4 tumoren van patiënten met M. Ollier. Hoewel er in de individuele tumoren wel veranderingen zijn gevonden, waren geen

van deze aanwezig in alle onderzochte tumoren, noch waren er specifieke afwijkingen in tumoren in de context van M. Ollier.
Met behulp van de onderzoeken zoals beschreven in de **hoofdstukken 4** en **5** zijn er geen aanwijzing gevonden dat veranderingen in de PTHLH signaaltransductie regelkring, of specifiek veranderingen in het *PTHR1* gen, verantwoordelijk zouden zijn voor het ontstaan van enchondromatose. RNA expressie studies (**hoofdstuk 6**) lieten zien dat er alleen zeer kleine (niet significante) verschillen zijn tussen graad II tumoren van patiënten met M. Ollier en solitaire graad II tumoren, hoewel sommige van deze verschillen mogelijk wel van betekenis zouden kunnen zijn als de groepen groter zouden worden gemaakt.
Uiteindelijk is er in geen van de uitgevoerde experimenten een verschil gevonden tussen enchondromatose gerelateerde tumoren en solitaire tumoren, wat erop wijst dat in beide gevallen dezelfde regelkringen zijn aangedaan.
De oorzaak van enchondromatose is nog altijd niet bekend. Het syndroom is niet erfelijk en het feit dat in sommige patiënten alleen bepaalde gedeeltes van het lichaam zijn aangedaan (bijvoorbeeld alleen de rechterkant) suggereert dat de oorzaak mogelijk in de vroeg embryonale ontwikkeling ligt. In dat geval zou in een vroeg stadium één cel kunnen veranderen, bijvoorbeeld een mutatie. Deze cel zou potentieel kunnen uitgroeien tot cellen die deel uitmaken van een bepaald deel van het lichaam wat ook wel mosaicisme genoemd wordt. Dit fenomeen wordt waargenomen bij andere aandoeningen zoals het McCune-Albright syndroom. De veranderde cellen zouden kwetsbaarder zijn voor een volgende verandering die zou kunnen leiden tot de ontwikkeling van enchondromen (Figuur 8.2). Nader onderzoek is nodig om deze hypothese te testen. De mutaties in het gen verantwoordelijk voor het McCune-Albright syndroom werden ontdekt door het zoeken naar klinische en biochemische overeenkomsten tussen dit syndroom en erfelijke aandoeningen. Hierbij kwam men uit bij Albright osteodystrophie, waarvan de veroorzakende mutaties reeds bekend waren. In beide aandoeningen bleek een en hetzelfde gen aangedaan te zijn namelijk *GNAS1*.
Helaas zal het moeilijk zijn om op dezelfde manier te werk te gaan in het geval van enchondromatose. Hoewel er wel overeenkomsten zijn met erfelijke aandoeningen, zijn deze syndromen nog zeldzamer dan M. Ollier of het Maffucci syndroom. Daarom is het waarschijnlijker dat de oorzaak zal worden gevonden aan de hand van uitgebreide studies naar RNA en eiwit expressie niveaus gebruikmakend van een grotere hoeveelheid M. Ollier gerelateerde tumoren dan in dit proefschrift gebruikt zijn.

V. Conclusies en samenvatting

Het doel van dit proefschrift was het onderzoeken van de moleculaire processen betrokken bij de ontwikkeling en progressie van enchondromen en conventionele centraal-gelegen chondrosarcomen, inclusief de subgroepen van M. Ollier gerelateerde tumoren en die gelokaliseerd in de botten van vingers en tenen.
Hoewel er duidelijk klinische verschillen te vinden zijn tussen deze subgroepen (M. Ollier gerelateerd, tumoren gelokaliseerd in de botten van vinger en tenen en de solitaire tumoren elders) zijn er geen duidelijke verschillen op moleculair niveau. Tumoren geassocieerd met M. Ollier zijn op moleculair niveau niet significant verschillend van solitaire tumoren. In het

geval van de tumoren van vingers en tenen vertoonde een gedeelte van de tumoren een verminderde PTHLH eiwitexpressie.
Ons doel was het verder verduidelijken van een stapsgewijs model voor tumorvorming en het identificeren van diagnostische en progressie gerelateerde moleculaire kenmerken.
Er is een model voor tumorvorming gemaakt, waarin de veranderingen aangaande maligne transformatie, progressie en die gerelateerd aan slechtere prognoses, zoals gevonden in dit proefschrift zijn verwerkt (Figuur 8.1). Gerelateerd met transformatie is een verhoogde eiwitexpressie van JUNB gevonden in laaggradige chondrosarcomen ten opzichte van enchondromen. Een slechtere prognose (en hogere histologische graad) gaat gepaard met hogere expressie niveaus van eiwitten in de PTHLH regelkring, alsmede hogere expressie van glycolyse geassocieerde genen gecombineerd met verlaagde expressie van oxidatieve fosforylering gerelateerde genen.
De analyse van de genomische veranderingen gecombineerd met de uitgebreide RNA niveau analyse leidde tot de identificatie van de kandidaat genen *RPS6* en *CDK4*. Deze genen maken deel uit van verder onderzoek, om hun rol in de pathogenese van enchondromen en conventionele centrale chondrosarcomen te verduidelijken.

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CURRICULUM VITAE

Leida B. Rozeman werd geboren op 8 april 1977 te Bilthoven. Na het behalen van het VWO diploma aan het "College Blaucapel" te Utrecht in 1995, begon zij in september 1995 met de studie Biologie aan de Universiteit van Utrecht. Zij liep stages bij Biologie, afdeling "ontwikkelings biologie" onder begeleiding van Dr. W. Dictus met als onderwerp "connexin-30 and –38 expression in Xenopus Leavis" en bij Geneeskunde, afdeling "Medische genetica" onder leiding van Prof. Dr. C. Wijmenga, met als onderwerp "Small nucleotide polymorphisms in Diabetes Melitus type II". In juni 2000 behaalde zij haar doctoraal.
Van januari 2001 tot en met december 2004 voerde zij het hier beschreven promotieonderzoek uit bij de afdeling Pathologie van het Leids Universitair Medisch Centrum, onder leiding van Dr. J.V.M.G. Bovée en Prof. Dr. P.C.W. Hogendoorn.

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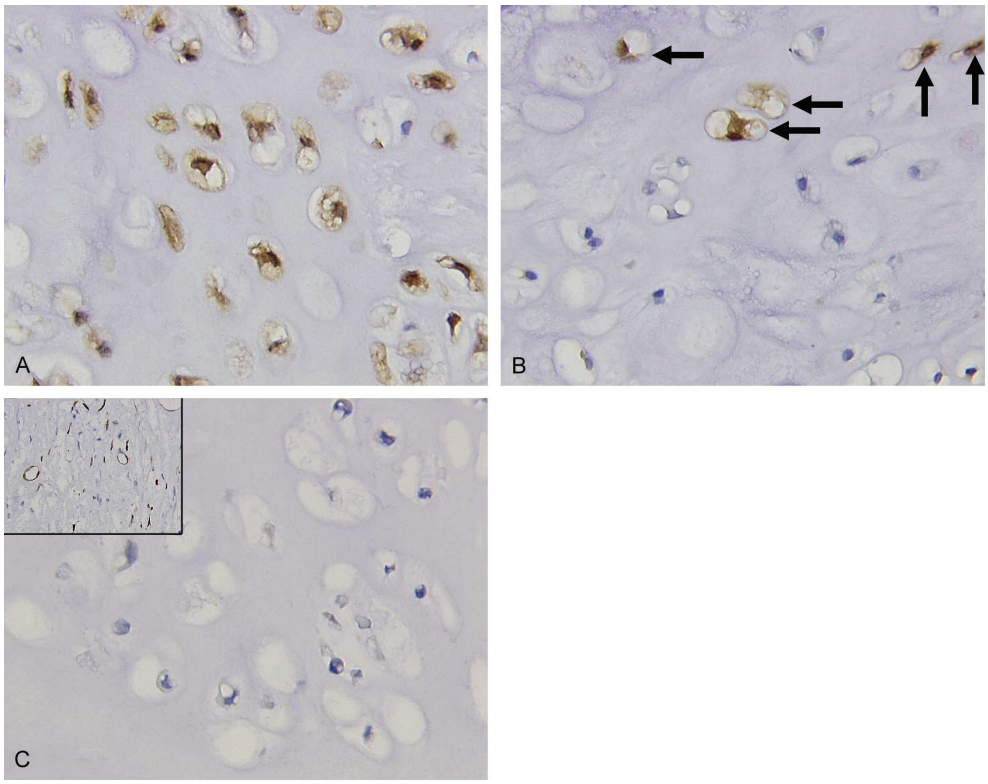

Figure 3.2: *Light micrograph showing the immunohistochemical nuclear and cytoplasmic localization of INK4A/p16. (A) Positive staining of INK4A/p16 in a grade II conventional central chondrosarcoma. (B) Some conventional central chondrosarcomas showed focal positive INK4A/p16 staining. (C) Fifteen per cent of the conventional central chondrosarcomas were entirely negative for INK4A/p16 staining; the inset shows the internal positive control of the tumour (endothelial cells)*

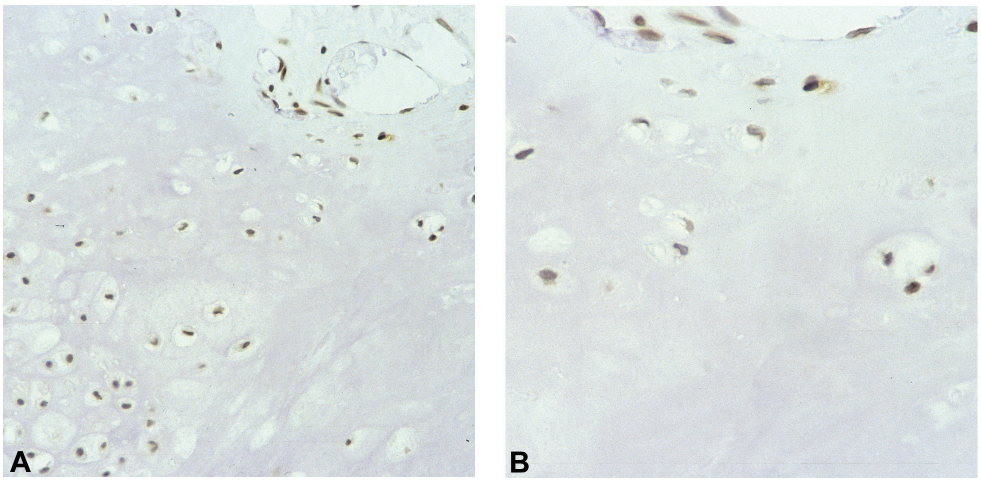

Figure 4.1: *Immunohistochemical staining for the PTHR1 protein. Sample 22.1, an enchondroma of the phalanx, showing positive PTHR1 expression in both nucleus and cytoplasm of tumor cells. The endothelial cells, serving as an internal control, also show PTHR1 expression.* ***A****: 50x,* ***B****: 100x.*

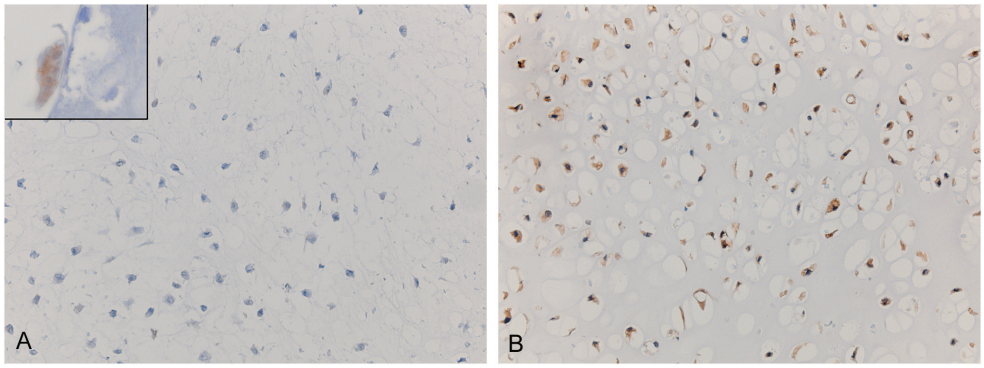

***Figure 5.2**: Lack of PTHLH expression in a subset of phalangeal chondrosarcomas. (A) PTHLH expression is absent in a phalangeal chondrosarcoma, with a positive internal control (inset), while (B) strong expression of PTHLH is found in grade II chondrosarcoma*

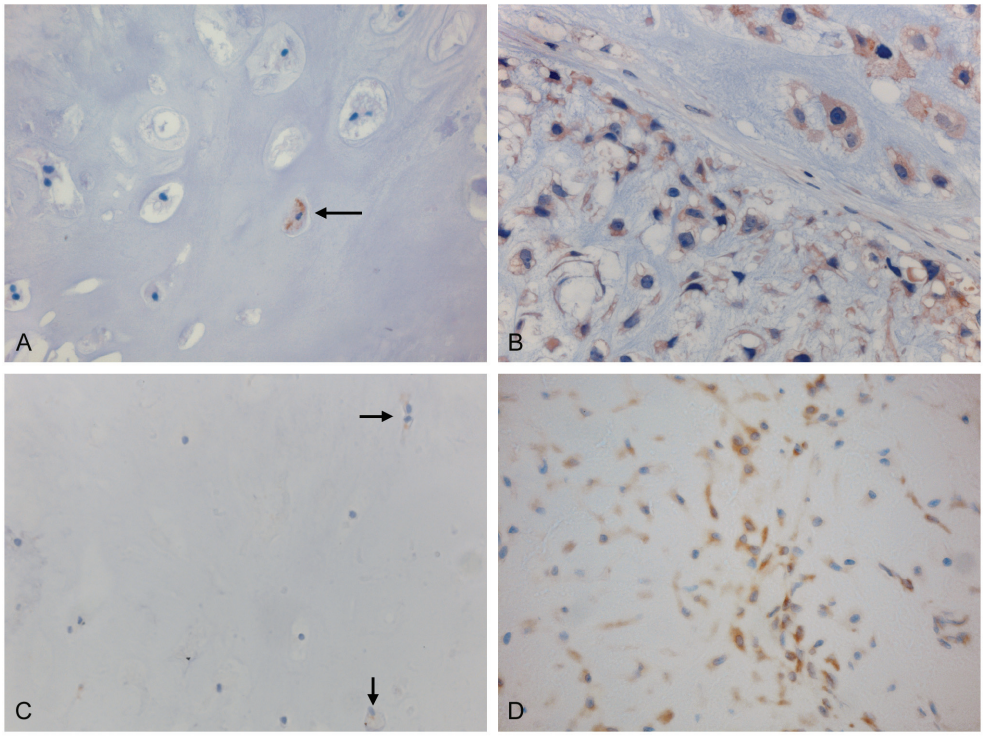

***Figure 5.3**: Immunohistochemical staining for BCL2 and PTHR1 in low- and high-grade conventional central chondrosarcoma. PTHR1 (A, B) and BCL2 (C, D) staining shows low intensity and a low percentage of positive cells in grade I conventional central chondrosarcomas (A and C, arrows), while increased staining is found in high-grade conventional central chondrosarcomas (B and D, grade III)*

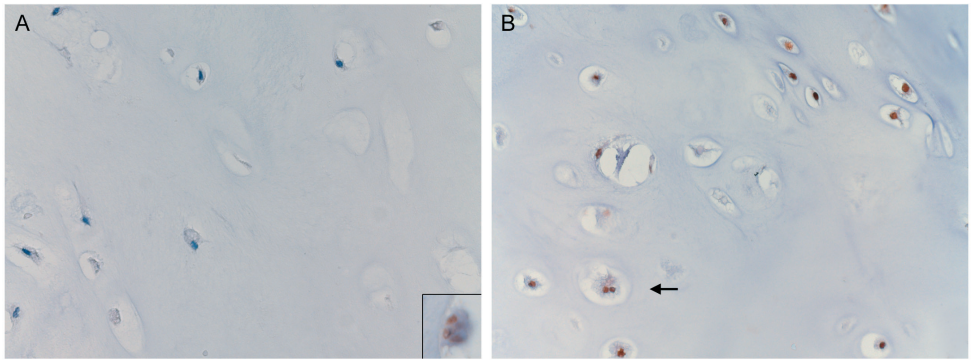

***Figure 6.3**: JunB protein expression in enchondromas and central chondrosarcomas. JUNB immunohistochemical (nuclear) staining demonstrates JunB expression to be absent or very low expressed in enchondromas (**A**), with positive internal controls (inset). Chondrosarcomas were positive in about 50% of the cases (**B**, grade I chondrosarcoma, with arrow pointing to double nucleus). Original magnification 40x*

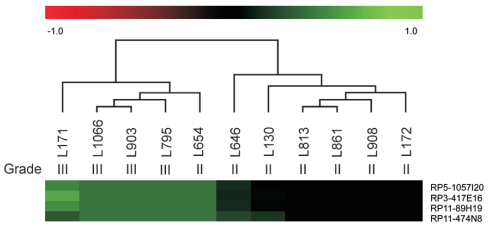

***Figure 7.2:** Hierarchical clustering of the four clones of chromosome 12 differentiating between grade II and grade III chondrosarcomas. Two clusters, one containing mainly grade III (amplified) samples and the other one containing grade II chondrosarcomas, can be discerned*