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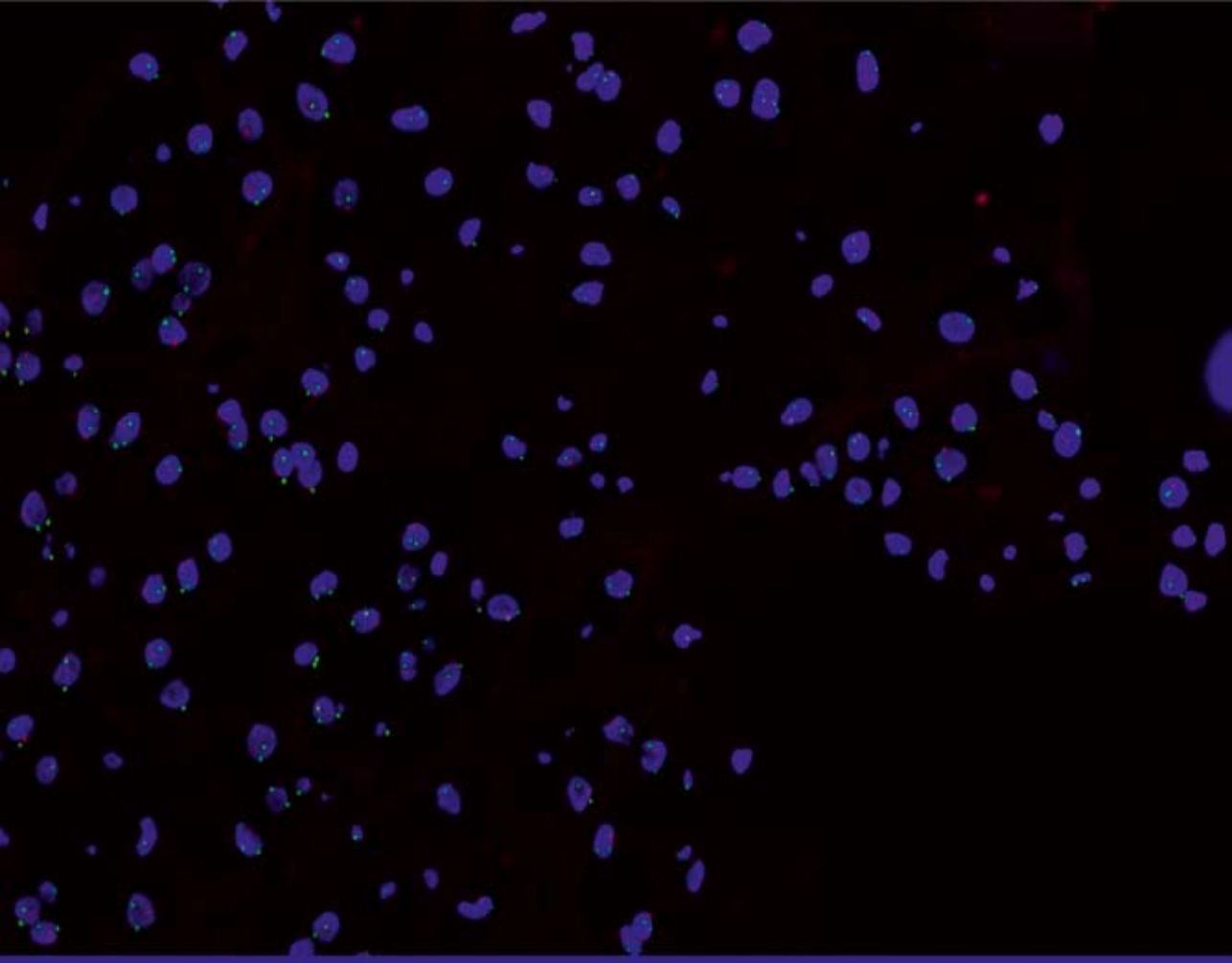

Genetics of Ollier disease and Maffucci syndrome

Twinkal C. Pansuriya

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To honor my parents

To my beloved Rohan

With love to my sisters, brother-in-law and family

Pansuriya, Twinkal Chandubhai

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Contents

Chapter 1

General Introduction

Genetic disorders of the skeleton known as skeletal dysplasia shows diverse manifestations (1). Skeletal dysplasia is a group of skeletal disorders that result from disturbances in the complex processes of skeletal development and growth which constitute a diagnostic challenge due to the rarity of these diseases (2). Mutations are reported either in regulators of skeletal organogenesis, such as cellular signaling (growth factors and its receptors), or in matrix components that affect cartilage and bone tissues (3). There are two main classes of skeletal dysplasia: osteochondrodysplasia and dysostosis. Osteochondrodysplasia develops due to the abnormal growth and development of bone and/or cartilage. In contrast, dysostosis is a developmental anomaly resulting from localized problems in the migration of mesenchymal cells and their condensation (3). The Nosology and Classification of Genetic Skeletal Disorders provides an overview of different diagnostic entities based on clinical and radiographic features and molecular pathogenesis (2). In 2010, in total 456 conditions were described which were divided into 40 different groups. Presently, for 316 of these 456 conditions, the underlying genetic defects are known (2).

Within this classification (2), enchondromatosis syndrome is recognized as a form of osteochondrodysplasia. Patients with enchondromatosis syndrome have multiple enchondromas (benign cartilage forming tumors in the medulla of bone) in their skeleton. The focus of our study concerns the two main subtypes of enchondromatosis syndrome known as Ollier disease and Maffucci syndrome. In addition to multiple enchondromas present in Ollier disease, soft tissue hemangiomas are present in Maffucci syndrome.

Ollier Disease

Ollier disease (OMIM 166000) is a rare, non-familial skeletal disorder (4). Ollier disease was first described by Louis Ollier, a French surgeon in 1889. The disorder is characterized by the presence of at least three enchondromas with an asymmetric distribution and extreme clinical variability (size, number, location, age of onset and requirement of surgery) (4-6).

Maffucci Syndrome

Maffucci syndrome (OMIM 166000) is characterized by presence of multiple enchondromas, resulting in bone deformities, together with soft tissue haemangiomas especially spindle cell hemangiomas or rarely lymphangiomas (4;7;8).

Chondrosarcomas

Enchondromas in Ollier disease and Maffucci syndrome can undergo malignant transformation towards chondrosarcoma. Chondrosarcomas are defined in the 2002 WHO classification as a ''Heterogeneous group of lesions with diverse morphological features and clinical behaviour'' (9). Chondrosarcoma is the third most frequent malignant bone tumor, in which the tumor cells deposit a hyaline cartilaginous matrix (9). The incidence of chondrosarcoma is slightly increased in males compared to females (9). The age of onset varies from 30-60 years (9). The pelvis is the most commonly affected site followed by femur, humerus, and ribs. The small bones of hands and feet, spine and craniofacial bones are rarely affected. Symptoms involve pain and swelling. Myxoid changes, calcification or ossification may be present. Radiographically, the development of chondrosarcoma is manifested as a lytic lesion, cortical erosion or destruction, soft tissue extension and irregularity or indistinctness of the surface of tumor (10).

MRI is used to identify soft tissue extension and the extent of tumor while CT scan can be helpful to see calcified matrix. As chondrosarcomas are highly resistant to chemo- and radiotherapy, surgery is the only option to cure the patients so far (11).

Chondrosarcoma subtypes

Chondrosarcomas can be divided mainly into 5 different subtypes including conventional (80-85%), dedifferentiated (6-10%), periosteal (2%), mesenchymal (2%) and clear cell chondrosarcomas (1%). Periosteal chondrosarcomas (juxtacortical chondrosarcomas) are malignant hyaline cartilage forming tumors located at the surface of the bone, arising from perichondrium and not connected to the original bone (9;12). Dedifferentiated chondrosarcoma contains two clearly distinct components: a well-differentiated cartilage tumor (either an enchondroma or a low grade chondrosarcoma), juxtaposed to a high-grade noncartilaginous sarcoma, with a sharp interface between the two components (13). Conventional chondrosarcomas are most frequent and arise de novo (primary) or from a benign precursor (secondary). Conventional chondrosarcomas are divided into two groups based on their anatomical location in the bone: i) secondary peripheral chondrosarcomas (10-15%) and ii) central chondrosarcomas (85-90%). Chondrosarcomas arising in patients with Ollier disease and Maffucci syndrome are classified as the conventional secondary central subtype.

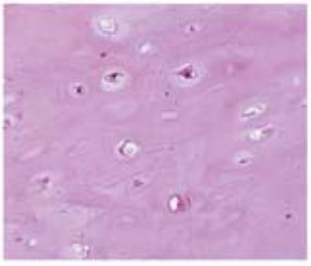
Conventional Chondrosarcomas

I) Secondary peripheral chondrosarcoma develop within the cartilage cap of a pre-existing osteochondroma. Osteochondroma, a benign tumor, is a cartilage capped bony projection arising at the surface of bone (14). Multiple osteochondromas (MO, also known as hereditary multiple exostoses) is an autosomal dominant skeletal dysplasia caused by mutations in *EXT1* or *EXT2* (15-18) and characterized by the presence of multiple osteochondromas (19). Although the involvement of *EXT1* or *EXT2* inactivation in osteochondroma formation is beyond dispute, *EXT1* and *EXT2* are not involved in the progression towards secondary peripheral chondrosarcoma (20). The most frequent location of secondary peripheral chondrosarcoma is ilium, followed by scapula, tibia, femur, pubic bone and ribs.

ii) Central chondrosarcoma arises *de novo* in the medulla of the bone (primary) or from a preexisting benign enchondroma (secondary). Most of the central chondrosarcomas are believed to arise primary. Clinical signs suggestive of malignancy are presence of pain, cortical erosion and extension of the tumor into soft tissues (6;21). Central chondrosarcomas can be found in almost all parts of the skeleton which are formed by enchondral ossification. The most preferential sites include femur, followed by ribs and ilium. The distinction between enchondroma and low grade chondrosarcoma is difficult at radiological as well as at the histological level (22) and slightly subjected to interobserver variability (23;24). Criteria to distinguish include mucoid matrix degeneration of more than 20% and/or presence of host bone entrapment (24). In Ollier disease and Maffucci syndrome more cellularity and atypia is tolerated, making the histological distinction even more difficult.

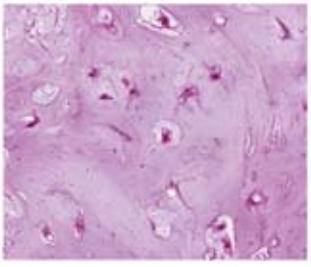
Histologically conventional chondrosarcomas can be divided into three grades (Grade I, II and III) (25) (Figure 1). Grading is so far the most important prognostic predictor for metastasis. The risk of developing metastasis increases with increase in tumor grade. Studies showed that metastases of grade I chondrosarcomas are rare or absent, while 10-33% of grade II and around 70% of grade III chondrosarcomas metastasize (25;26).

Figure 1 Histology of enchondroma and different grades of chondrosarcomas



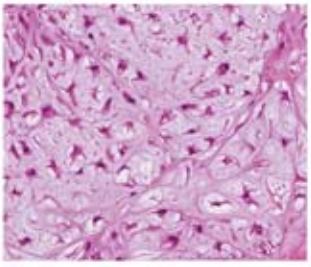
Enchondroma

encasement
low cellularity
no atypia



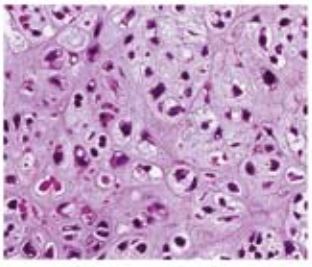
Grade I chondrosarcoma

bone entrapment of preexisting host bone
presence of small, densely staining nuclei
chondroid or myxoid matrix



Grade II chondrosarcoma

nuclei are of moderate size
increased cellularity
myxoid matrix
presence of mitosis



Grade III chondrosarcoma

pleomorphic nuclei
high cellularity
myxoid matrix
presence of mitosis
spindle cell shaped cells at the edge of lobules

Approaches to understand complex diseases

Complex diseases are caused by a combination of genetic and environmental factors. For most of these diseases it is difficult to identify the cause as they do not obey standard Mendelian patterns of inheritance. Genomics is the field of study helping to understand a role of the genome in the development of particular diseases. The Human Genome Project has changed the view of researchers to gain new insights in pathogenesis of diseases. The known role of copy-number alterations in sporadic genomic disorders, combined with emerging information about inherited copynumber variation, indicate the importance of assessing copy-number variants (CNVs), including common copy-number polymorphisms, involved in disease (27). In principle, complex disease can be explained by the presence of particular single nucleotide polymorphisms (SNPs) or CNVs or variation in noncoding sequences associated with diseases. As Ollier disease and Maffucci syndrome are non-inherited disorders, we first started off with a genome-wide analysis to search for candidate regions involved in these disorders. We used high resolution SNP array combined with expression arrays.

Genome-wide approach

Single Nucleotide Polymorphism (SNP) Arrays

SNP arrays offer an opportunity to identify copy number changes together with loss of heterozygosity (LOH) events occurring in the tumor, throughout the genome. SNPs are variable positions in the genome with two different allelic types when the frequency of the minor allele exceeds 1% in at least one population (28;29). SNP arrays are an ideal platform to map somatic and germline genetic alterations (30-33). Only SNPs which are heterozygous in germline are informative as an indication of possible LOH. Paired normal DNA is not always available and therefore, an advantage of SNP arrays are that it provide marker densities that enable the identification of LOH regions, without using germline paired DNA. SNP frequency has an uneven distribution throughout the whole genome. Including copy number variation (CNV) probes to a SNP array platform like Affymetrix SNP 6.0 array can compensate for the unevenness of the SNPs. SNPs are much less frequent inside coding areas including exomic regions, therefore the detection rate of genetic changes that encompass only one or a few exons especially for small genes is rather limited. Also, balanced translocations and point mutations can not be detected by SNP arrays.

Gene Expression Array

Microarrays are used as a routine tool for molecular profiling, identification of new targets and biomarker discoveries in biomedical research (34). Microarray technology has allowed the abundance of thousands of different mRNAs to be measured simultaneously in a given sample using a single hybridization reaction (35;36). Therefore, analysis of individual genes has provided an opportunity to analyze large sets of genes and relationships in their expression (35). Interpretation of the results to gain insight into biological mechanisms is still challenging. There are some critical issues such as correct selection of samples, proper experimental design, sample collection, preparation of targeted RNA, integrity and purity of RNA related to microarrays in order to guarantee the quality and reproducibility of the obtained data (36).

Genomic changes (amplification or deletions) often comprise 10s 100s of genes, therefore integration of copy number changes and expression arrays might be more informative.

Expression changes (up or down regulation) of genes residing in copy number alteration (CNA) regions of the genome might identify genes important in the pathogenesis of tumors.

Methylation array

DNA methylation plays a critical role in regulating gene expression and cellular functions during normal development as well as in carcinogenesis (37). Methylation is largely known as epigenetic modification of DNA (38). Methylation of DNA occurs exclusively in 5-cytosine and in mammals, the majority of cytosine methylation occurs in CpG sites. Non-CpG methylation is rare (39). CpG islands are present in ~70% of human promoters (40).

Epigenetic changes (which alter the gene expression) have been recognized as one of the most important molecular signatures of the tumors in recent years. These alterations comprise of hypermethylation of tumor suppressor genes or hypomethylation of oncogenes (38). The exact mechanism of aberrant methylation is still unknown. Methylation profiling helps to understand the nature of gene regulation in cells, and also the epigenetic mechanisms of interactions between cells and environment (37). There are three methods available for DNA methylation profiling which includes I) discrimination of bisulfite induced C to T transition, ii) cleavage of genomic DNA by methylation sensitive restriction enzymes and iii) immunoprecipitation with methyl-binding proteins or antibodies against methylated cytosine (37).

Some of these approaches permit the investigation of the limited number of methylated regions at a time. Whereas, microarray and sequencing based DNA methylation profiling technologies have been developed in order to assess methylation status for a large number of genes or even the entire genome.

Hypothesis driven approach

Based on the literature related to chondrocyte differentiation and enchondral bone formation, one could postulate a number of candidate genes for Ollier disease and/or Maffucci syndrome.

1. *NDST1*

EXT1 or *EXT2* are known to be involved in osteochondroma (14-16). While it is evident that inactivation of *EXT1* or *EXT2* is the driving force for the development of benign peripheral cartilaginous tumors, they are not involved in central chondrosarcoma and expression of these genes was comparable to the growth plate (41). The EXT proteins are glycosyltransferases responsible for the elongation of heparan sulfate (HS) chains (42;43). HS is a large complex carbohydrate that binds various growth factors and enzymes and its assembly involves three steps I) chain initiation ii) chain elongation iii) chain modification.

I) chain initiation occurs when four sugars are attached to specific serine residue of the core proteins. ii) elongation steps involve N-acetyl glucosamine and glucoronic acids which are alternatively added by copolymerases encoded by EXT1 or EXT2. iii) chain modification involves Ndeacetylation/ N-sulfation (NDST1), C5-epimerization, 2-O sulfation of uronic acids and 3-O and 6-O sulfation of glucosamine residues. Presto et al. proposed a GAGosome model in which cells over-expressing NDST1 and EXT2, NDST1 competes with EXT1 to bind to EXT2 and will form heteroduplex (44). Binding of more NDST1 to EXT2 might alter formation and localization of HS. The role of EXT1, NDST1 and HS is unknown in enchondromas and chondrosarcomas related to Ollier disease and Maffucci syndrome.

2. *PTH1R*

Enchondroma might arise as a result of abnormal regulation of pathways involved in chondrocyte proliferation and differentiation. One of the most important signaling pathways is the Indian Hedgehog/Parathyroid Hormone Like Hormone IHH/PTHLH negative feed back loop (45). Prehypertrophic chondrocytes secrete IHH which will bind to its receptor Patched (PTCH) which will result in increased secretion of PTHLH. PTHLH will bind to its receptor PTH1R which will inhibit further differentiation of chondrocytes by up-regulating BCL2, resulting in less IHH producing cells (46). PTH1R and IHH pathways are tightly coupled and therefore reduced PTH1R signaling could lead to impaired chondrocyte proliferation and differentiation.

Previously, a R150C *PTH1R* (3p22 (47)-p21.1) point mutation was reported in two out of six patients with constitutively active IHH signaling (48) but an elaborative study on 28 Ollier patients failed to detect any mutations in *PTHR1* by our group (49). G121E PTH1R, A122T *PTH1R* and R255H *PTH1R* mutations were subsequently found in 3 out of 14 Ollier patients (50). Two heterozygous mutations, G121E *PTH1R* and A122T *PTH1R* were present only in enchondroma from an Ollier patient while R255H *PTH1R* was present in tumor as well as in leukocyte DNA. All these mutations were claimed to alter the ligand affinity of the receptor as well as its expression at the cell surface and ultimately impaired its function (50). In total four different heterozygous mutations were reported. Thus, heterozygous *PTHR1* mutations may contribute or act as a modifier in small subset of Ollier patients (48-50).

3. *IDH1* and *IDH2*

Gliomas are the most frequent non-cartilaginous tumors associated with Ollier disease (51;52). Also, six patients with Maffucci syndrome having a glioma have been reported in the literature (52-57). Glioma is the most common type of primary brain tumor (58). Heterozygous mutations at the R132 codon of isocitrate dehydrogenase 1 (*IDH1*) and R172 codon of isocitrate dehydrogenase 2 (*IDH2*), mutations were described for up to 70% of gliomas (59). Mutations in *IDH1* and *IDH2* are mutually exclusive with rare exceptions, which suggest mutation in either of these two isoforms is sufficient to confer growth advantage and/or cell survival (60). Mutations in *IDH1* or *IDH2* were also reported for solitary central and periosteal chondrosarcomas as well as for few patients with enchondromatosis syndrome (61).

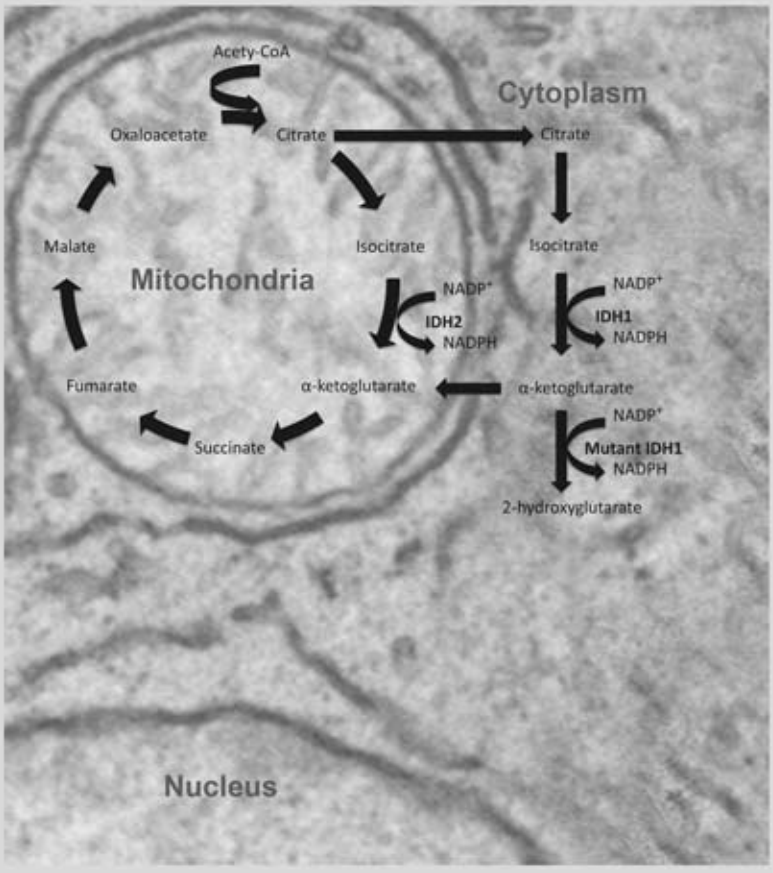
IDH catalyzes the oxidative decarboxylation of isocitrate to α- ketoglutarate (α-KG) and reduce NAD(P+) to NAD(P)H (Figure 2). IDH1 is located in the cytoplasm and in the peroxisome while IDH2 is located in the mitochondria. They are involved in lipid metabolism and in the Krebs cycle (Figure 2). Mutant IDH1 or IDH2 leads to gain of function by producing 2- hydroxyglatarate (2HG), a structural analogue of α-KG (62) and ultimately lead to reduction in α-KG production (Figure 2). Based on the increased incidence of gliomas and *IDH* mutations in solitary central cartilaginous tumors, we hypothesized that *IDH1* and/or *IDH2* mutations may occur in patients with Ollier disease and/or Maffucci syndrome.

4. *GNAS*

Like gliomas, juvenile ovarian granulosa cell tumors show an increased incidence in patients with Ollier disease and Maffucci syndrome (52;53;63;64).

Mutations in *GNAS* at R201 were reported in 30% of juvenile ovarian granulosa cell tumors (65). Apart from this, somatic mosaic *GNAS* mutations are also found in McCune-Albright syndrome, which is a non hereditary disorder characterized by polyostotic fibrous dysplasia combined with endocrinopathies (66). Based on the association of juvenile ovarian granulosa cell tumors with Ollier disease and Maffucci syndrome, we hypothesized that *GNAS* mutations might be present in these patients.

Figure 2 Role of IDH1 and IDH2



Enzymes of TCA cycle in the mitochondria and mutated IDH in the cytosol are represented. Mutant IDH1 produces 2HG and reduces the amount of α -KG.

Aim of the investigation and outline of the thesis

The main purpose of the studies described in this thesis is to find the genetic deficit in Ollier disease and Maffucci syndrome and understand their functional consequences. As Ollier disease and Maffucci syndrome are very rare, non-inherited syndromes with a unilateral predominance of the multiple enchondromas, we hypothesized the presence of somatic mosaicism with an early post zygotic mutation resulting in asymmetric involvement of skeletal structures, similar to McCune Albright syndrome caused by somatic mosaic *GNAS* mutations, (67) .

We first searched the literature and present a detailed overview of all different subtypes of enchondromatosis syndrome in **Chapter 2.**

In **Chapter 3** and **4**, the Affymetrix Genome-Wide Human SNP Array 6.0 platform was used to identify candidate gene/genes for Ollier disease **(Chapter 3)** and Maffucci syndrome **(Chapter 4)**. The arrays contain 1.8 million reporters, including more than 906,600 SNPs and 946,000 probes for the detection of CNV. We compared genotypes between tumor and paired blood or saliva DNA. We have integrated copy number variation results with Illumina genome-wide expression v3 array and selected few candidate genes for enchondroma development.

In **Chapter 5** and **6**, a hypothesis driven approach was used to study the five genes as outlined above. As described earlier in detail, *NDST1* was selected as a candidate gene and other components of heparan sulfate pathway were analyzed in **Chapter 5**. Since a small subset of patients with Ollier disease showed mutations in *PTH1R*, we also performed mutation analysis as described in **Chapter 6**. Based on the increased incidence of gliomas and juvenile granulosa cell tumors in Ollier disease and Maffucci syndrome, and mutation studies on non-syndromal chondrosarcomas, we evaluated the occurrence of *IDH1, IDH2* and *GNAS* mutations. In addition we performed epigenetic studies to investigate the mechanism of enchondroma development using Illumina HumanMethylation arrays in Ollier disease and Maffucci syndrome as described in **Chapter 6**. Finally, results are summarized and discussed in **Chapter 7**.

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Chapter 2

Enchondromatosis: insights on the different subtypes

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Abstract

Enchondromatosis is a rare, heterogeneous skeletaldisorder in which patients have multiple enchondromas. Enchondromas are benign hyaline cartilage forming tumors in the medulla of metaphyseal bone. The disorder manifests itself early in childhood without any significant gender bias. Enchondromatosis encompasses several different subtypes of which Ollier disease and Maffucci syndrome are most common, while the other subtypes (metachondromatosis, genochondromatosis, spondyloenchondrodysplasia, dysspondyloenchondromatosis and cheirospondyloenchondromatosis) are extremely rare. Most subtypes are non-hereditary, while some are autosomal dominant or recessive. The gene(s) causing the different enchondromatosis syndromes are largely unknown. They should be distinguished and adequately diagnosed, not only to guide therapeutic decisions and genetic counseling, but also with respect to research into their etiology. For a long time enchondromas have been considered a developmental disorder caused by the failure of normal endochondral bone formation. With the identification of genetic ab-ormalities in enchondromas however, they were being thought of as neoplasms. Active hedgehog signaling is reported to be important for enchondroma development and *PTH1R* mutations have been identified in ~10% of Ollier patients. One can therefore speculate that the gene(s) causing the different enchondromatosis subtypes are involved in hedgehog/PTH1R growth plate signaling. Adequate distinction within future studies will shed light on whether these subtypes are different ends of a spectrum caused by a single gene, or that they represent truely different diseases. We therefore review the available clinical information for all enchondromatosis subtypes and discuss the little molecular data available hinting towards their cause

Keywords.

Ollier disease, Maffucci syndrome, enchondroma, metachondromatosis, enchondromatosis, central chondrosarcoma

Introduction

Enchondromas are common, benign, and usually asymptomatic hyaline cartilage forming neoplasms in the metaphyses and diaphyses of the short and long tubular bones of the limbs, especially the hands and feet [1,2]. They usually occur as a single lesion (solitary enchondroma) which is most often found incidentally when radiographic studies are performed for other reasons. Occasionally patients present with multiple enchondromas causing severe deformity of the affected bones, generally defined as enchondromatosis [2,3]. The distribution of the enchondromas, and other accompanying symptoms as well as the mode of inheritance define the different subtypes of enchondromatosis (Figure 1), which mainly includes Ollier disease, Maffucci syndrome, metachondromatosis, genochondromatosis, spondyloenchondrodysplasia, cheirospondylo-enchondromatosis and dysspondyloenchondromatosis. These subtypes should be distinguished and adequately diagnosed, not only to guide therapeutic decision and genetic counseling, but also to enable future studies to shed light on whether these are different ends of a spectrum caused by a single gene. or that they represent true different diseases. We therefore review the available clinical information for all enchondromatosis subtypes and discuss the little molecular data available hinting towards their cause.

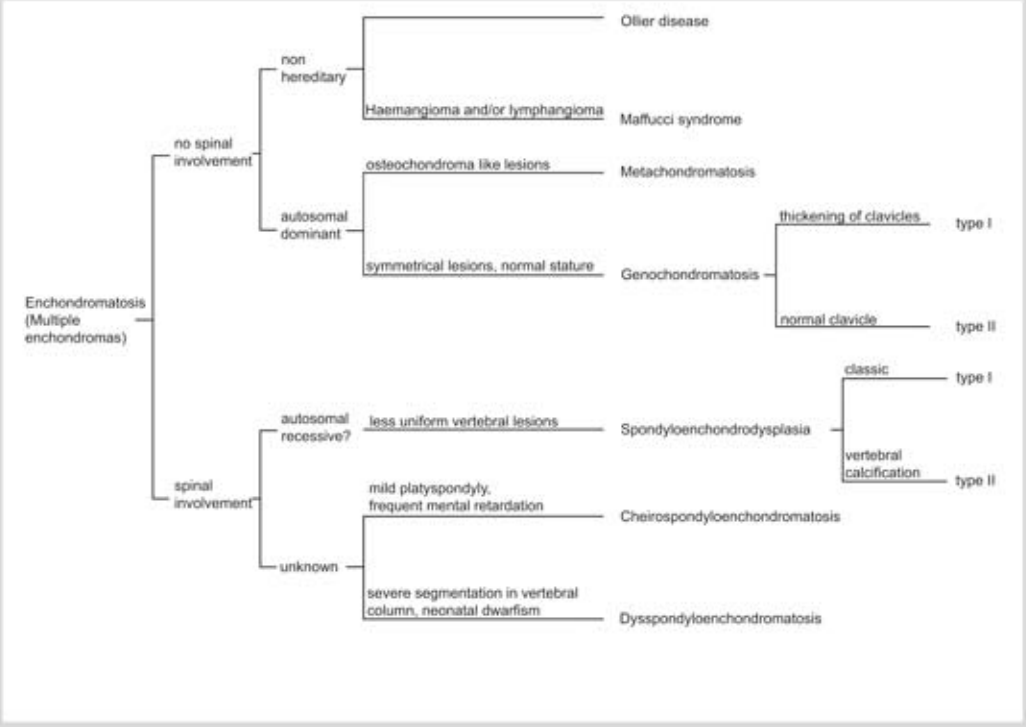
Enchondroma

On conventional radiographs enchondromas present as multiple, oval-shaped, linear and/or pyramidal osteolytic lesions with well-defined margins in the metaphysis and/or diaphysis of the long tubular and in the flat bones [4]. Magnetic resonance (MR) studies demonstrate lobulated lesions with intermediate signal intensity on T1-weighted images and predominantly high signal intensity on T2-weighted sequences [5]. Histologically enchondromas show low cellularity with an abundant hyaline cartilage matrix sometimes with extensive calcifications [1]. When enchondromas are located in the phalangeal bones or when they occur in enchondromatosis patients, cellularity is increased and more worrisome histological features are tolerated, since they are not correlated with malignant behavior in this specific context (Figure 2) [1]. Treatment of solitary enchondroma is surgical but only in case of complains or cosmetic deformity [6]. In case of enchondromatosis, the deformities as well as malignant progression of enchondromas may require multiple surgical interventions [7-12].

Secondary central chondrosarcoma

While solitary enchondromas almost never progress to secondary central chondrosarcoma, malignant transformation in enchondromatosis is estimated to occur in 25-30% of the patients [1]. Central chondrosarcoma is a malignant bone tumor forming hyaline cartilage and arising centrally in the medullary cavity of bone [13]. The distinction between enchondroma and low grade chondrosarcoma is difficult on conventional radiographs [14]. Fast contrast-enhanced dynamic MRI is more helpful in this regard [15].

Figure 1 Enchondromatosis sub-types



Classification diagram for patients with multiple enchondromas based on spinal involvement and genetic transmission.

At the histological level the distinction can also be very difficult and is subject to interobserver variability [16] [17]. Low grade chondrosarcomas (grade I) can be treated surgically with local curettage combined with cryosurgery or phenol treatment while resection and reconstruction is obligatory in case of grade II or III chondrosarcoma [18].

Enchondromagenesis

The underlying mechanism for enchondroma development is largely unknown. Several cytogenetic/genetic reports are present in the literature using solitary enchondromas, suggesting these lesions to be neoplastic (http://atlasgeneticsoncology.org//Tumors/EnchondromaID5333.html). Enchondromas in Ollier disease are comparable to solitary enchondromas at m-RNA expression level [19].

Since enchondromas arise in the metaphysis in close proximity to the growth plate, they may result from failure of terminal differentiation of growth plate chondrocytes. In support of this, transgenic mice expressing the hedgehog (Hh) regulated transcription factor *Gli2* in chondrocytes, which mimics activated Hh signaling, develop lesions similar to human enchondromas [20]. Hedgehog signaling is a crucial regulator of normal chondrocytes proliferation and differentiation within the normal growth plate. Enchondromas indeed demonstrate levels of hedgehog signaling that are comparable to normal growth plate [20-23].

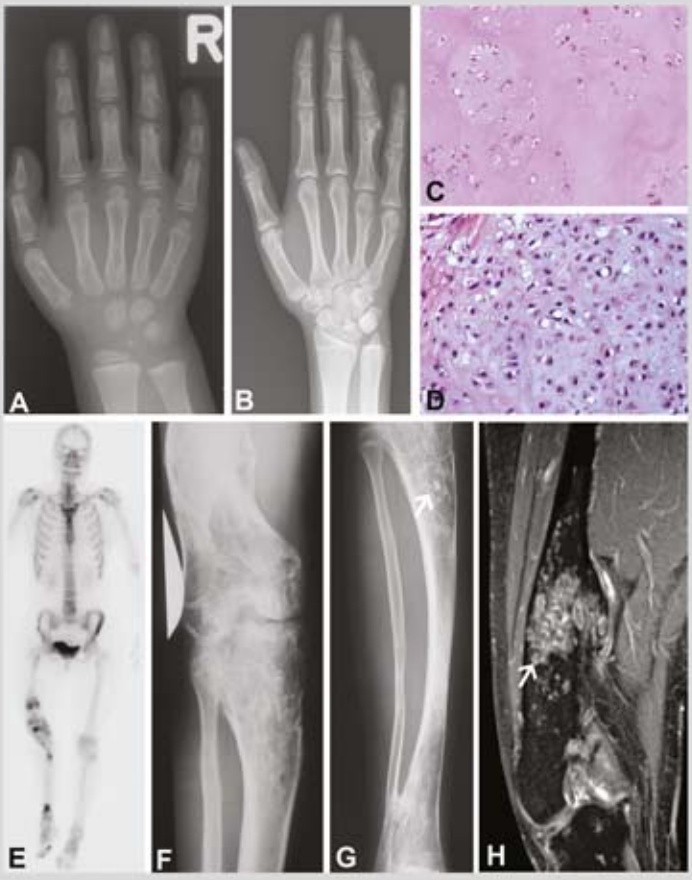
Additionally, ten percent of patients with en-chondromatosis harbour a mutation in the PTHLH receptor, *PTH1R*, in their tumor tissue [20,22,23]. The mutations were shown to decrease the function of the PTHLH receptor with ~30% [22]. PTH1R is a receptor for parathyroid hormone and for parathyroid hormone-related peptide which acts in a negative feedback loop with Indian Hedgehog (IHH) regulating normal endochondral bone formation [24-26]. PTHLH signaling is active in solitary enchondromas and in chondrosarcomas [27]. In parallel, multiple osteochondromas (MO) syndrome (multiple benign cartilaginous tumors arising from the surface of bone) is an auto-somal dominant disorder caused by mutations in the *EXT1* and *EXT2* genes, leading to dis-turbed hedgehog signaling based on their involvement in heparan sulphate (HS) biosynthesis [28-30]. The *EXT* genes are not affected in central chondrosarcomas and their m-RNA expression is normal [21]. It may be hypothesized that the genes causing the different enchondro-matosis subtypes also affect the HS dependent signaling pathways.

Ollier disease

Ollier disease (also known as dyschondroplasia, multiple cartilaginous enchondromatosis, enchondromatosis Spranger type I), is the most common subtype, first described in 1889. It is defined by the presence of multiple enchondromas with asymmetric distribution (Figure 2) [4,31-34]. Ollier disease is non-familial and mostly encountered early in childhood, affecting both sexes equally. The estimated prevalence of Ollier disease is 1/100,000 [2]. The true incidence can be higher since mild phenotypes without skeletal deformities can go undetected. Few cases of familial occurrence have been reported (OMIM 166000) [35-37] There is large clinical variability with respect to size, number, location, age of onset, and requirement of surgery [1,2,4,34]. Lesions are usually distributed unilaterally and may involve the entire skeleton, although the skull and vertebral bodies are very rarely involved. Sometimes lesions are bilateral or present in only one extremity [4].

Malignant transformation of one or more enchondromas towards secondary central chondrosarcoma is estimated to occur in 5-50% of the patients and can be life threatening [38-42]. Malignant transformation most frequently occurs in long tubular and flat bones while this is far less common in the small bones of hands and feet (Figure 2). This is interesting since enchondromas preferentially occur at the hands and feet. In addition to the risk of developing chondrosarcoma, Ollier patients also seem to have an increased risk for the development of non-skeletal malignancies as reported in Table 1, especially intracranial tumors of glial origin [43]. There is at present no curative or preventive treatment option for patients with Ollier disease. The underlying cause of Ollier disease is so far unknown. The nonhereditary asymmetrical polyostotic distribution of the lesions might suggest a somatic mosaic mutation [14]. This is similar to McCune-Albright syndrome / polyostotic fibrous dysplasia in which an activating mutation in *GNAS1* occurs during early em-bryogenesis leading to a somatic mosaic state resulting in fibrous dysplasia affecting several bones [44,45].

Figure 2 Ollier disease



(A) 4-year-old female patient with Ollier disease. Multiple enchondromas, manifesting as central end eccentric osteolytic lesions and deformities in the metacarpals and phalanges of the fourth and fifth ray of the right hand. (B) Same patient as in Figure 2A 13 years later. The enchondromas have increased in size and some are more evidently visible compared to the previous study. This has resulted in deformity of the fourth finger. (C) Histology of enchondroma of long bone from Ollier patient showing moderate cellularity and abundance of hyaline cartilage matrix (200 times magnification). (D) Histology of chondrosarcoma grade II of the long bone from Ollier patient showing increased cellularity and atypical chondrocytes (200 times magnification).

(E) Technetium-99m bone scintigraphy, anterior projection, demonstrates shortening of the right lower limb. Varus deformity of the femur and valgus deform-ity of the tibia. Multiple areas of focally increased uptake of the tracer in femur and tibia. (F-G) Anteroposterior radiograph of the right knee and lower leg of same patient as 2E. Deformity of both the distal femur and the tibia and fibula. Structural changes in the marrow cavity and cortical bone of femur and tibia consisting of osteolysis and osteosclerosis. Specifically in the proximal tibia areas with mineralization in the sense of calcifications can be appreciated (arrow). The appearance is consistent with multiple chondromatous tumors. (H) Coronal fatsuppressed T1-weighted magnetic resonance image after intravenous contrast administration of the femur. Varus deformation of the femur. Multiple, partially lobulated, areas with increased signal intensity due to enhancement of the chondromatous lesions. Large lesion in the distal diaphysis of the femur, of which histology showed a chondrosarcoma (arrow). The enhancement demonstrates rings and arcs (also known as septal or nodular enhancement) consistent with the chondromatous nature of the lesions.

Not many genetic studies are reported for Ollier tumors due to the rarity of the disease (summarized in Table 2). As discussed above, four different heterozygous mutations, affecting either the germ line or only the tumor tissue, were found in the *PTH1R* gene (R150C, R255H, G121E and A122T) [20,22,23] in 5 of 48 Ollier patients (~10%). Thus, *PTH1R* mutations may contribute to the disease in a small subset of Ollier patients but is probably not causative for the disease [22].

Table 1. Non-cartilaginous malignancies associated with Ollier disease

Associated tumors	No. of patients	References
Glioma	17	[38,43,90-99]
Juvenile granulosa cell tumor	7	[38,100-105]
Non-small cell lung cancer	1	[106]

Table 2 Genetic findings in Ollier enchondromas and chondrosarcoma

Tumor per patient	Technique used	Results of chromosomal abnormality	References
low grade CS	cytogenetics	deletion at 1p	[108]
high grade CS	microsatellite marker, SSCP, IHC	LOH at 13q14 (RB1), 9p21 and over expression of TP53	[109]
Enchondroma	array-CGH	noalteration	[110]
Enchondroma	array-CGH	deletion of 6	
CS II	array-CGH	gain at 1, 2, 5, 7, 8, 9, 15, 16, 17, 18, 19, 20, 21 and 22	[110]
CS II	array-CGH	gain at 6, 7, 12, 14, 15, 16, 17, 18, 19 and loss at 1, 3, 4, 6, 9, 10, 13, 15, 16 and 22	[110]

Maffucci syndrome

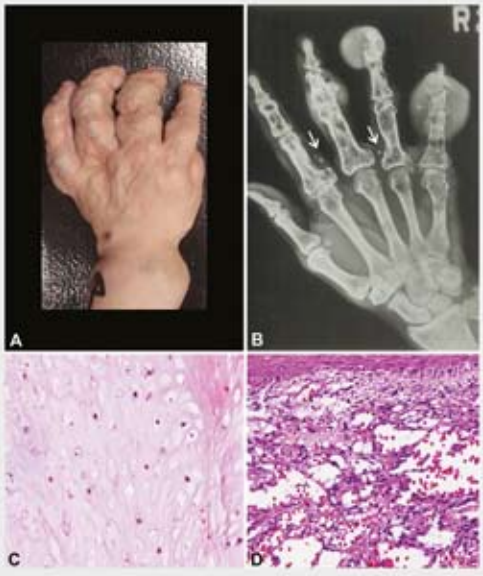
Maffucci syndrome (also known as dyschondrodysplasia with haemangiomas, enchondromatosis with multiple cavernous haemangiomas, Kast syndrome, haemangiomatosis chondrodystrophica, enchondromatosis Spranger type II) was first described in 1881[32,46,47]. It is non-hereditary and characterized by the presence of multiple enchondromas combined with multiple haemangiomas of soft tissue or, less commonly, lymphangiomas (Figure 3) [34, 48]. Lesions are asymmetrically distributed and there is no gender discrimination. The disease appears to develop in 25% of cases from the time of birth or during the first year of life, in 45% of cases symptoms start before the age of 6 and in 78% of cases symptoms developed before puberty [49,50]. Lewis et al reviewed ninety-eight cases and showed that hand, foot, femur, tibia, and fibula were most frequently affected by enchondromas [50].

Haemangiomas are benign vascular tumors which often protrude as bluish or reddish soft nodules. They can be found anywhere in the body. In addition to the enchondromas, radiographs can show phleboliths, associated with soft tissue calcifications in haemangiomas. Histologically, haemangiomas can be of the capillary or cavernous subtype. Spindle cell haemangioendothelioma is more specific for Maffucci syndrome [51,52]. Both the enchondromas and the vascular lesions may progress to malignancy. The risk of malignancy is higher than in Ollier disease [2,49]. When considering intracranial tumors, the majority is of mesenchymal origin and includes secondary central chondrosarcoma and angiosarcoma associated with Maffucci syndrome [43]. Non-mesodermal tumors associated with Maffucci syndrome are summarized in Table 3.

While in Ollier disease intracranial tumors other than chondrosarcomas of the cranium are exclusively of glial origin, in Maffucci syndrome different tumor types are encountered [43]. Also, patients with Maffucci syndrome are almost 10 years older when developing intracranial tumors, and are more likely to live in Asia or South America as compared to Ollier disease [43].

Genetic studies on Maffucci syndrome are sparse. An inversion of chromosome bands p11 and q21 of chromosome 1 were reported in one patient with Maffucci syndrome [53]. Robinson et al showed an increased number of nerve fibers in tumors as well as in normal tissue of Maffucci patients, while in enchondroma and haemangioma tissue numerous methionine enkephalin positive nerves were detected, serving as a growth factor for cartilage proliferation [54]. In total, 26 patients with Maffucci syndrome were screened for mutations in *PTH1R* and revealed absence of mutation [23,55].

Figure 3 Maffucci Syndrome



(A) Hand of a patient with Maffucci syndrome showing deformities due to multiple enchondromas and a superficial haemangioma. (B) Radiograph of a patient with Maffucci syndrome. Multiple enchondromas with and without soft tissue extension in the second to fifth digit and the fifth metacarpal bone. In addition phleboliths in the soft tissue at the basis of the second and fourth finger (arrows) indicating haemangiomas. (C) Histology of enchondroma and (D) Haemangioma (400 times magnification).

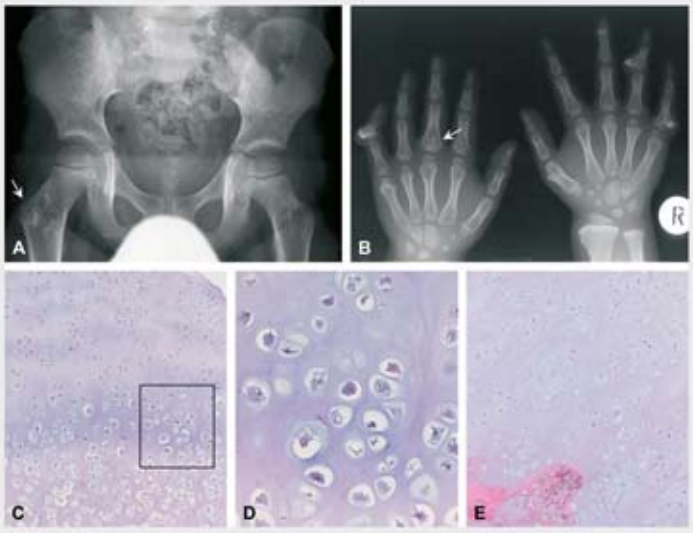
Table 3 Non-cartilaginous and non-vascular neoplasms associated with Maffucci syndrome

Associated tumors	No. of patients	References
Astrocytoma	6	[38,50,111-113]
Pituitary adenoma	4	[50,114-116]
Juvenile granulosa cell tumor	6	[50,117-120]
Pancreatic adenocarcinoma	3	[38,50,121]
Adrenal adenoma	1	[50]
Intracranial chordoma	1	[122]
Biliary adenocarcinoma	1	[38]
Olfactory neuroblastoma	1	[123]
Paraganglioma	1	[124]
Fibrosarcoma	1	[50]
Thyroid adenoma	1	[51]
Hepatic adenocarcinoma	1	[121]
Fibro adenoma breast	2	[125]
Breast carcinoma	1	[114]
Squamous cell carcinoma	1	[126]
Fibroadenoma of thorax and canalicular adenoma	1	[127]
Acute myeloblastic leukemia	1	[51]
Lymphoid leukemia	1	[128]
Ovarian fibrosarcoma	1	[129]

Metachondromatosis

This rare hereditary condition displays a combination of multiple enchondromas with multiple osteochondroma-like lesions [32,56,57] (MIM 156250, enchondromatosis Spranger type III). The enchondromas mainly involve the iliac crests and metaphyses of the long bones of the lower extremities while the osteochondroma-like lesions are mainly located in hands and feet [58]. The syndrome manifests early in childhood [59,60]. In contrast to conventional osteochondromas, the osteochondroma-like lesions in metachondromatosis point towards the joint, do not cause bone deformities, and may regress spontaneously [3,59] (Figure 4). Importantly, malignant transformation has not been reported. Avascular necrosis of the femoral epiphysis can be found due to interference with the integrity of the lateral epiphyseal vessels by enchondromas [59,61-63].

Figure 4 Metachondromatosis



(A) Radiograph of the pelvis with an enchondroma in the right proximal femur (arrow) adjacent to the apophysis of the greater trochanter; (B) radiograph of both hands showing multiple osteochondromas pointing towards the epiphyses. Enchondroma in the proximal phalanx of the left third digit (arrow); (C, E) micrographs of osteochondroma-like lesions (D) magnification of C. These lesions are histologically indistinguishable from conventional osteochondroma recapitulating the normal growth plate architecture (reproduced with permission from [64]).

The mode of inheritance is autosomal dominant but the underlying gene has not been identified so far, due to the extreme rarity of the disease. In a single case, mutations in the EXT genes causing multiple osteochondromas were absent and EXT mRNA expression levels were normal. IHH/PTHLH signaling was normally active in two cases. These data suggest that EXT related pathways are not involved in the pathogenesis of metachondromatosis [64].

Genochondromatosis

Genochondromatosis is an extremely rare autosomal dominant disorder manifesting itself in childhood [65](MIM 137360). Patients have normal stature and enchondromas are distributed symmetrically with characteristic localization in the metaphyses of the proximal humerus and distal femur. Two subtypes are distinguished: genochondromatosis type I includes the presence of a chondroma on the medial side of the clavicle while in type II the short tubular bones of the hand, wrist and feet are affected while the clavicle is normal [65-68]. The enchondroma like lesions will not lead to any bone deformities, may be discovered accidentally, and tend to regress in adulthood [65] in which they differ from the enchondromas in the other subtypes. Moreover, no malignancies associated with genochondromatosis have been described in the literature. No spinal involvement is reported which emphasizes it being different from spondyloenchondrodysplasia, cheirospondyloenchondromatosis and dysspondyloenchondromatosis [3]. No genetic studies have been reported for this rare subtype.

Spondyloenchondrodysplasia

Spondyloenchondrodysplasia (SPENCD, enchondromatosis Spranger type IV) is an autosomal recessive inherited disorder characterized by vertebral dysplasia combined with enchondroma like lesions in the pelvis or long tubular bones (MIM 271550) [32,69-81]. Estimated prevalence is higher in Israel [77]. The spinal aberrations include platyspondyly; flat, often rectangular vertebral bodies are seen at radiography with irregular areas of increased and decreased mineralization, and short broad ilia. Spondyloenchondrodysplasia can manifest itself from birth to later infancy [71]. Patients usually have a short stature (short limbs), with increased lumbar lordosis, barrel chest and kyphoscoliosis, genua valga or vara, facial anomalies, and may show clumsy movements [3]. Type I is classic, and type II also affects the central nervous system including spasticity, developmental delay, and lateonset cerebral calcifications [76,81]. In addition, clinical manifestation of autoimmunity can be seen. The spine is less severely affected as compared with dysspondyloenchondromatosis and cheirospondyloenchondromatosis, in which the vertebral lesions are less uniform and the ilia are not short [3].

The clinical features of spondyloenchondrodysplasia are highly variable within and between the families; neurological and autoimmune manifestations were seen in different combinations within one single family suggesting remarkable pleiotropic manifestations of a single disease [81]. In addition, there are two reports suggesting an association of spondyloenchondromatosis with D-2-hydroxyglutaric aciduria, a neurometabolic disorder [82,83]. Although the disease is thought to be autosomal recessive, an autosomal dominant inheritance pattern has also been described [77,80].

Cheirospondyloenchondromatosis

Cheirospondyloenchondromatosis (generalized enchondromatosis with platyspondyly, enchondromatosis Spranger type VI) combines symmetrically distributed multiple enchondromas with marked involvement of metacarpals and phalanges resulting in short hands and feet with mild platyspondyly [3,32]. It occurs at very early age. There is mild to moderate dwarfism and joints, especially of the fingers, become enlarged. Mental retardation is frequently seen. Genetic transmission is unknown [3].

Dysspondyloenchondromatosis

Dysspondyloenchondromatosis (enchondromatosis with irregular vertebral lesions, enchondromatosis type V) is a nonhereditary disorder characterized by spondyloenchondromatosis combined with malformation of the spine [3,32,70,80,84]. The irregularity of the vertebral anomalies differentiates dysspondyloenchondromatosis from other enchondromatosis with spinal involvement. Severe segmentation abnormalities and secondary deformities of the vertebral column can be seen [3]. In addition, neonatal dwarfism, unequal limb length or asymmetric limb shortening, a flat midface with a frontal prominence, and progressive kyphoscoliosis can be found [3,80,85]. Multiple enchondromas are present in the long tubular and flat bones while the bones of hands and feet are not or only mildly affected. The disease manifests itself at birth. Haga et al reported a case with dysspondyloenchondromatosis along with Maffucci syndrome in one patient [86] which suggests that these two different syndromes may be part of one spectrum caused by a pleiotropic gene. Malignant transformation is not yet reported in the literature.

Other less well delineated subtypes

Halal and Azouz provisionally added three more subtypes to the Spranger classification including generalized enchondromatosis with irregular vertebral lesions (type VII), generalized enchondromatosis with mucopolysaccharides (type VIII) and enchondromatosis with concave vertebral bodies (type IX), all of which are non-hereditary [87]. Gabos and Bowen describe 8 patients with extensive unilateral involvement of epiphyseal and metaphyseal regions by enchondromas appearing before growth plate closure leading to severe deformity. It is however unclear whether this is a variant of Ollier disease, or that it should be classified separately as epiphysealmetahyseal enchondromatosis as proposed by the authors [88]. In addition, metaphyseal chondrodysplasia, Vandraager Pena type may also be considered an enchondromatosis subtype (MIM 250300) [89] although it is more often considered a subtype of metaphyseal dysplasia. It is an autosomal recessive disorder in which the metaphyses of the long bones have an extensive sponge like appearance at radiographs while histologically numerous islands of cartilage reminiscent of enchondromatosis are seen [89].

Conclusions

Many different syndromes are present with multiple enchondromas, and most of them are extremely rare. None of these syndromes seem to be determined by a simple mendelian manner, and it is unclear whether they represent separate entities, or that they are manifestations of a single causal process. Since they all share the occurrence of multiple enchondromas, this may suggest that the same gene or gene family may be involved in at least a proportion of the different types of enchondromatosis.

It can be expected that within the upcoming era of next generation sequencing approaches, elucidating the genes causing rare genetic disorders, also the gene(s) that causes the different enchondromatosis subtypes may be identified. Adequate classification of the different enchondromatosis subtypes as reviewed here is not only relevant for clinical management with regards to genetic counseling and the risk of malignant transformation, but also to allow future molecular studies to reveal whether (a proportion of) the different subtypes are different ends of a spectrum caused by a pleiotropic single gene, or that they truly represent separate disease entities.

Note added in proof

Sobreira et al recently reported mutations in the *PTPN11* gene in two metachondromatosis families (PLOS Genet, 2010;6:6). Future studies should reveal whether this gene is also involved in other enchondromatosis subtypes.

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Chapter 3

Genome-wide analysis of Ollier disease: Is it all in the genes?

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Abstract

Background: Ollier disease is a rare, non-hereditary disorder which is characterized by the presence of multiple enchondromas (ECs), benign cartilaginous neoplasms arising within the medulla of the bone, with an asymmetric distribution. The risk of malignant transformation towards central chondrosarcoma (CS) is increased up to 35%. The aetiology of Ollier disease is unknown.

Methods: We undertook genome-wide copy number and loss of heterozygosity (LOH) analysis using Affymetrix SNP 6.0 array on 37 tumours of 28 Ollier patients in combination with expression array using Illumina BeadArray v3.0 for 7 ECs of 6 patients.

Results: Non-recurrent EC specific copy number alterations were found at *FAM86D*, *PRKG1* and *ANKS1B*. LOH with copy number loss of chromosome 6 was found in two ECs from two unrelated Ollier patients. One of these patients also had LOH at chromosome 3. However, no common genomic alterations were found for all ECs. Using an integration approach of SNP and expression array we identified loss as well as down regulation of *POU5F1* and gain as well as up regulation of *NIPBL*. None of these candidate regions were affected in more than two Ollier patients suggesting these changes to be random secondary events in EC development. An increased number of genetic alterations and LOH were found in Ollier CS which mainly involves chromosomes 9p, 6q, 5q and 3p.

Conclusions: We present the first genome-wide analysis of the largest international series of Ollier ECs and CS reported so far and demonstrate that copy number alterations and LOH are rare and non-recurrent in Ollier ECs while secondary CS are genetically unstable. One could predict that instead small deletions, point mutations or epigenetic mechanisms play a role in the origin of ECs of Ollier disease.

Background

Enchondroma (EC), a benign cartilage forming tumor in the medulla of the bone, is thought to be a precursor of secondary central chondrosarcoma (CS). EC develops either as a single, solitary lesion or as multiple lesions in the context of Ollier disease [1]. Ollier disease is the most common subtype of enchondromatosis and shows multiple ECs with marked unilateral predominance [1,2]. The risk of malignant transformation towards central CS is up to 35% in Ollier disease [1,3]. There is no marker that would indicate progression towards malignancy, thus there is a vital need to understand the genetics of these tumors which may help to develop markers for early diagnosis. A comprehensive understanding of the molecular events in ECs and central CS also enables the identification of possible targets for treatment [4]. While the genetics of enchondroma is poorly understood, the involvement of the EXT genes is well established in the development of solitary as well as hereditary multiple osteochondromas (MO) (OMIM 133700), benign cartilage tumors at the surface of bone [5]. The lack of EXT function seems to disturb hedgehog signalling in MO, while activated hedgehog signalling in mice seems to underlie the development of the Ollier related phenotype [4]. Heterozygous mutations in *PTH1R* are found in a small subset of Ollier patients [6-8]. It is however unclear whether these mutations in *PTH1R* are causing or modifying the disease [7], and since ~90% of Ollier patients lack *PTH1R* mutations, we aimed to study Ollier related ECs by mapping genetic changes using genomic arrays.

We hypothesized Ollier disease to be a germ-line mosaic condition due to the fact that it is non-hereditary and because of its unilateral predominance feature [3,9]. An early postzygotic mutation resulting in asymmetric involvement of skeletal structures can be expected, as was also shown for polyostotic fibrous dysplasia [10]. One could speculate that an inactivating mutation in a tumor suppressor gene, similar to EXTs in osteochondroma, would have occurred in the developing mesoderm early after gastrulation. In case of a tumor suppressor gene, later on, an additional hit may be required for the formation of ECs with subsequent genetic changes causing progression towards central CS. We tested this hypothesis using high-resolution SNP array combined with expression array on DNA derived from tumor tissue and, whenever available, normal DNA from Ollier patients.

SNP arrays provide an excellent possibility for large scale, genome-wide, high-resolution analysis of both DNA copy number alterations (CNA) and loss of heterozygosity (LOH) in cancer cells. It also provides a feasible means of detecting genotyping alterations in the tumors of individual patients and, in principle enables the identification of new areas with common allelic imbalance that could harbor potential tumor suppressor genes which helps in the identification of novel candidate genes affected by genomic abnormalities [11,12]. In the present study, we used Affymetrix Genome-Wide Human SNP Array 6.0 to obtain a comprehensive registry of genetic aberrations in 37 tumors of 28 patients with Ollier disease and correlate it with gene expression using Illumina Human-6 v3 Expression Array and qRT-PCR and protein expression using tissue microarray (TMA). Based on the obtained genomic profiles with limited and non-recurrent genetic changes in Ollier ECs, we conclude that they are genetically heterogeneous and that the reported CNA in this study are likely to be secondary random events in ECs.

Materials and Methods

Patient materials and reference samples

Fresh frozen tissues from 37 tumors from 28 patients diagnosed with Ollier disease were collected from the EuroBoNet consortium (http://www.eurobonet.eu) (Table 1): Leiden University Medical Center, The Netherlands (12 tumors), Leuven University, Belgium (5 tumors), Rizzoli Institute, Italy (6 tumors), Royal Orthopaedic Hospital, United Kingdom (7 tumors), Lund University, Sweden (2 tumors), Netherlands Committee on Bone Tumors, The Netherlands (2 tumors), Heidelberg University, Germany (1 tumor), University of Ghent, Belgium (1 tumor) and Groningen University Medical Center, The Netherlands (1 tumor). All samples were derived from primary tumors, not from recurrent tumors, and all were graded according to Evans et al [13]. Diagnoses were originally made in the multidisciplinary teams of the centers of origin. The histology was revised and representativity was assessed on the available paraffin or frozen tissue by one experienced bone tumor pathologist. For SNP array analysis, 14 Ollier ECs, and 23 Ollier CS (13 grade I, 8 grade II, 2 grade III) from 28 Ollier patients were used. As controls, normal DNA derived from fresh frozen muscle tissue ($n = 3$), peripheral blood lymphocytes ($n = 4$) or saliva ($n = 4$) was available for 11 Ollier patients and 3 patients with unrelated bone diseases. We used blood lymphocyte DNA from 12 healthy controls and 1 HapMap sample. We also isolated DNA from saliva for 3 of these controls to validate the use of saliva DNA in this study. Twenty eight of these thirty controls and DNA from 10 additional HapMap samples were used in MLPA. RNA from 4 articular cartilage, 2 growth plates and 7 ECs was used for expression array and 3 articular cartilage, 2 growth plates and 8 ECs were used in qRT-PCR.

Five tissue micro array (TMA) blocks containing 86 tumors were constructed, of which 65 were Ollier related and 21 were solitary central tumors (ECs and CS) from both the EuroBoNet and the European Musculoskeletal Oncology Society (EMSOS) networks (Table S1): Leiden University Medical Center, The Netherlands (27 tumors), Rizzoli Institute, Italy (12 tumors), Copenhagen University, Denmark (9 tumors), University clinic of Orthopaedic Surgery and Medical university of Graz University, Austria (6 tumors), Bern University, Switzerland (5 tumors), University of Navarra, Spain (4 tumors), Netherlands Committee on Bone Tumors (21 tumors), Istanbul University, Turkey (2 tumors). All the samples were obtained according to the ethical guidelines of the host institution. Samples were coded 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).

DNA and RNA isolation

Tumor samples were selected that contained more than 80% of tumor cells, as estimated on haematoxylin and eosin-stained frozen sections. Most of the samples were macro dissected and L2099 sample was micro dissected to enrich the tumor percentage [14]. DNA from fresh frozen tissue was isolated using the wizard genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer instructions. Blood DNA was isolated as described by Miller et al [15].

Table 1 Clinicopathological data of the Ollier patients

Patient ID	Sample	Tumor Grade	Tumor location	Gender	Age	Application
17	L1083	CS I	metacarpal	M	48	1,3
17	L2218	CS I	phalanx	M	49	1,3
20	L286*	CS II	femur	F	23	1,3
21	L204*	CS I	femur	M	26	1,3
21	L253*	CS I	tibia	M	26	1,3
22	L206	EC	phalanx	F	26	1,2,3,4
22	L910	EC	phalanx	F	16	1,2,3,4
23	L813	CS II	humerus	M	68	1,3
25	L1251*	EC	phalanx	M	15	1,2,3,4
25	L2220*	EC	metacarpal	M	14	1,3,4
26	L1974	CS II	scapula	M	48	1,3
27	L1975*	CS II	femur	M	31	1,3
28	L1976*	CS II	tibia	M	41	1,3
29	L1977*	CS I	tibia	M	41	1,3
29	L1978*	EC	foot	M	38	1,3
30	L1980	CS II	knee	F	63	1,3
31	L810	CS III	unknown	M	-	1,3

Table 1(Continue)

Patient ID	Sample	Tumor Grade	Tumor location	Gender	Age	Application
33	L1685	CS I	pubic bone	F	23	1,3
34	L1687	CS I	phalanx	M	18	1,3
34	L1686	EC	phalanx	M	18	1,2,3,4
35	L2386	CS I	phalanx	F	13	1,3
36	L2463*	EC	tibia	F	12	1,3
38	L1629	EC	unknown	M	36	1,3
38	L1630	EC	Iliac bone	M	36	1,2,3,4
42	L2098	CS II	humerus	F	15	1,3
43	L2099	CS II	humerus	F	49	1,3
47	L2103a	EC	phalanx	M	39	1,2,3,4
47	L2103b	CS I	phalanx	M	39	1,3
48	L2104a	CS III	tibia	M	35	1,3
48	L2104b	EC	femur	M	35	1,3
50	L2221*	CS I	femur	F	42	1,3
52	L1513*	CS I	femur	F	23	1,3
54	L1490*	EC	phalanx	F	12	1,3
61	L2205	EC	ilium	M	6	1,3
64	L1683	EC	metacarpal	F	29	1,2,3,4
68	L2280*	CS I	acromion	F	24	1,3
69	L2513	CS I	pelvis	M	33	1,3

* Normal DNA available enabling paired analysis. Application - 1: sample used for SNP array, 2: expression array, 3: MLPA and 4: qRT-PCR.

Saliva DNA was isolated using the Oragene DNA kit (DNA Genotek Inc Ontario, Canada) according to the protocol provided by the supplier and DNA was precipitated using sodium acetate precipitation. DNA concentration was quantified spectrophotometrically using Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and the fragment sizes were determined on 1% agarose gel.

RNA isolation from the fresh frozen tissue was performed as described previously [16]. RNA concentration was measured spectrophotometrically and the fragment sizes were determined by RNA 6000 Nano LabChip kit using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). DNA and RNA from all the samples were good enough to continue with the experiment.

SNP Array and Data Analysis

We used the Affymetrix Genome-Wide Human SNP Array 6.0. Genomic DNA preparation, labeling and hybridization were performed according to Affymetrix's recommended protocols (Affymetrix, Santa Clara, CA, USA). Then, arrays were scanned with GeneChip™ GSC3000 7G Whole-Genome Association System (Affymetrix). Overall hybridization quality was estimated by the genotype call rate using the Birdseed genotype calling algorithm in Genotyping Console (version 3.0.2, Affymetrix). Average call rate was 97.83%. To analyze the data we used statistical language R version 2.8 and Nexus software version 4.1 (BioDiscovery, CA, USA). We did not use HapMap samples as baseline in this study to avoid the bias for the experiment performance at different labs, batch effect and hybridization quality. The analysis was performed on a subset (30 controls, 14 ECs and 23 CS of Ollier patients, Table 1) of a larger experiment of 92 samples including samples unrelated to Ollier disease to achieve a larger set of common controls. Than we performed copy number analysis using 92 samples as a baseline in R software and only 29 control samples of high quality as a baseline in Nexus software. Results that we obtained using different softwares and different baselines were comparable. We used CEL files in R software. For the genomic analysis using R, we did genotyping using the CRLMM algorithm in the Oligo package [17], copy number analysis using the aroma affymetrix package [18], and we constructed LAIR plots to visualize regions of LOH and allelic imbalance [19]. Chromosome-X was not analyzed to avoid gender-related issues [20]. In Nexus, we performed copy number analysis using CNCHP log-ratio files generated by genotyping console using 29 controls as a baseline. Hidden Markov model (HMM) based SNP-FASST segmentation was used to identify aberrant genomic regions. Here we considered at least 5 probes for each segment. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/ accession number GSE22965).

Multiplex Ligation-Dependent Probe Amplification (MLPA)

MLPA was used to confirm copy number gains and losses found within the selected candidate genes by SNP array. MLPA probes were designed using NCBI Build 36.1. We used two probes for *TCRA* and one probe for *POU5F1*, *ANKS1B*, *FAM86D* and *PRKG1*. Probe sequences can be obtained upon request. MLPA was performed as described previously [21]. Sample series of SNP array (Table 1) and in addition to that DNA from ten HapMap samples was used. Data analysis was done using SoftGenetics GeneMarker version 1.70. We set 1.2 and 0.8 as a threshold to detect the gains and losses respectively.

Expression Array and Data Analysis

In total, we analyzed 7 ECs while 2 growth plate and 4 articular cartilage samples served as a control (Table 1). Expression array was performed using Illumina Human- 6 v3.0 Expression BeadChips (Illumina Inc., San Diego, CA). For Illumina BeadArray assay, cRNA was synthesized with an Illumina RNA Amplification Kit (Ambion, Austin, TX, USA), purified, labeled and hybridized as per the manufacturer's instructions. Then, arrays were scanned with Illumina BeadArray reader (500GX, Illumina) and scanned images were imported in BeadStudio software version 3.1.3.0 (Illumina). For the expression array, unprocessed data were collected from BeadStudio and processed using VST [22] and quantile normalization with the R bead array package [23]. The processed data was analyzed using Linear Models for Microarray Data (LIMMA) analysis which uses moderated t-test to detect differentially expressed genes between two groups by taking into account natural variance within these groups and correcting for multiple testing using false discovery rate [24].

Real-time quantitative Reverse Transcriptase PCR (qRT-PCR)

qRT-PCR was used to confirm the results obtained by the integration of SNP and expression array. Here we have used 5 controls (2 growth plates and 3 articular cartilage) and 8 ECs (Table 1). RNA used for expression array was also used for the qRT-PCR. cDNA was synthesized using 1 μg of total RNA with AMV reverse transcriptase (Roche Applied Science, Almere, The Netherlands) in combination with oligo-dT and random hexamer priming and qPCR was performed as described elsewhere [25]. Primer details can be provided upon request. Expression of the genes of interest (*FAM86D, POU5F1* and *ANKS1B*) was normalized by geometric averaging of multiple internal control genes using the geNorm program [26]. Out of four normalization genes the best three were selected within this program: *GPR108, SRPR* and *TBP.* Relative quantification was performed using standard curves, followed by adjustment with the normalization factor calculated by geNorm program [25]. The average relative expression of gene of interest in ECs was compared to controls. To see the EC specific gene expression changes, relative expression of ECs with change in copy number was compared with ECs without change in copy number.

Tissue Microarray (TMA)

Five TMA blocks were constructed using TMA master (Zeiss, 3D Histech, Hungary) and each block contain maximum 70 cores including seven control tissues (growth plate, articular cartilage, breast carcinoma, prostate, colon, skin and tonsil) for orientation purpose. In total 5 TMA blocks contain 86 tumors, of which 65 Ollier related and 21 solitary ECs and CS (Table S1). The TMA blocks contain 2-mm cores of each tumor in triplicate.

Immunohistochemistry

Tissue sections were cut from TMA blocks and dried overnight at 60°C. Slides were kept in Xylol for 20 minutes. Immunohistochemistry was performed as described earlier [27]. Detailed information on the antibodies used to check protein expression of POU5F1 and NIPBL are given in table S2. We used power vision (poly-HRP-GAM/R/R, Immunologic) as a secondary antibody. Visualization was carried out with liquid Dab+ substrate chromogen system (DAKO, Glostrup, Denmark).

As a negative control primary antibody was omitted. Immunostained TMA slides were scanned using a high resolution Mirax Desk instrument (Zeiss, Mirax 3D Histech, Hungary) and scored independently by two observers (JVMGB and TCP) with the Mirax viewer TMA module software version 1.1.12 (Zeiss) and discrepancies were discussed. In brief, the intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong) and percentage of positive tumors cells (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74%, 4 = 75-100%) were assessed. Cores with a negative internal control and loss of tissue were excluded from the analysis. A sum score ≥ 4 was considered positive. Statistical analysis was done using Pearson Chi-Square test in SPSS (version 16.0, Chicago, Illinois, USA).

Results

Genome-wide detection of copy number alterations and loss of heterozygosity using SNP array

Samples L1975, L1974, L1980, L813, L1083 and L286N were excluded from further analysis because of quality issues and low call rates. All samples including 29 controls and 32 tumors (14 ECs, 18CS) showed DNA copy number aberrations mainly restricted to known variable regions (Figure 1)[28,29]. CNA were more frequently found in CS II and CS III as compared to ECs and CS I (Figure 1). The number of copy number changes in saliva and blood DNA were comparable.

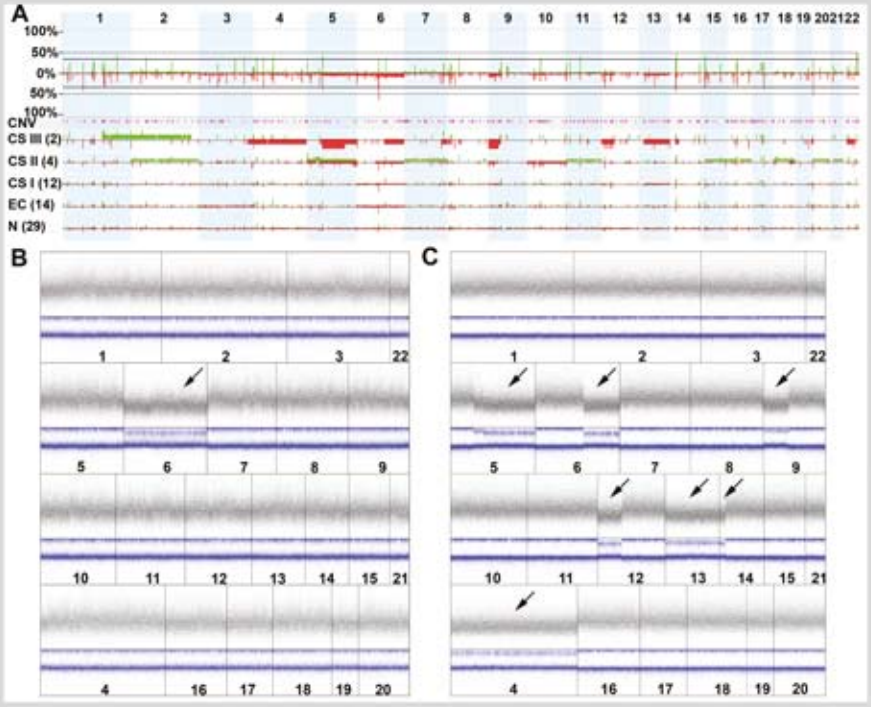
Genetic alterations in Ollier Enchondromas

We used paired analysis which is based on the comparison of tumor and corresponding normal DNA (available for 5 ECs from 4 patients) to study LOH and CNA. Although sequences of homozygous SNPs were identified that could indicate LOH, these same sequences were also observed in the corresponding normal sample. We could not find any LOH in these 5 ECs using both R and Nexus softwares. We have identified 7 EC specific CNA by paired approach (Table 2). Selection of candidate genes *TCRA, ANKS1B* and *PRKG1* for further validation is based on copy number change in minimum 10 probes within the gene.

Unpaired analysis revealed absence of LOH in the majority of ECs. We confirmed the loss of chromosome 6 with LOH in L206 (Figure 1B) which was in agreement with the results published previously by our group using array CGH [30] proving validity of the assay. Also loss of one copy of chromosome 3 and 6 with LOH was found in L1683. These results were confirmed using R and Nexus software. Furthermore, an unpaired approach (29 controls as a baseline) was used to find most common copy number gains and losses in at least 5 out of 14 ECs (Table S3, Additional file 1) using Nexus. None of these were confirmed in paired comparison which suggests that they are not tumor specific changes. When ECs are located in the phalangeal bones, cellularity is increased [1]; we could not find differences between ECs or CSI of the hand versus those of long bones at the genomic level although the sample sizes are small.

Homozygous deletion of *FAM86D* at chromosome 3p12.3 was found in two ECs in one patient (L206 and L910) and selected for further validation (Figure 2). Interestingly both ECs were located in different digits of the hand. It was not possible to get normal DNA from the same patient to investigate tumor specific loss of *FAM86D*.

Figure 1 Genome-wide copy number alterations in all 22 chromosomes



A) Copy number alterations in controls, Ollier enchondromas (Ecs) and chondrosarcoma (CS) grade I, II, III. The upper panel shows genome-wide frequency plots of gains and losses in 29 controls and 32 Ollier tumors. Gains are plotted in green above 0% baseline and losses are plotted in red below 0% baseline. The X-axis corresponds to the genomic region from chromosomes 1 to 22 and the Y-axis represents the percentage of gains and losses of all selected samples at the specific location in genome. The lower panel shows frequency plot of 29 controls, 14 ECs, 12 CS I, 4 CS II and 2 CS III. The number and size of genomic alterations increases with increasing tumor grade. Enchondromas and control samples show a comparable number and size of genomic alterations, which can be attributed to common copy number variation. B) An example of copy number alterations in Ollier enchondroma (L206). This figure shows copy number alterations in all 22 chromosomes of enchondroma (L206). The black band indicates the number of copies of the chromosomes. The blue bands show the unpaired LAIR value. The lower band of this contains the originally uninformative homozygous SNPs. The top band indicates heterozygous informative SNPs. With LOH or imbalances between the alleles, the position of this band will decrease. As a loss of chromosome 6 these heterozygous SNPs are becoming homozygous showing the LOH. C) An example of copy number alterations in Ollier chondrosarcoma grade III (L810). Copy number loss with LOH is present at chromosome 4, 5q, 6q, 9p, 12p, 13 and 14q.

Table 2 Paired copy number alterations in Ollier enchondromas

Patient ID	Sample	Cytoband	Copy number event in EC	Region
29	L1978	14q11.2*	gain	*TCRA*
25	L2220	12q23.1*	loss	*ANKS1B*
25, 54	L2220, L1490	10q11.22	loss	intergenic region
54	L1490	1p31.3	loss	intergenic region
54	L1490	2q11.2	gain	intron of *VWA3B*
54	L1490	5q13.2	loss	intergenic region
54	L1490	10q11.23*	gain	intron of *PRKG1*

* Candidate regions selected for further validation based on minimum 10 affected probes within the gene.

Genetic alterations in Ollier chondrosarcomas

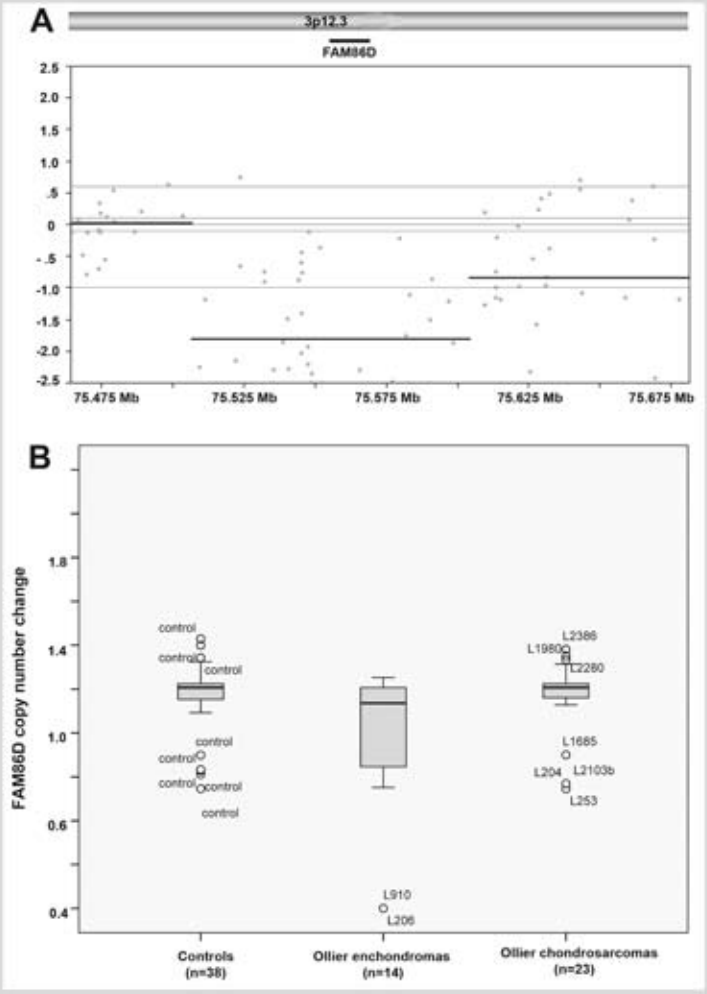
Ollier CS showed large genomic alterations and LOH at various locations in the genome (Table 3, Figure 1C). Frequent recurrent CNA involves chromosomes 3p, 5q, 6q and 9p.

Verification of gene copy number alterations by MLPA

To validate regions identified by SNP arrays, we performed MLPA on 37 Ollier tumors (Table 1) and 38 controls for the candidate genes *FAM86D, TCRA, ANKS1B* and *PRKG1* (Table 4). In addition, a probe was designed at *POUF51* which is at chromosome 6p21.31 in order to confirm loss of chromosome 6 in L206 and L1683. We have used 38 controls as a baseline. Results of validation experiments are summarized in Table 4. In short we confirmed three (*FAM86D, PRKG1* and monosomy 6) out of five candidate regions. We confirmed the homozygous loss of *FAM86D* in two ECs of the same patient (Figure 2). Gain at *PRKG1* in L1490 is confirmed which seems to be EC specific in this case but was not found in other ECs and CS. Loss at chromosome 6 using probe at *POU5F1* was confirmed in L206 while loss was not confirmed in L1683 at given thresholds. *TCRA* region at chromosome 14q11.2 is known as a highly polymorphic region [31] and its frequent rearrangements are observed in blood lymphocytes [32]. We used majority of blood samples as a baseline and found copy number gain in all ECs at *TCRA* as a result of SNP array unpaired analysis. Paired analysis with SNP array showed copy number gain at *TCRA* in L1978 and was not confirmed with MLPA.

Indeed this gain was due to loss in the corresponding normal DNA. Loss at *ANKS1B* in L2220 was not confirmed by MLPA at given thresholds however the peak was lower in L2220 compared to corresponding normal.

Figure 2 *FAM86D*



A) Homozygous loss of FAM86D in L206. A 200 Mb region containing the FAM86D gene at 3p12.3 is shown (X-axis). The gene lies in a ~100 Mb homozygous deleted region, within a larger area of hemizygous deletion. The individual copy number probes are shown as the log ratio of the intensity and zero is two copies. The horizontal lines are segments with identical copy number as identified by the HMM SNP-FASST algorithm.
B) MLPA for 38 controls, 14 Ollier enchondromas (ECs) and 23 Ollier chondrosarcomas (CS). The Y-axis shows ratio profile and 1.0 indicates two copies of a given chromosomal locus. Homozygous copy loss of the FAM86D as shown by SNP array was confirmed in L206 and L910. Most of the controls and tumors show either two copies or hemizygous loss of this gene. Note that only outliers are displayed with ID numbers.

Table 3 Genetic alterations in Ollier chondrosarcomas

Sample	Tumor Grade	Gain	Loss	LOH	Copy neutral LOH
L2218	CS I	-	-	-	-
L204	CS I	1q	3p,4q	3p,4q	-
L253	CS I	1q	6p,6q,9p,12q,13	6p,6q,9p,12q,13	-
L1977	CS I	-	-	-	-
L1685	CS I	-	2q	2q	-
L1687	CS I	3p,7q,8q	-	-	-
L2386	CS I	-	6p,11q	-	-
L2103b	CS I	-	-	-	-
L2221	CS I	14q,17q	-	-	-
L1513	CS I	-	-	-	-
L2280	CS I	-	-	-	-
L2513	CS I	-	-	-	-
L286	CS II	5p,11p,11q,18p	3p,5p,5q,6q,9p, 11p,18p,18q	3p,5p,6q,9p,1 1p,18p,18q	-
L1976	CS II	8q	-	8q	-
L2098	CS II	2,5,7,15,16,17q,18,20,21	10,17q	10,17q	11
L2099	CS II	-	-	-	-
L810	CS III		4,5q,6q,9p,12p,13,14q	4,5q,6q,9p,12p,13,14q	-
L2104a	CS III	1q,2p,2q,12p,14q	3q,5q,7q,9p,12p, 22	3q,5q,7q,9p,12p,22	2q

Some of the regions with loss do not show loss of heterozygosity (LOH). This could be explained by the loss of alleles in an aneuploid background.

Expression array and its integration with copy number alterations

Expression array was performed using 7 ECs and 6 controls with Illumina Human-6 v3.0 (Table 1). We performed function based analysis by integrating the gene expression results with SNP array results. In total 1044 genes were differentially expressed in ECs compared to controls (adj. p-value < 0.01, Table S4). We considered all up regulated genes (881 genes, adj. p-value < 0.01) with presence of gain in at least one EC and all down regulated genes (163 genes, adj. p-value < 0.01) with presence of loss in at least two ECs. We found *NIPBL* which was gained as well as up regulated while *POU5F1* that was lost as well as down regulated in ECs compared to controls. The same pattern was found in CS.

Validation of integration approach of SNP and expression array using qRT-PCR

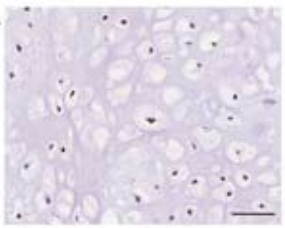
Using qRT-PCR, we confirmed lower expression of *ANKS1B, FAM86D* and *POU5F1* (Table 4). L2220 showed one copy loss of *ANKS1B* and its expression was decreased in this EC as compared to the average of relative expression of other 7 ECs however there is no difference in the expression comparing all ECs versus controls. SNP array results revealed homozygous loss in L206, L910 and one copy loss in L2103 for *FAM86D*. *FAM86D* expression was decreased in ECs compared to controls. There was no expression of *POU5F1*.

Protein expression

Using a TMA we demonstrated that POU5F1 protein expression was absent in all Ollier tumors which is in line with the expression array and qRT-PCR results. For NIPBL, 30% of Ollier tumors were positive (Figure 3, Table 4). Only 34/65 Ollier and 11/21 solitary tumors could be analyzed since cores from the rest of the tumors were lost during the immunohistochemistry. There was no significant difference in the expression of NIPBL within the tumors of different grades in Ollier disease (Pearson Chi-Square, p-value = 0.1).

Figure 3 NIPBL protein expression

A



B

Tumor grade	Ollier	Solitary
EC	9/22	2/2
CS I	1/8	1/1
CS II	0/4	2/3
CS III	-	4/5

A) Example of nuclear expression of NIPBL in Ollier enchondroma (400 times magnification).
B) Number of Ollier and solitary tumors with nuclear NIPBL expression.

Table 4 Summary of validation experiments for the candidate genes

Candidate genes	Technique used	Summary of validation
PRKG1	MLPA	No copy number change in controls. Gain in tumor (1/37)
FAM86D	MLPA, qRT-PCR	Loss in controls (5/38), gain in controls (1/38), HMloss in tumors (2/37) loss in tumours (6/37). Lower m-RNA expression in Ecs
POU5F1	MLPA, qRT-PCR, IHC	Gain in controls (3/38), loss in tumors (4/37), gain in tumors (2/37). Its mRNA and protein expression was absent in tumors
ANKS1B	MLPA, qRT-PCR	No copy number changes in controls and tumors. Lower m-RNA expression in L2220 compared with other 7ECs
TCRA _probe1	MLPA	Loss in controls (4/38), gain in tumors (2/37)
TCRA _probe2	MLPA	Loss in controls (4/38), gain in tumors (2/37)
NIPBL	IHC	30% Ollier tumors showed protein expression

Discussion

The origin of both solitary and Ollier related ECs is largely unknown. To address this, we performed genomewide analysis of ECs occurring in non-hereditary Ollier disease. Since these tumors are polyostotic, with a unilateral predominance, manifesting early in life, we postulated that there may be a germ-line mosaicism. We attempted to find causative genes for ECs of the Ollier disease using a high-resolution SNP array containing 1.8 million markers combined with expression array and obtained comprehensive genetic profiles of 37 Ollier disease related tumors. This is the first and largest genomewide molecular study on Ollier disease reported so far, which was possible through the collaboration of many different institutes within the EuroBoNeT Network and the European Musculo-Skeletal Oncology Society (EMSOS).

In general, the obtained genomic profiles showed absence of large genetic aberrations in Ollier ECs except loss of chromosome 6 in two ECs from two unrelated Ollier patients. Small non-recurrent genetic changes combining the SNP and expression array at *FAM86D, PRKG1, ANKS1B, NIPBL* and *POUF51* were found in ECs. Most of these genes are not known to play an important role in cartilage formation. We found homozygous loss of *FAM86D* in two ECs of the same patient. Function of *FAM86D* is still unknown. We confirmed intronic gain at *PRKG1* in one EC, which is involved in fatty acid metabolism [33]. We found loss at *ANKS1B*, while overexpression of *ANKS1B* is reported in pre-B cell acute lymphocytic leukaemia [34].

Gain at *NIPBL* was found in L2205 while at the protein level only 30% of Ollier ECs and CS expressed NIPBL. Inactivating mutations in *NIPBL* are associated with Cornelia de Lange syndrome and one of the characteristic features of this syndrome is reduction in limb growth (OMIM 122470). Loss of *POU5F1* was found in two ECs with monosomy at chromosome 6 and its mRNA and protein expression was absent in all Ollier and solitary ECs and CS. The transcription factor *POU5F1* (OCT3/4) is involved in regulating pluripotency and is normally expressed during early embryogenesis in embryonic stem and germ cells [35].

Here we studied extensively candidate genes obtained from paired analysis of enchondromas. Normal DNA was available from 4 Ollier patients enabling paired analysis. Despite the low number of paired samples our data suggest that no common CNA are associated with EC development. Extending the analysis with the unpaired samples we could not see any common CNA in all ECs. All aberrations we obtained in at least 5 out of 14 ECs are reported as common copy number variants in DGV database (http://projects.tcag.ca/variation/). Loss of chromosome 6 was the only recurrent change in ECs of two unrelated Ollier patients. Therefore, relatively small numbers of copy number alterations that we found per ECs are more likely to be secondary random genetic changes. Although SNP array technology is a powerful analysis tool, it can not detect balanced chromosomal translocations, inversions and whole-genome ploidy changes. However, balanced translocations and inversions have not been reported for the Ollier tumors in the literature so far [2].

Previously, *PTH1R* was reported to be the gene causing Ollier disease [6]. However, in subsequent studies it was shown that *PTH1R* is only mutated in a sub group of patients (~10%) decreasing receptor function to ~70%, suggesting that it may contribute to the disease but is probably not causative [7]. PTH1R is a key player within the IHH pathway which is crucial for endochondral ossification. The presence of known point mutations (R150C, R255H, G121E and A122T) in *PTH1R* was excluded in the present series (unpublished data). Also, we could not find a deletion or LOH at the 3p21.31 region harboring the *PTH1R* gene. Recently, inactivating mutations in *PTPN11* are reported in another enchondromatosis subtype called metachondromatosis in which multiple ECs are combined with osteochondroma- like lesions [2,36]. In our series we could not detect any CNA or LOH at *PTPN11* by SNP array. Also, expression of *PTPN11* was not decreased in ECs as compared to controls in expression array.

Large gains, losses and LOH were seen more often in Ollier CS as compared to ECs, which is in line with increased genetic instability and aneuploidy seen in solitary central chondrosarcoma progression. Most common CNA involve 3p, 5q, 6q and 9p in Ollier CS. However, we could not detect recurrent CNA in all Ollier CS that could have been responsible for malignant transformation of ECs. For Ollier CS, a deletion at the short arm of chromosome 1 [37], LOH at *RB1* at chromosome 13 and p15/p16 loci at the short arm of chromosome 9 [38] were reported in single cases each. Our results show very few copy number alterations in ECs and an increased number of variable genomic alterations in CS. This is in support of the multistep model for CS development [39].

In conclusion, we present genome-wide copy number and expression profiles of the largest international series of Ollier ECs and CS reported so far. We show absence of recurrent CNA and LOH in majority of ECs suggesting that instead point mutations or other copy number neutral structural changes (inversions, insertions, balanced translocations) or deletions below the resolution of this platform involving a single or a few exons only [40] might have an important role in EC pathogenesis. This opens the possibility to study these tumors further using a next generation sequencing approach. An increased number of genetic alterations are found in Ollier CS, supporting the multistep genetic progression model.

Abbreviations

EC: Enchondroma; CS: Chondrosarcoma; SNP: Single nucleotide polymorphism; LOH: Loss of heterozygosity; CNA: Copy number alterations; TMA: Tissue microarray; MLPA: Multiplex Ligation Dependent Probe Amplification

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Authors' contributions

The study was designed by TCP, JVMGB, PCWH, JO and KS. Data analysis was done by TCP and JO. Tissue microarray was constructed by TCP and TK. RS, LS, AHMT, SHMV were responsible for acquisition of patient material and patient data. The manuscript was written and approved by all the coauthors.

Competing interests

The authors declare that they have no competing interests.

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Table S1 Clinicopathological data of 86 tumors used in TMA

	Ollier	Solitary
Total number of patients	43	21
Total number of tumor samples	65	21
EC	39	6
CS I	17	4
CS II	-	5
CS III	9	6
Male:Female	20:19*	13:8
Median age at diagnosis years (range)	19 (3-63)	55 (29-84)

* Gender information was not available for four Ollier patients.

Table S2 Antibody information

Protein	Antibody Identification	Dilution	Antigen retrieval	Positive control	Staining	Company
NIPBL	250133	1:800	citrate, microwave	normal lung	nuclear	Abbiotec
POU5F1	ab73099	1:3	citrate, microwave	testicular tumor	cytoplasm	Abcam

Table S3 Unpaired copy number changes in 35% of EC (min. 5 out of 14)

Number of ECs	Region	Cytoband	Copy number event	Genes	% of CNV overlap
5	chr1:0-218,741	p36.33	gain	6	99.8
5	chr1:142,693,888-143,978,071	q21.1	gain	11	100
5	chr1:16,758,739-16,878,452	p36.13	gain	3	100
5	chr1:194,993,359-195,068,330	q31.3	gain	2	100
5	chr1:25,468,751-25,519,541	p36.11	gain	1	100
7	chr4:69,056,055-69,170,240	q13.2	gain	1	100
5	chr7:142,155,398-142,171,867	q34	gain	1	100
5	chr8:12,286,694-12,287,223	p23.1	gain	1	100
5	chr9:44,889,904-47,006,984	p11.2 - p11.1	gain	3	100
5	chr9:65,336,138-70,168,361	q12 - q13	gain	23	100
5	chr10:46,401,243-46,481,060	q11.22	gain	1	100
5	chr11:18,905,781-18,918,566	p15.1	gain	1	100
5	chr11:5,745,410-5,759,390	p15.4	gain	1	100
6	chr11:55,125,250-55,213,752	q11	gain	4	100
5	chr14:18,072,112-19,492,811	q11.1 - q11.2	gain	9	92.32
5	chr15:18,671,839-20,157,067	q11.2	gain	11	100

Table S3 Unpaired copy number changes in 35% of EC (min. 5 out of 14)

Number of ECs	Region	Cytoband	Copy number event	Genes	% of CNV overlap
5	chr16:32,859,978-33,530,318	p11.2	gain	4	100
5	chr16:34,320,629-34,620,525	p11.2 - p11.1	gain	2	100
5	chr19:47,950,927-48,049,905	q13.31	gain	2	100
5	chr20:1,509,277-1,530,137	p13	gain	1	100
5	chr21:9,985,800-10,195,652	p11.2 - p11.1	gain	6	100
6	chr8:7,225,806-7,830,758	p23.1	loss	26	100
5	chr10:27,265,953-27,269,233	p12.1	loss	1	100
5	chr14:18,072,112-19,495,051	q11.1 - q11.2	loss	9	92.32
5	chr20:1,509,277-1,543,885	p13	loss	1	100

Table S4 List of first 50 up and down regulated genes in enchondromas compared to controls using expression array (adj. p-value<0.001)

Gene	Probe ID	Log fold change	Adjusted p-value	Up or down regulated in ECs compared to controls
RPL5	830609	1.498288353	1.23E-05	up
SEPHS1	5700612	0.882184	1.44E-05	up
FBXL7	6980056	0.411650382	2.24E-05	up
PENK	6220019	6.282102003	2.87E-05	up
HNRPA1L-2	7320424	1.445469169	2.87E-05	up
KLHDC2	2810364	1.249636151	2.87E-05	up
RPS15A	2370193	1.70815712	3.15E-05	up
PSAT1	4850674	3.132269511	3.27E-05	up
NARS	5220653	1.472789211	3.27E-05	up
DDOST	6450605	1.169205597	3.27E-05	up
SUB1	1660661	0.812277392	3.81E-05	up
MORF4L1	4900343	1.892734965	5.16E-05	up
SERP1	1770541	1.250963064	5.85E-05	up
HNRPDL	2570358	0.910611746	5.85E-05	up
RGMB	840553	1.178803561	6.55E-05	up
ARPP19	6620356	0.916295729	6.55E-05	up
NDUFB10	1820482	1.467683024	6.64E-05	up
PCBP2	6480411	1.296922763	6.64E-05	up
RPS3A	6380255	0.995648646	6.64E-05	up
UBE4B	2140563	0.851349469	6.64E-05	up
WRB	4590102	0.563758352	6.64E-05	up
RAPGEF6	3390504	-0.672882046	6.64E-05	down
RPS15	5490603	1.512440234	6.76E-05	up
MBNL2	990128	1.603622517	7.16E-05	up
SDC2	2690026	1.600929972	7.16E-05	up

Table S4 (Continue)

Gene	Probe ID	Log fold change	Adjusted p-value	Up or down regulated in ECs compared to controls
CNPY2	1510564	1.281962896	7.16E-05	up
MAGED2	3420487	1.247397333	7.16E-05	up
RPS3A	6560164	1.230142924	7.16E-05	up
GOLT1B	2100368	0.862688589	7.16E-05	up
SUCLG2	4060692	1.144442877	7.65E-05	up
C17orf79	3390477	0.920304871	7.65E-05	up
RILPL1	5220309	1.001129512	8.42E-05	up
RPS3A	3180438	1.267085346	9.56E-05	up
SNAPIN	870041	0.448976723	0.000100663	up
FASTK	770687	0.914367321	0.0001063	up
CAV2	5910553	0.879047002	0.0001063	up
PRKRA	770021	0.834038593	0.0001063	up
FAM155A	3710048	0.75233413	0.0001063	up
YY1	610279	1.128257546	0.000107097	up
COL8A2	70196	2.609508099	0.000107799	up
DDAH1	3170292	1.740797333	0.00011324	up
DAG1	2060091	0.840321903	0.000122272	up
GABARAP	6290132	0.673758241	0.000122272	up
UBE3B	5420025	0.321545224	0.000130534	up
NELL1	1990731	2.608889501	0.000137633	up
NMD3	4010048	1.069344286	0.000137633	up
B9D1	6040465	0.44041778	0.000137633	up
TMEM45A	6280520	1.546856993	0.000138337	up
RPL7	2680082	1.196436462	0.000152905	up
C12orf62	6620360	0.75248323	0.000152905	up

For more detail please see *Orphanet J Rare Dis.* 2011, 14;6:2.

Chapter 4

Maffucci Syndrome: A Genome-Wide Analysis Using High Resolution Single Nucleotide Polymorphism and Expression Arrays on Four Cases

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Abstract

Ollier disease and Maffucci syndrome are rare, nonhereditary skeletal disorders characterized by the presence of multiple enchondromas with (Maffucci) or without (Ollier) co-existing multiple hemangiomas of soft tissue. Enchondromas can progress toward central chondrosarcomas. *PTH1R* mutations are found in a small subset of Ollier patients. The genetic deficit in Maffucci syndrome is unknown. Here, we report the first genome-wide analysis using Affymetrix SNP 6.0 array on Maffucci enchondromas (n = 4) and chondrosarcomas (n = 2) from four cases. Results were compared to a previously studied cohort of Ollier patients (n = 37). We found no loss of heterozygosity (LOH) or common copy number alterations shared by all enchondromas, with the exception of some copy number variations. As expected, chondrosarcomas were found to have multiple genomic imbalances. This is similar to conventional solitary and Ollier-related enchondromas and chondrosarcomas and supports the multistep genetic progression model. Expression profiling using Illumina BeadArray-v3 chip revealed that cartilaginous tumors in Maffucci patients are more similar to such tumors in Ollier patients than to sporadic cartilage tumors. Point mutations in a single gene or other copy number neutral genomic changes might play a role in enchondromagenesis.

Introduction

Enchondromatosis patients have multiple skeletal enchondromas. Enchondromas are benign hyaline cartilage-forming tumors arising within the medulla of the bone (Lucas and Bridge, 2002; Bovee et al., 2010). Ollier disease is rare, nonhereditary, and characterized by the often unilateral occurrence of multiple enchondromas. Maffucci syndrome is extremely rare, also nonhereditary, and demonstrates multiple vascular tumors of soft tissue in addition to enchondromas (Maffucci, 1881; Mertens and Unni, 2002; Auyeung et al., 2003; Bovee et al., 2010; Pansuriya et al., 2010). According to Spranger et al. (1978), Ollier disease (type I) and Maffucci syndrome (type II) are the most common enchondromatosis subtypes. Diagnosis is based on a combination of clinical, radiological, and histological features (Lewis and Ketcham, 1973). Both the enchondromas and the vascular lesions may progress to malignancy, and the malignant transformation rate is the highest (25–100%) in Maffucci syndrome (Zwenneke et al., 2001; Silve and Juppner, 2006).

Because the genetic background of Maffucci syndrome is unknown and genetic studies are limited to a single case report (Matsumoto et al., 1986), we set out to perform whole-genome analysis on tumors from four Maffucci patients collected from the EuroBoNeT consortium (www.eurobonet.eu). We used a high-resolution Affymetrix SNP 6.0 array to detect copy number alterations (CNA) as well as loss of heterozygosity (LOH) and Illumina expression array to study six tumors of four patients.

Materials And Methods

Clinical Information

Patient 1

A 43-year-old man was originally diagnosed with Ollier disease, which was changed to Maffucci syndrome when later developing hemangiomas. An enchondroma in the femur had transformed to a chondrosarcoma grade II (L2195), which was removed by en bloc resection. Subsequently, he developed an enchondroma that was curetted from his finger. The patient is still alive without any sign of metastasis.

Patient 2

A 10-year-old girl was initially diagnosed with Ollier disease based on multiple enchondromas. An enchondroma from her left femur and 4 years later in the fourth and fifth digits of the left hand were curetted. At the age of 17 and 18, amputations of the fifth digit and of the left second toe, respectively, were required for histologically established grade I chondrosarcoma. Six months later, a biopsy from her left proximal tibia (L2097a) and her left fifth toe (L2097c) confirmed the presence of enchondromas. At the age of 19, she developed multifocal lesions involving her left ankle and a biopsy demonstrated cavernous spaces with intervening spindle cell proliferation mixed with some inflammatory cells. The diagnosis of hemangiomas associated with Maffucci syndrome was made. At 21 years, biopsies of the left fifth toe and fourth finger, respectively, revealed atypical enchondroma. One year later, her left hallux was curetted demonstrating an enchondroma.

Patient 3

A 29-year-old man was diagnosed with Maffucci syndrome. Radiologically, bilaterally distributed lesions involving the pelvis, femur, metacarpals, phalanges, carpal bones, metatarsals, and foot phalanges were found. A biopsy of the left distal femur demonstrated chondrosarcoma and an above the knee amputation was performed. A large expanding mass involving the metaphysis and epiphysis of the distal femur was seen; the diagnosis was grade II chondrosarcoma (L2102). In the same year, a biopsy of the left ileum showed an enchondroma (L2101). In the following years, biopsies confirmed enchondromas in multiple digits of the left hand as well as of the right distal femur. In addition, a spindle-cell hemangioendothelioma was excised from the hand, supporting the diagnosis of Maffucci syndrome (Figure 1). A grade II chondrosarcoma of the distal phalanx of the fifth digit of the right hand required amputation.

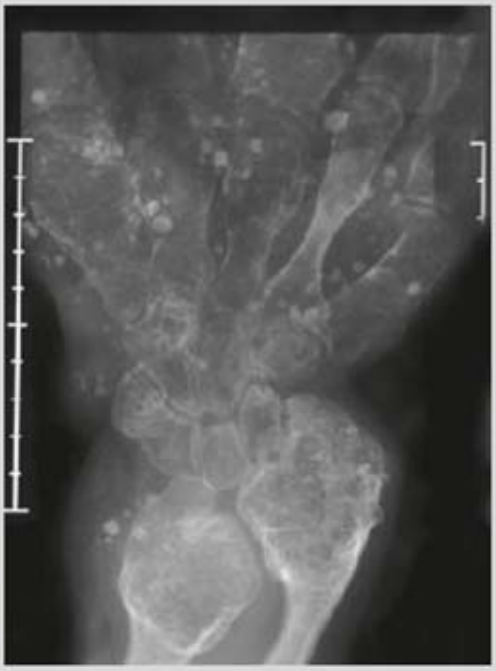
Patient 4

A 37-year-old woman was diagnosed with Maffucci syndrome based on the presence of histologically proven spindle-cell hemangiomas and multiple enchondromas. Histologically confirmed enchondromas were present in the phalangeal bones (L1684). In addition, the patient had superficial hemangiomas in the left thigh. At age 37, an enchondroma was curetted from her left third finger. The patient is alive and has not developed chondrosarcomas or lung metastases.

Sample Preparation

Fresh frozen tissues from six tumors and one normal muscle of the four patients were collected from the EuroBoNet network (Table 1). All samples were obtained according to the ethical guidelines of the host institution. Samples were coded, 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). All samples derived from primary tumors and all were graded (Evans et al., 1977).

Figure 1 Radiographic images from patient 3



Patient 3 with Maffucci syndrome. In addition to the multiple enchondromas, soft tissue calcifications are seen representing phleboliths in hemangiomas.

Single Nucleotide Polymorphism Array, Expression Array, and Data Analysis

DNA and RNA were isolated from tumors containing a minimum of 80% tumor cells. Affymetrix Genome-Wide Human SNP Array 6.0 was performed as described earlier (Pansuriya et al., 2011). The average call rate was 98.3%. Data analysis was performed using statistical language R version 2.8 and Nexus software version 4.1 (BioDiscovery, CA) on six tumors (Table 1) and 29 controls as described previously (Pansuriya et al., 2011). Sample preparation, hybridization to Illumina Human-6 v3.0 BeadChips, and data analysis were performed as previously described (Table 1) (Pansuriya et al., 2011). Data files are publicly available at NCBI's Gene Expression Omnibus under accession number GSE26675 (www.ncbi.nlm.nih.gov/geo/, accession number GSE26675).

Table 1 Clinicopathological Data of the Patients

Patient ID	Tumor	Diagnosis	Tumor location	Age	Gender	Application
Patient 1	L2195a	CSII	Femur	43	M	1,2,3
Patient 2	L2097a	EC	Tibia	18	F	1,2
Patient 2	L2097c	EC	Toe	18	F	1,2
Patient 3	L2102	CSII	Femur	29	M	1,2,3
Patient 3	L2101	EC	Ilium	29	M	1
Patient 4	L1684	EC	Phalanx	37	F	1,2,3

*Normal DNA available enabling paired analysis. Applications: samples used for (1) single nucleotide polymorphism array, (2) MLPA, (3) expression array. EC, enchondroma; CS, chondrosarcoma.

Validation with Multiplex Ligation-Dependent Probe Amplification and Fluorescence In Situ Hybridization

Because of the shared homologous sequences between the chromosomes 13 and 21 centromeric regions and the short arms of chromosomes 13 and 21, we performed MLPA to confirm the single nucleotide polymorphism (SNP) array results and performed FISH on metaphases of a normal individual to map the presently assigned 21p11.2 locus sequences by using BAC probes selected from the UCSC database. MLPA was performed for the *TPTE* locus (CTCACCTGTCATTGGGGCCGAGCTCAATGATGACTCCCGCCAGGTCAGTCGGATCAGGACTAAAGGACA) using 38 normal controls and five Maffucci tumor samples. There was not enough DNA from L2101 to allow further studies. Data analysis was done as described (Pansuriya et al., 2011) using SoftGenetics Gene Marker version 1.70. Apart from the region on chromosome arm 21p, candidate regions (*FAM86D* and *PRKG1*) that we found in Ollier tumors were also screened in Maffucci tumors (Pansuriya et al., 2011). FISH was performed on normal metaphase and interphase cells using blood derived from normal donors as reported (Pajor et al., 1998) to confirm the location of the *TPTE* and *BAGE* genes at chromosome arm 21p. We used BAC clone RP11-95E16 (Cy3 labeled) covering *TPTE* region, FITC-labeled L1.26 alphoid repeat probe specific for chromosome 13 and 21 centromeres, and a whole chromosome paint (wcp) probe specific for the long arm of chromosome 21 (Cy5 labeled).

Results

Genetic Alterations in Maffucci enchondromas and Chondrosarcomas

Using an unpaired approach (29 control samples as a baseline) for enchondromas, we found in total nine regions with copy number gain and eight regions with copy number loss in a minimum of two patients (Table 2). Most of the identified regions were known to have common copy number variants in the DGV (Database of Genomic Variants) database. Selection of candidate regions for validation was done based on the copy number gain or loss in three or more enchondromas, with the same copy number event occurring in chondrosarcomas. One of the regions mapped to 21p11.2 containing the *TPTE* gene and a gene cluster (*BAGE2, BAGE3, BAGE4, BAGE5,* and *BAGE*) in three and four enchondromas, respectively.

There was no common genomic region with LOH (Figure 2) or CNA in all Maffucci enchondromas, consistent with previous findings in Ollier enchondromas (Pansuriya et al., 2011). Paired LOH and CNA analysis based on chondrosarcoma DNA and corresponding normal DNA was only possible for patient 1. This analysis revealed LOH with copy number loss at small regions of chromosome arms 1q, 7p, 7q, 8q, 12p, 12q, 13q, 14q, 21p, 21q, and copy neutral LOH at chromosome arm 9p. Copy number gains involving small regions were found at 7q and 15q. Unpaired analysis on the chondrosarcoma of patient 3 revealed LOH with copy number loss at small regions of chromosome arms 3p, 6q, 9p, 9q, 12q, 13p, 13q, and 14q. Copy number gains were observed for small areas of chromosome arms 16p and 16q.

Expression Array Findings

Expression array analysis demonstrated that the *TPTE* and *BAGE* genes were not highly expressed in the tumor samples. Because we had only one Maffucci enchondroma on the expression array, comparison between Ollier and Maffucci enchondromas was not possible. There were no significantly differentially expressed genes between grade II chondrosarcomas from Maffucci ($n = 2$) and Ollier patients ($n = 4$), possibly explained by small sample sizes; the top 50 genes with the lowest p-values are listed in Supporting Information Table 1. We performed cluster analysis on Ollier ($n = 16$), Maffucci ($n = 3$), and solitary ($n = 19$) tumors using the 100 most significantly differentially expressed genes, obtained by LIMMA analysis on Ollier versus solitary tumors (unpublished data). As expected, the Ollier and solitary tumors show good separation. The Maffucci tumors cluster together with Ollier tumors (Figure 3).

Verification of Gene CNA by MLPA and Location by FISH

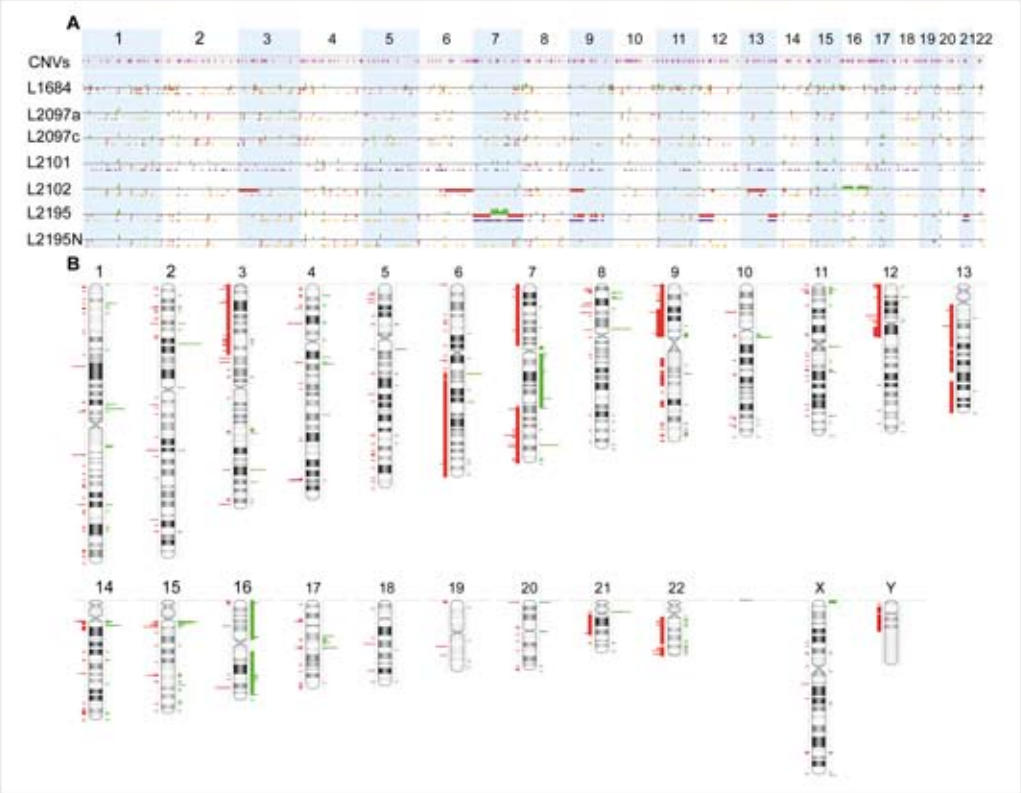
MLPA analysis did not confirm gain of *TPTE* at 21p11.2 containing *TPTE* genes in any of the Maffucci tumors at given threshold (0.8 loss, 1.2 gain). In addition, we screened Maffucci tumors for the candidate genes previously identified in Ollier disease (Pansuriya et al., 2011), and no copy number alteration (CNA) affecting *FAM86D* and *PRKG1* was seen. FISH results in line with the data from human genome sequencing project reporting polymorphic genome variant (DGV database: http://projects.tcag.ca/variation/) proved that indeed 21p11.2 region containing *TPTE* gene is polymorphic, as specific signals were observed both at chromosome 21 and 13 close to the centromeric signals (Supporting Information Figure 1).

Table 2 Regions with Copy Number Gains in Minimum Two Maffucci Patients with Enchondromas (Aggregate ¼ 50% EC)

Location	Start	End	Length	Event	Genes	Enchondromas	Patient ID	% of CNV Overlap
1q21.1	142,693,888	144,042,778	1,348,890	CN gain	12	L1684, L2097c	4,2	100
1q31.3	194,993,359	195,081,241	87,882	CN gain	2	L1684, L2097a	4,2	100
2p11.2–11.1	90,982,622	91,669,499	686,877	CN gain	3	L2097a,L2101	4,3	100
8p23.1	12,259,626	12,521,958	262,332	CN gain	3	L1684, L2097a, L2097c	4,2	100
8p11.23–11.22	39,347,411	39,506,777	159,366	CN gain	2	L2097a, L2097c, L2101	2,3	100
8p23.1	7,202,562	7,841,769	639,207	CN gain	26	L1684, L2097a, L2097c	4,2	100
11q11	55,133,136	55,209,319	76,183	CN gain	3	L1684, L2101	4,3	100
15q11.2	18,453,455	20,084,073	1,630,618	CN gain	11	L1684, L2101	4,3	100
21p11.2–11.1	9,758,730	10,195,652	436,922	CN gain	6	L1684, L2097a, L2097c	4,2	100
1q21.3	150,822,228	150,853,170	30,942	CN loss	2	L1684, L2101	4.3	100
2p24.1	20,614,169	20,751,564	137,395	CN loss	3	L1684, L2097a	4,2	29
12p11.21	31,005,629	31,040,097	34,468	CN loss	1	L1684, L2097a	4,2	100
15q23	65,858,075	65,937,824	79,749	CN loss	2	L1684, L2097a	4,2	0
17p11.2	18,299,660	18,352,373	52,713	CN loss	1	L1684, L2101	4,3	100
20p13	1,508,963	1,543,885	34,922	CN loss	1	L1684, L2101	4,3	100
22q13.31	44,356,090	44,430,291	74,201	CN loss	1	L1684, L2101	4,3	6
22q13.32	47,291,203	47,498,786	207,583	CN loss	1	L1684, L2097a	4,2	7

% of CNV overlap is according to DGV (Database of Genomic Variant) and calculated according to Nexus software. These regions are annotated using NCBI 36.1 (hg18).

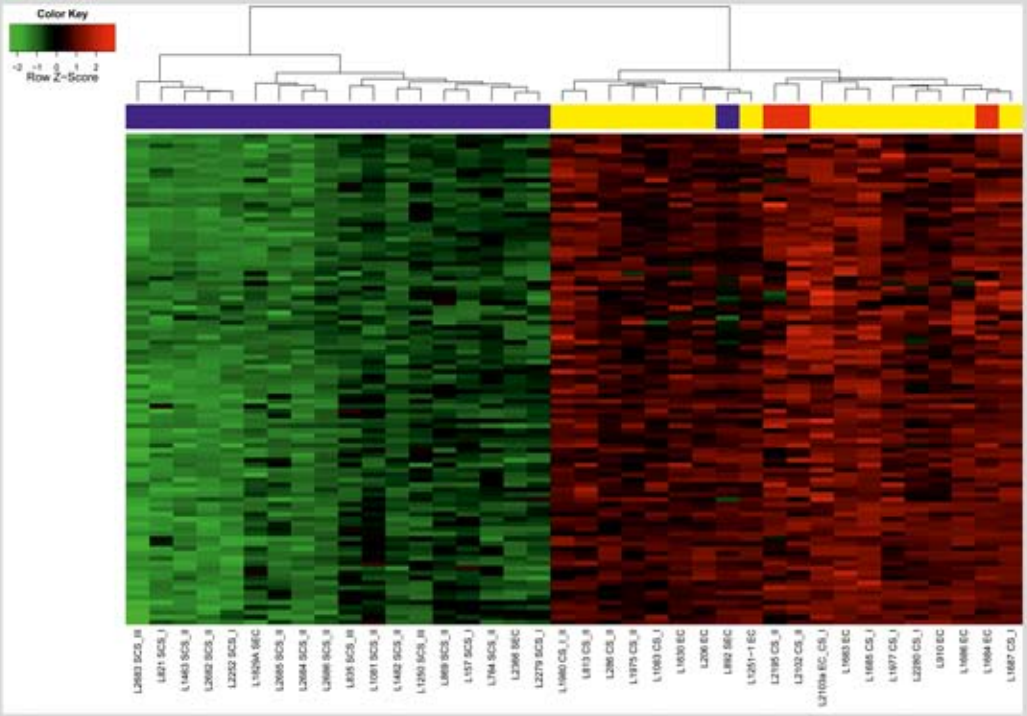
Figure 2 Genome-wide copy number alterations in the tumors and normal of Maffucci patients



A: Copy number alterations in enchondromas, chondrosarcomas, and one normal tissue of Maffucci patients. Gains are plotted in green above the baseline, and losses are plotted in red below the baseline. The X-axis corresponds to the genomic region from chromosomes 1 to 22, and the Y-axis represents the percentage of gains and losses of samples used in this study at the specific location in genome. The number and size of genomic alterations increases with increasing tumor grade. Also, the number and size of genomic alterations in enchondromas and in controls are comparable, which can be attributed to common copy number variation.

B: An overview of genomic aberrations observed in four enchondromas and two chondrosarcomas of Maffucci patients. The idiogram shows copy number gains in green on the right side of the chromosomes and copy number losses in red on the left side of the chromosomes in Maffucci enchondromas and chondrosarcomas.

Figure 3 Supervised clustering of enchondromas and chondrosarcomas occurring in context of solitary, Ollier and Maffucci syndrome



Heatmap depicting supervised clustering of Ollier (yellow, n = 16), Maffucci (red, n = 3), and solitary (blue, n = 19) tumor samples using the 100 most significantly differentially expressed genes (unpublished data), obtained by LIMMA analysis on Ollier versus solitary tumors (all genes have Benjamini and Hochberg FDR-adjusted P values < 0.00001). The heatmap was generated using the function heatmap 2 of R package *gplots* under standard settings. Negative z-scores are shown in green, while positive z-scores are shown in red. Maffucci tumors clustered together with Ollier tumors, indicating that the Maffucci tumors are more similar to tumors of Ollier disease than to solitary tumors. The solitary enchondroma L892 clusters together with the rest of the Ollier and Maffucci tumors, possibly because of misdiagnosis due to the undetected lesions. This cannot be proven, because the patient was lost to follow up.

Discussion

Enchondromatosis is a rare skeletal disorder for which seven subtypes have been described among which Ollier disease and Maffucci syndrome are the most common (Pansuriya et al., 2010). Both disorders can be differentiated from most of the other enchondromatosis subtypes by their sporadic nature and the predominantly unilateral occurrence of enchondromas. It is unclear whether these are different ends of a spectrum caused by mutations in a single gene or whether they represent different diseases. Some of the other enchondromatosis subtypes seem to be caused by genes involved in skeletal development and be inherited as Mendelian disorders, for example, metachondromatosis (*PTPN11*) (Sobreira et al., 2010; Bowen et al., 2011) and spondyloenchondrodysplasia (*ACP5*) (Briggs et al., 2011; Lausch et al., 2011) (Table 3). Two of our patients were originally diagnosed as having Ollier disease, developing hemangiomas only later in life, demonstrating that these two disorders might be closely related. Therefore, Ollier patients should be carefully checked for the presence of hemangiomas.

Table 3 Summary of Genetic Findings in Subtypes of Enchondromatosis

Candidate genes	Enchondromatosis type	Mutation type	References	Single nucleotide polymorphism array Maffucci (Present study)	Sequencing Maffucci (Present study)
PTH1R	Ollier disease	R150C, A1222T, R255H, G121E	Hopyan et al., 2002; Couvineau et al., 2008	-	Absent (unpublished data)
PRKG1	Ollier disease	copy number gain	Pansuriya et al., 2011	absent	-
FMA86D	Ollier disease	homozygous loss	Pansuriya et al., 2011	absent	-
PTPN11	Metachondromatosis	loss of function mutations	Sobreira et al., 2010; Bowen et al., 2011	absent	Absent (Bowen et al., 2011)
ACP5	Spondyloench-ondromatosis	homozygous loss	Lausch et al., 2011a; Briggs et al., 2011	absent	-
PTHLH	Symmetrical enchondromatosis	duplication	Collinson et al., 2010	absent	-

The pathogenesis underlying enchondroma development as well as the genetic cause of the different enchondromatosis subtypes are so far unknown, with the exception of *PTPN11* mutations causing autosomal dominant metachondromatosis in which enchondromas are combined with osteochondroma-like lesions (Sobreira et al., 2010; Bowen et al., 2011).

We excluded the presence of *PTPN11* mutations in our four patients (Bowen et al., 2011). Duplication involving *PTHLH* was recently reported for a patient with symmetrical enchondromatosis (Collinson et al., 2010). We did not see any copy number gain at the *PTHLH* locus at 12p11 in enchondromas from Maffucci patients. Four point mutations in *PTH1R* (R150C, A1222T, R255H, and G121E) leading to impaired function have been reported in 8% of patients with Ollier disease (Hopyan et al., 2002; Rozeman et al., 2004; Couvineau et al., 2008). PTH1R is involved in the IHH-PTHLH negative feedback loop regulating endochondral bone formation. In total, 26 patients (17 tumors and 9 blood samples) with Maffucci syndrome have been screened for *PTH1R* mutations, but none was detected (Rozeman et al., 2004; Couvineau et al., 2008). We screened our four patients for the four known *PTH1R* mutations and did not find any of these point mutations (data not shown). Nor were there any CNA in other genes involved in the IHH-PTHLH pathway in the tumors from our Maffucci patients.

Overall, the enchondromas did not show any large genomic alterations. The 21p11.2 region is known to contain copy number variants, and copy number changes are therefore not specific for the disease. Using FISH, we showed that the current annotation of the pericentromeric regions of acrocentric chromosomes might not be correct and should be handled with care. Two chondrosarcomas grade II showed more genomic aberrations. CNA at *FAM86D* and *PRKG1*, which we previously identified in a subset of Ollier enchondromas (Pansuriya et al., 2011), were absent in Maffucci enchondromas.

In conclusion, we report the first genome-wide study of enchondromas and chondrosarcomas from four individuals with Maffucci syndrome. Similar to Ollier-associated enchondromas, Maffucci associated enchondromas do not show LOH or common CNA. For the enchondromagenesis, small mutations and/or copy number neutral genomic alterations might be causative, which can be further investigated using a next-generation sequencing approach. An increased number of genetic alterations are found in Maffucci chondrosarcomas, supporting the multistep genetic progression model for chondrosarcomagenesis.

Acknowledgments

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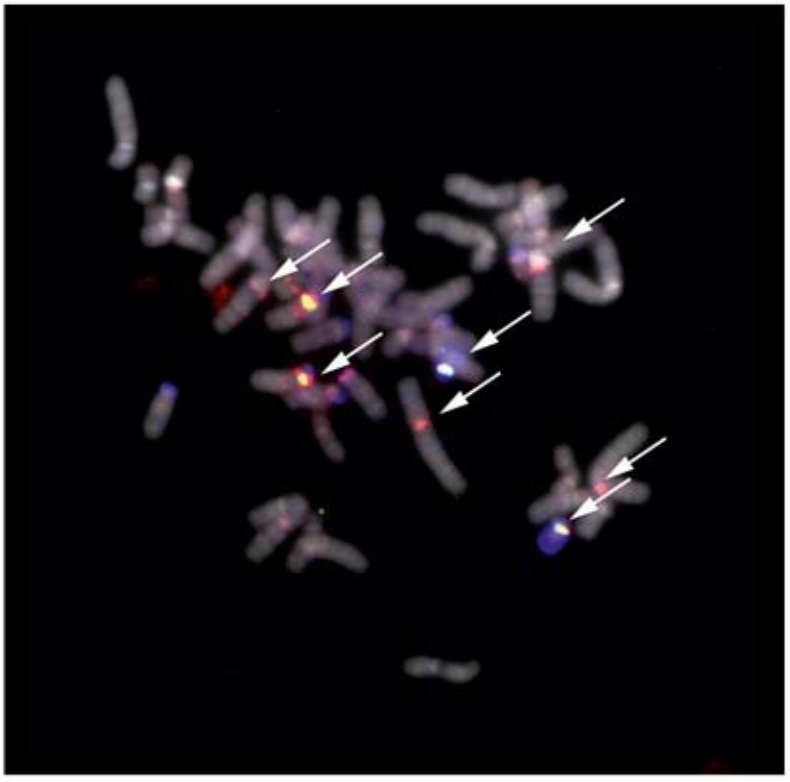
Figure S1 FISH of chromosomes 13 and 21



FISH results indicated chromosomes 13 and 21 in metaphase. Probe RP11-95E16 (Cy3 labeled, red) and centromeric probe binds to the chromosome 13 and 21 (FITC, green) and wcp 21 (blue). A colocalization of all 4 red and green signals was seen indicating the presence of RP11-95E16 signals both at chromosome 21 and 13; in addition several secondary signals of the RP11-95E16 clone were observed at other chromosomes.

Table S1 Top fifty genes with the lowest p-value between Ollier and Maffucci chondrosarcoma grade II

Gene Symbol	Probe ID	logFC	p-value	adj. p-value
C11orf87	7510619	-1.04410334	3.89E-06	0.111094124
NOMO2	2650040	0.765193013	1.25E-05	0.15298495
ZP4	7400392	-0.49531778	1.61E-05	0.15298495
ARL4A	7560615	-0.92348809	2.50E-05	0.157254509
PTGFR	2480139	-2.1611789	2.96E-05	0.157254509
PTGFR	770551	-2.00072338	3.93E-05	0.157254509
CNTNAP2	1400520	-2.48996817	4.16E-05	0.157254509
TNFSF10	870202	-0.83818461	4.41E-05	0.157254509
CSPG5	160397	-0.52438993	6.53E-05	0.20696256
TCF7L1	5050678	-0.31782816	8.05E-05	0.229817111
SELP	4810468	-0.70240586	9.35E-05	0.242399961
RORB	2370561	-0.68969674	0.0001544	0.367116161
CASP4	1450136	-0.91852242	0.0001706	0.374490797
SLC26A7	6760221	-0.48107142	0.000241	0.469874082
GORAB	830441	-0.93710499	0.000247	0.469874082
FLJ14213	4610672	-0.62513607	0.0002934	0.515246514
ADAMTS18	3290133	-0.29706855	0.000307	0.515246514
C6orf141	3450632	-0.26495298	0.0003508	0.556052553
THNSL1	5260520	-0.42022175	0.000379	0.56094917
PRG2	1580195	-0.54328552	0.0003932	0.56094917
SLCO1C1	2510554	-0.43268572	0.0004278	0.581158031
PLOD2	460338	1.312897386	0.0004555	0.582134774
MAP3K1	4230373	-0.65515382	0.0004703	0.582134774
TP63	6060131	-0.90703834	0.0005088	0.582134774
LOC25845	2760068	-0.72352259	0.0005494	0.582134774

Table S1 (Continue)

Gene Symbol	Probe ID	logFC	p-value	adj. p-value
EYA1	5720040	-1.61785141	0.0005548	0.582134774
CES1	2680056	-0.99532331	0.000556	0.582134774
KCTD14	2940632	0.567228862	0.0005999	0.582134774
CNR1	4010152	-0.28482701	0.0006165	0.582134774
SALL4	5050270	-0.45354179	0.0006362	0.582134774
ARL4A	1410113	-2.26725707	0.0006541	0.582134774
CCL20	4220246	-2.39865682	0.0006695	0.582134774
FAM148B	5860093	-1.72158352	0.0006733	0.582134774
EYA1	4850377	-0.20387975	0.0007209	0.587165546
PTPN13	3060609	-0.7534637	0.000749	0.587165546
TCP11L1	1070753	0.401831792	0.0007517	0.587165546
LOC649553	4040563	0.190343381	0.0007615	0.587165546
GGT5	670538	-1.20784023	0.0008607	0.639070504
HOXA3	2900048	-0.18387521	0.0008736	0.639070504
EMID1	6100487	-1.45511301	0.0009094	0.648682531
MYL10	4730427	-0.18551934	0.0010966	0.729648862
NOSTRIN	7210113	-1.245946	0.0011105	0.729648862
HES5	6590300	-0.4334798	0.0011293	0.729648862
MEOX1	1230594	-0.79694545	0.0011413	0.729648862
GRB10	5960725	0.385577604	0.0011508	0.729648862
KCNB1	6900196	-0.3336151	0.0012007	0.74472513
MAGEC1	7650731	-0.28558901	0.0013058	0.78699823
SLC22A3	6200333	-0.23877922	0.001324	0.78699823
SNTB2	840053	-0.33481726	0.0014166	0.797789001
ABCG2	7160220	-0.28215736	0.0014351	0.797789001

Chapter 5

Analysis of NDST1, EXT1 and HS in central chondrosarcoma and Ollier related tumors

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Abstract

Enchondromatosis is a rare skeletal disorder characterized by multiple enchondromas which may in some occasions progress towards central chondrosarcoma. In a some what related syndrome, multiple osteochondromas (peripheral tumors), mutations were found in *EXT1* (exostosin 1) and *EXT2* (exostosin 1), involved in HS (heparan sulfate) biosynthesis facilitating normal growth plate signaling. Apart from *EXT1* and *EXT2* other genes are also important in HS biosynthesis and one of them is *NDST1* (N-deacetylase/N-sulfotransferase). Interactions between EXT1 and EXT2 as well as NDST1 and EXT2 are reported in the literature. To explore possible parallels with multiple osteochondromas we studied EXT1, HS and NDST1 in enchondromatosis. We performed immunohistochemistry for EXT1, HS and NDST1 using a large series (n=65) of tumors related to enchondromatosis syndrome. Enchondromas and chondrosarcomas normally expressed EXT1 protein and HS irrespective of whether they occurred solitary or in the context of enchondromatosis, suggesting normal synthesis of heparan sulfate proteoglycans in these tumors. The level of NDST1 protein expression was almost identical to that in normal articular cartilage and growth plate. In total 98% of enchondromatosis related, 69% of solitary central and 67% of peripheral tumors showed NDST1 protein expression. NDST1 mutation analysis was performed and we found silent mutations (F185F and N343N) in two different exons of NDST1 in two unrelated tumors. An unknown variant (G to A) was found in exon 7 of 5/18 cases. The frequency of this variant found in cases (5/36) was not significantly different from the controls (10/164). Therefore, this unknown variant (G to A) can be a rare SNP unrelated with tumorigenesis. Overall, our results suggest normal function of EXT1, NDST1 and HS with absence of *NDST1* mutations in central cartilaginous tumors. Thus, no role of *NDST1* associated with enchondroma formation both within the context of enchondromatosis as well as in solitary cartilaginous tumors was identified.

Introduction

Enchondromas can present as a solitary lesion or as multiple lesions within the context of the enchondromatosis syndrome (1). Enchondromas are benign cartilaginous neoplasms in the medulla of bone. The enchondromatosis syndrome includes several different subtypes (1;2). Ollier disease (enchondromatosis subtype 1) is a rare, non-hereditary disorder in which patients have multiple enchondromas often with a unilateral predominance. The conditions in which multiple enchondromas are associated with hemangiomas of the soft tissue and osteochondromalike lesions are called Maffucci syndrome (enchondromatosis subtype 2) and metachondromatosis (type 3), respectively (1-4). Secondary central chondrosarcomas (CS) can develop from a preexisting enchondroma. The elucidation of the genetic deficit underlying these rare enchondromatosis subtypes has been hampered by their rarity. Genetic screens showed four different heterozygous parathyroid hormone-related peptide receptor (*PTH1R*) point mutations in a subgroup of Ollier patients (8%) (5-7) and *PTPN11* mutations are reported for metachondromatosis syndrome (8;9). Osteochondroma is a benign outgrowth of bone with a cartilage cap at the surface of the bone (10). Similar to enchondromas, osteochondromas can occur as solitary or multiple lesions within the context of the hereditary syndrome known as multiple osteochondromas (MO)(previously called hereditary multiple exostoses (HME)) (11). MO is an autosomal dominant syndrome caused by mutations in *EXT1* and *EXT2* (12;13). The EXT proteins are glycosyltransferases responsible for the elongation of heparan sulfate (HS) chains (14;15).

Central (CS) and peripheral chondrosarcomas (PCS) are similar at the histological level despite their different origin. Schrage et al. previously investigated involvement of EXT related pathways in central chondrosarcomas (16) and demonstrated that mutations in *EXT1 or EXT2* were absent and that the level of gene expression was comparable to the growth plates. Presto et al. proposed GAGosome model in which in cells over-expressing NDST1 and EXT2, NDST1 competes with EXT1 to bind to EXT2 and will form hetero-duplex (17). Binding of more NDST1 to EXT2 might alter formation and localization of HS.

We here further explore the parallels between central and peripheral chondrosarcoma and analyzed three different major components (NDST1, EXT1 and amount of HS) in a large series of enchondromatosis related tumors as well as solitary central tumors. NDST1 is involved in chain elongation step of HS synthesis. Based on the absence of mutations, normal expression of *EXTs* in central tumors and the GAGosome model, we hypothesized that *NDST1* might be a candidate gene for central cartilaginous tumors.

Materials and Methods

Patient Material

Five tissue microarrays (TMAs) containing 86 paraffin embedded tumors of which 65 are Ollier related and 21 solitary central tumors (18) and whole sections from 32 solitary central cartilaginous tumors (8 ECs, 8 CSI, 8 CSII and 8 CSIII) were included to study protein expression of EXT1, HS and NDST1. For mutation analysis, fresh frozen tumor tissues (n = 15, Table 1) were used. Detailed clinical information about the samples is described earlier (18). Samples were collected from EuroBoNet (www.eurobonet.eu) as well as contributors via EMSOS (European Musculo-Skeletal Oncology Society (http://www.emsos.org/)) networks. All chondrosarcomas were graded according to Evans et al (19) and coded according to the ethical guidelines "Code for Proper Secondary Use of Human Tissue in The Netherlands" (Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Five TMAs (18) were assessed for expression of the EXT1 protein and of HS. Immunohistochemistry using EXT1 antibody (Aviva System Biology, San Diego, CA, USA, 1:400 dilution, EDTA antigen retrieval) was performed as described (20). Heparan sulfate was assessed using the 10E4 antibody (US Biological, Marblehead, MA, USA, 1:400 dilution) and staining was performed as described (21). Placenta and skin were used as positive controls for EXT1 and 10E4, respectively.

We used primary NDST1 antibody (ab55296, Abcam Inc., dilution 1:800, EDTA antigen retrieval) to stain 5 TMAs and 32 whole section slides. Ileum was used as a positive control and primary antibody was omitted as a negative control. The specificity of NDST1 antibody for nuclear staining was checked using a tissue microarray containing 79 soft tissue tumors of 28 different entities, as described previously (22) and 24 peripheral cartilaginous tumors (5 OCs, 8 PCSI, 7 PCSII and 2 PCSIII). Three cartilage and three growth plates were used as normal controls. Immunohistochemistry procedures were performed as described previously (18).

Data Analysis

TMA slides were scanned using a high resolution Mirax Desk scanner (3D Histech, Hungary) and scored using the Mirax viewer TMA module software version 1.1.12 (3D Histech, Hungary) while the whole sections were scored manually. Percentage of positive tumor cells in case of EXT1 and 10E4 staining was estimated by two observers (CEA and TCP) (20). Most of the cores contained internal positive controls (vessel walls) and therefore cases without positive staining of vessel walls were excluded from further statistical analysis since prolonged decalcification may have destroyed the antigen. We took the average of percentage of positive cells from three cores of the same tumor present on TMA. In brief for NDST1 protein, the intensity (0=no staining, 1=weak, 2=moderate, 3=strong) and percentage of positive tumor cells (0=0%, 1=1-24%, 2=25-49%, 3=50-74%, 4=75-100%) were assessed. A sum score ≥4 was considered positive for scoring of cytoplasmic staining and nuclear staining was scored as present or absent by three independent observers (JVMGB, TCP and YS). Statistical analysis was done using Oneway ANOVA to see the difference in protein expression between the tumor grades of each group (Ollier disease, solitary central tumors and peripheral tumors) was performed in SPSS (version 16.0, Chicago, Illinois, USA). Spearman's rank correlation coefficient was calculated to verify statistical dependence between two variables (EXT1 and HS expression) in SPSS.

Table 1 Clinical information of the cases used for of *NDST1* mutation Screening

Cases	Diagnosis	Material	Gender	Age	Tumor location	Silent Mutation	Unknown variation (Ex7)
L1684	Maffucci syndrome	EC	F	37	Phalanx	-	G>GG
L2102	Maffucci syndrome	CS II	M	29	Femur	-	G>GG
L2195	Maffucci syndrome	CS II	M	43	Knee	Phe185Phe (Ex 2)	G>GG
L1490 II	Ollier disease	EC	F	12	Phalanx	-	**G>GA**
L1977	Ollier disease	CS I	M	41	Tibia	-	G>GG
L2280	Ollier disease	CS I	F	24	Acromion	-	G>GG
L286	Ollier disease	CS II	F	23	Femur	-	G>GG
L172	Ollier disease	CS II	M	40	Scapula	-	G>GG
L157	Solitary central chondrosarcoma	CS I	M	54	Humerus	Asn343Asn (Ex 3)	G>GG
L178	Solitary central chondrosarcoma	CS II	M	57	Tibia		G>GG
L182 (II)	Solitary central chondrosarcoma	CS I	F	34	Rib		G>GG
L185	Solitary central chondrosarcoma	CS III	F	47	Femur		G>GG
L19 (II)	Solitary central chondrosarcoma	CS II	F	48	Femur		**G>GA**
L247	Solitary central chondrosarcoma	CS I	F	39	Tibia		**G>GA**
L319	Solitary central chondrosarcoma	CS I	F	37	Tibia		**G>GA**
80078 N	Metachondromatosis*	blood	M	4	-		G>GG
L1345 N	Metachondromatosis	blood	M	6	-		G>GG

* sample with *PTPN11* mutation.

DNA isolation

The percentage of tumor cells was estimated using frozen sections and only blocks containing >70% of tumor cells were used for DNA isolation. DNA from fresh frozen tumor tissue was isolated using a Wizard genomic DNA purification kit (Promega Benelux, Leiden, The Netherlands), according to the manufacturer's instructions (Table 1). DNA concentration was measured by NanoDrop spectrophotometer (Isogen, The Netherlands) and in total 10ng of DNA was used for sequencing.

Mutation screening

DNA isolated from 15 tumors (Table 1) and blood DNA of two metachondromatosis patients was included. Primer sequences for exons 1b, 2b, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14 of *NDST1* were used as described earlier (23) while the remaining primers to cover the full *NDST1* gene were designed using Primer3 program. Primer sequences used in this study are noted in table 2. Sanger Sequencing was performed as described by van Eijk et al (24). In short, M13 tails were added to enhance PCR and facilitate Sanger sequencing. Approximately 10 ng of PCR product was sequenced with M13 forward (TGTAAAACGACGGCCAGT) and/or reverse (CAGGAAACAGCTATGACC) primer on an ABI 3700 DNA Analyzer using BigDye Terminator Chemistry (Applied Biosystems, Carlsbad, CA) at the Leiden Genome Technology Center (www.lgtc.nl). Reverse sequencing was performed to confirm mutations and polymorphisms. Sequences were analyzed with Mutation Surveyor DNA variant analysis software) version 3.0.24 (Softgenetics, State college, PA).

To validate the allelic distribution of GA instead of GG allele present in *NDST1* exon 7 of 5 cases, high resolution melting curve analysis (HRM) was performed using a light scanner (Idaho Technology, Salt Lake City, UT) as described earlier (24). To check the frequency of GA allele in all the cases (n=18), HRM analysis was performed using blood DNA from normal healthy controls (n=82).

Results

EXT1 protein expression

Around 50% of tumors related to Ollier disease showed EXT1 expression and in case of solitary tumors, expression was more variable (Table 3). There was no significant difference between different grades of Ollier (Oneway ANOVA, p=0.9) related and solitary tumors (Oneway ANOVA, p=0.08). Average percentage of positive cells from three cores of the same tumor is plotted for each grade in figure 1.

Evaluation of heparan sulfate (HS)

Percentages of positive tumors expressing HS in cytoplasm are given in table 3, figure 1. There was no correlation with histological grade in patients with Ollier disease (Oneway ANOVA, p=0.6) nor in solitary tumors (Oneway ANOVA, p=0.08). There is no correlation with histological grade. One Ollier CSII showed HS expression in the matrix, membrane and in the cytoplasm. One Ollier enchondroma and solitary CSIII showed membranous and cytoplasmic expression of heparan sulfate.

Table 2 Primer sequences used for *NDST1* mutation analysis

Primer position	Sequence (5' - 3')	Tm °C
*NDST1*_Ex1A_F	CCGGTGGCCAAGGTCTC	> 75
*NDST1*_Ex1A_R	CCCAGTTGCGAGTAGAGGC	> 75
*NDST1*_Ex1B_F	TGCCACTCAAGCCTGTGCAG	74.9
*NDST1*_Ex1B_R	TGCAGAGGGGGCTCTGAACT	74.9
*NDST1*_Ex2A_F	CCTTTGGGGTTCTGGATGTG	73.8
*NDST1*_Ex2A_R	GTTGTTGCCAAACAGCACGC	73.8
*NDST1*_Ex2B_F	TCTGAGTCCATCCCACACCT	73.8
*NDST1*_Ex2B_R	TGAAGGCTGAAGCTTGCCAG	73.8
*NDST1*_Ex3_F	ACTCATTCCTTTCTCCCCTG	72.7
*NDST1*_Ex3_R	TCCTGGAAGTTGCTAGTGAG	72.7
*NDST1*_Ex4_F	CAGTGGGTGGTTCTGAGCTG	74.9
*NDST1*_Ex4_R	CTCCAGCCCAGCCCTTAG	> 75
*NDST1*_Ex5_F	CTCTCCCATTCTACAAAGGG	72.7
*NDST1*_Ex5_R	AGACTGTGCTCTCCATTCTC	72.7
*NDST1*_Ex6_F	CAGAAGGCACCATAGCTCCT	73.8
*NDST1*_Ex6_R	TGTGCAGCAGCCCCTTCTCA	74.9

Tm: melting temperature. M13F and M13R sequence were added to the primers.

Table 2(Continue)

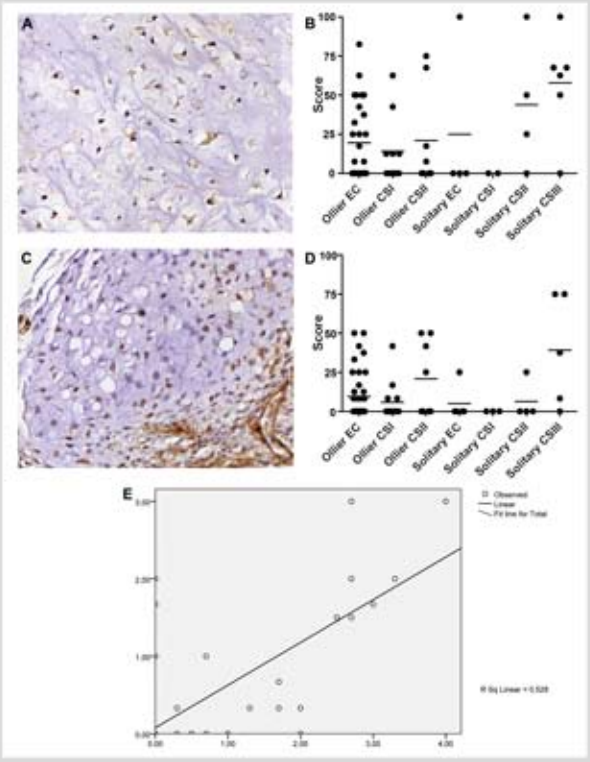
Primer position	Sequence (5' - 3')	Tm °C
*NDST1*_Ex7_F	AGGGTGGCTCAGACACTGAT	73.8
*NDST1*_Ex7_R	TCCATCCCTGTAGCAACCTG	73.8
*NDST1*_Ex8_F	AGTCCACTGACTGGGTTCTG	73.8
*NDST1*_Ex8_R	GGTCAAGAGCAGAGAGACCT	73.8
*NDST1*_Ex9_F	CTGGGTCTCAGGTGTCTACC	74.9
*NDST1*_Ex9_R	TCCCGCTGTGGACAGAAAGG	74.9
*NDST1*_Ex10_F	TGAGTTTTGTCTGTGAGCCG	72.7
*NDST1*_Ex10_R	GAGTAACTGAGTGTCAGACG	72.7
*NDST1*_Ex11_F	GCATGCTGACCCTCTTTCC	73.9
*NDST1*_Ex11_R	CCTCACAAGGGTCAGGG	74.2
*NDST1*_Ex12_F	ATCCCCTTTCTCCCTTTCCA	72.7
*NDST1*_Ex12_R	AGACTTTGGCTTTGTTGCCC	72.7
*NDST1*_Ex13_F	TCCCATCCAAAGACTTTCCC	72.7
*NDST1*_Ex13_R	TATGCAGGTGCTACAGGTAG	72.7
*NDST1*_Ex14_F	ACACAAGGTCTGAGCTTTCC	72.7
*NDST1*_Ex14_R	TCACAAACGTTCAGTCTGGC	72.7

Tm: melting temperature. M13F and M13R sequence were added to the primers.

Correlation between EXT1 and heparan sulfate

In enchondromas and chondrosarcomas related to enchondromatosis syndrome, correlation was found between EXT1 and HS protein expression (Figure 1).

Figure 1 Analysis of EXT1 and HS



A) Immunohistochemical staining for EXT1 protein (400x magnification)

B) Scoring of cytoplasmic expression of EXT1 protein. On the X-axis different tumor grades of Ollier and solitary tumors are given. Y-axis indicates the percentage of positive cells in each tumor.

C) 10E4 staining showing cells with and without cytoplasmic expression of HS.

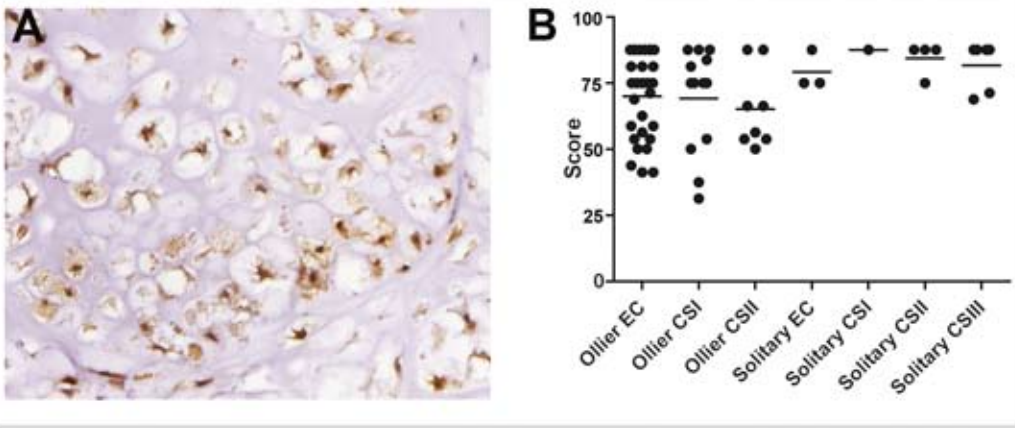
D) Scoring of cytoplasmic expression of HS. On the X-axis different tumor grades of Ollier and solitary tumors are given. Y-axis indicates the percentage of positive cells in each tumor.

E) In enchondromas and chondrosarcomas of enchondromatosis, a correlation was found between EXT1 and HS expression (R Square = 0.52, Spearman Correlation test).

NDST1 Protein Expression

Chondrocytes in articular cartilage and in the normal growth plates demonstrated cytoplasmic expression of NDST1. In total 98% of tumors related to Ollier disease and 69% of solitary tumors showed NDST1 protein expression as outlined in table 3. In tumors, cytoplasmic staining was highly variable ranging from weak to strong (Figure 2, Table 3). In total, 26 of 146 (18%) tumors were non evaluable due to the core losses or lack of positive internal control (vessel wall) and were therefore excluded from further analysis. The peripheral tumors that we included for comparison demonstrated cytoplasmic NDST1 expression in 1/5 (20%) osteochondromas and 10/13 (76%) peripheral chondrosarcomas. Few tumors showed nuclear staining. Interestingly, among 28 different types of soft tissue tumors that we stained on TMA to assess specificity of nuclear staining, only synovial chondromatosis showed nuclear staining.

Figure 2 Analysis of NDST1



A) Ollier chondrosarcoma showing cytoplasmic NDST1 protein expression in tumor cells (400x magnification).

B) Scoring of cytoplasmic expression of NDST1 protein. On the X-axis different tumor grades of Ollier and solitary tumors are given. Y-axis indicates the percentage of positive cells in each tumor.

Table 3 NDST1, EXT1 and Heparan sulfate protein expression

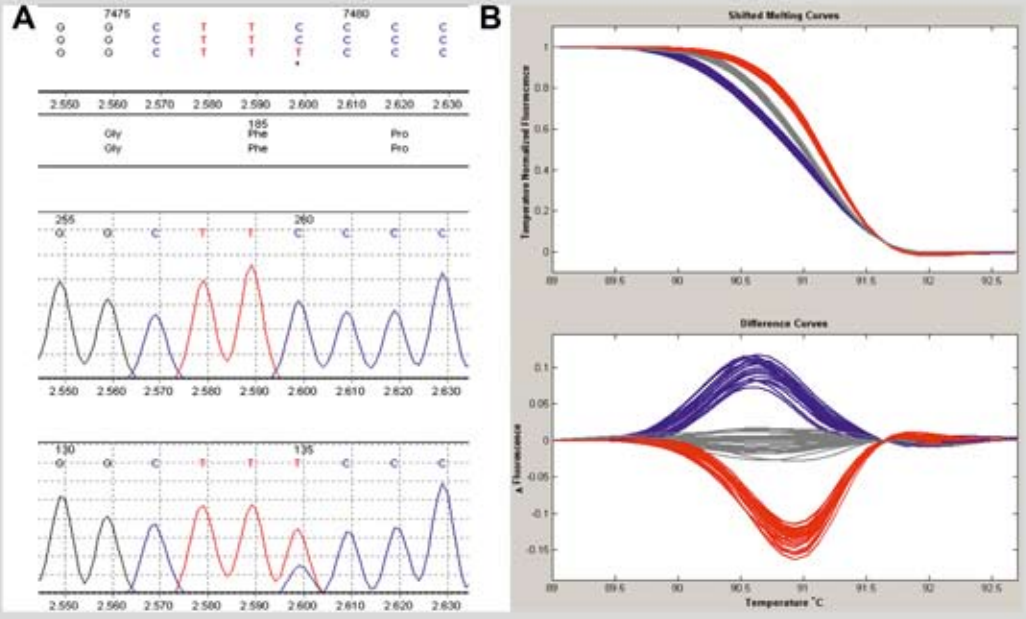
Disease	Tumor Grade	NDST1 expression	EXT1 expression	HS expression
Ollier	EC	28/28 (100%)	15/29 (51%)	15/38 (39%)
	CSI	12/13 (92%)	5/10 (50%)	4/13 (30%)
	CSII	8/8 (100%)	4/8 (50%)	4/8 (50%)
Solitary	EC	5/5 (100%)	1/4 (25%)	1/5 (20%)
	CSI	3/9 (33%)	0/2	0/3
	CSII	9/10 (90%)	3/4 (75%)	1/4 (25%)
	CSIII	9/14 (64%)	5/6 (83%)	4/5 (80%)

NDST1 mutation analysis

As we found expression of NDST1 in the majority of our tumors, we sequenced whole gene in selected tumors to evaluate the possible presence of activating point mutations. Two silent mutations (TTC→TTT; Phe185Phe in exon 2A and AAC→AAT; Asn343Asn in exon 3) were found in 2/18 (L2195, L157) cases which were confirmed using reverse and forward sequencing with M13 primers (Figure 3). Two known heterozygous SNPs (rs2273235 and rs2273234) in exon 2 were observed in 10/18 tumors. In exon 7, we found a known heterozygous SNP (rs1290147) with allele distribution GG (4); GC (14); CC (0) and an unknown variant with allele distribution GG (13); GA (5); AA (0) so GG is converted to GA in 5/18 cases (Table 1). This unknown variant found in five cases was confirmed by sequencing from both directions.

To validate the finding of this unknown variant (G to A) and to estimate the frequency of the GA variant in the normal population we performed HRM analysis for these 18 cases (36 alleles), along with 82 DNA from healthy controls (164 alleles) (Figure 1). The frequency of the A allele was higher in the patient group (5/36 = 13.8%) compared to the control group (10/164 = 6.09%). However, this difference was not statistically significant (ChiSquare, Fischer exact's test, $p = 0.153$) and therefore the A allele does not seem to be related to the disease.

Figure 3 *NDST1* mutation analysis



A) *NDST1* mutation analysis by Sanger sequencing. Output of Mutation surveyor software indicating Phe185Phe heterozygous silent mutation in Maffucci chondrosarcoma grade II (L2195). Top panel indicates three letter code for amino acid and middle panel indicates the reference sequence while lower panel indicates the sequence of L2195.

B) High resolution melting curve analysis for genotyping. Blue colour indicates genotype (GC GA), gray indicates (GC GG) and red indicates (GG GG).

Discussion

The GAGosome concept was introduced by Esko and Selleck, where they speculated that the enzymes are assembled into complexes, GAGosomes, responsible for elongation and modification of the HS chain (25). Different interactions between EXT1 and EXT2 (15), C5-epimerase with 2-Osulfotransferase (26) and xylosyltransferase with galactosyltransferase-I (27) were described. Presto et al. proved interactions between NDST1 and EXT2 (17). They showed that in cells overexpressing NDST1 and EXT2, NDST1 competes with EXT1 to bind to EXT2 and will form a hetero-duplex (17).

In this study, we analyze expression of three components of the GAGosome (EXT1, HS and NDST1) in central chondrosarcomas and a relatively large series of tumors occurring in the context of enchondromatosis, which we were able to collect all across Europe through the EuroBoNeT and EMSOS networks thus fairly excluding a population bias.

Only 40- 50% of the tumors expressed EXT1 and HS. We speculate based on our findings for the protein expression of EXT1 and HS that central tumors produce heparan sulfate when needed. This might explain why not all cells stain equally. Furthermore, we found a correlation between EXT1 and HS protein expression. The percentage of EXT1 positive cells is slightly higher than HS positive cells. The cellular environment might influence the activity of EXT1 in a cell. Alternatively, short HS chains may be produced that are not detected by the 10E4 antibody. We previously observed a similar discrepancy in the balance between EXT1 and 10E4 immuno staining in normal cartilage and growth plates (20).

In enchondromatosis related tumors, we found high expression of NDST1. NDST1 is widely expressed in central tumors. The nuclear localization of NDST1 that we found is puzzling and its nuclear function is not reported in the literature so far. Based on the high expression of NDST1 and the proposed GAGosome model, we hypothesized that *NDST1* might be a candidate gene for enchondroma or central chondrosarcoma development. There were no copy number alterations of *NDST1, EXT1* or *EXT2* on SNP array which was performed on tumors involved in Ollier disease, sporadic solitary central and Maffucci syndrome (18;28). Previously, total 30 genes related to HSPG biosynthesis pathways including *EXT* and *EXT*-like genes were checked for genomic losses and gains in seven solitary central chondrosarcomas and did not show any copy number alterations (16). Two metachondromatosis cases also showed no point mutations in *NDST1*. One of these two cases was positive for *PTPN11* mutations (9).

In summary, we present the evaluation of NDST1 in central cartilaginous tumors (Ollier diseases, Maffucci syndrome and solitary central chondrosarcomas) and showed high expression of NDST1 as well as absence of *NDST1* mutations. In conclusion, we demonstrate no role of NDST1 in enchondromas or central chondrosarcomas.

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Chapter 6

Somatic mosaic *IDH1* and *IDH2* mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome

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Abstract

Ollier disease and Maffucci syndrome are non-hereditary skeletal disorders characterized by multiple enchondromas (Ollier disease) combined with spindle cell hemangiomas (Maffucci syndrome). We report somatic heterozygous mutations in *IDH1* (encoding R132C and R132H substitutions) or *IDH2* (R172S) in 87% of enchondromas (benign cartilage tumors) and in 70% of spindle cell hemangiomas (benign vascular lesions). In total, 35 of 43 (81%) subjects with Ollier disease and 10 of 13 (77%) with Maffucci syndrome carried *IDH1* (98%) or *IDH2* (2%) mutations in their tumors. Fourteen of 16 subjects had identical mutations in separate lesions. Immunohistochemistry to detect mutant IDH1 R132H protein suggested intraneoplastic and somatic mosaicism. *IDH1* mutations in cartilage tumors were associated with hypermethylation and downregulated expression of several genes. Mutations were also found in 40% of solitary central cartilaginous tumors and in four chondrosarcoma cell lines, which will enable functional studies to assess the role of *IDH1* and *IDH2* mutations in tumor formation.

Enchondromas are benign, cartilage-forming tumors within the medullary cavity of the bone[1–3]. Individuals with enchondromatosis syndrome, which encompasses seven major subtypes, develop multiple enchondromas. The most common subtypes are non-hereditary Ollier disease (subtype I) and Maffucci syndrome (subtype II), the latter distinguished by spindle cell hemangiomas that occur in addition to the multiple enchondromas[1,3]. Malignant transformation of enchondromas to chondrosarcomas occurs in >30% of these individuals[3,4].

To date, genome-wide screens have not identified a causative gene for Ollier disease or Maffucci syndrome[5–9]. Individuals with these diseases have an increased incidence of gliomas[3,10] and juvenile granulosa cell tumors[3,11–13]. *IDH1* and, more rarely, *IDH2* mutations in gliomas[14–16] and *GNAS*-activating mutations in juvenile granulosa cell tumors[17] have been reported. Notably, *IDH1* and *IDH2* mutations were recently reported in solitary central and periosteal enchondromas and chondrosarcomas, including in a few tumors from individuals with enchondromatosis[18]. The possibility that *GNAS* mutations are present in enchondromas and chondrosarcomas has not previously been explored.

We therefore assessed whether mutations in *IDH1*, *IDH2* or *GNAS* may cause enchondroma and spindle cell hemangioma formation in Ollier disease and Maffucci syndrome. Sequence analysis of hotspot mutation sites was performed using lesional tissue from 43 individuals with Ollier disease, and this analysis revealed in 33 subjects (78%) the presence of heterozygous mutations in *IDH1* of c.394C>T (encoding an R132C substitution) or c.395G>A (encoding R132H) (NM_005896.2 for both) or in *IDH2* of c.516G>C (encoding R172S) (NM_002168.2) (Supplementary Figure 1a–c). Among the individuals with Maffucci syndrome, 7 of 13 subjects (54%) carried *IDH1* mutations encoding the R132C substitution. Mutations were absent in DNA isolated from the blood, muscle or saliva of the subjects (Supplementary Figure 1b). Mutations in *GNAS* were absent in the tissues examined.

An additional eight tumors had sub-threshold peaks at the position in *IDH1* expected to encode mutations resulting in R132C or R132H substitutions, suggesting that the mutant allele might be present in a small subpopulation of the tumor cells at the limit of or below the level of detection of Sanger sequencing. We therefore performed a hydrolysis probe assay, which is capable of detecting mutant allele frequencies as low as 1%, to look for *IDH1* mutations encoding R132C or R132H[19,20]. Mutations were confirmed in seven of eight tumors (Supplementary Figure 1d–g), and there was insufficient DNA from the eighth tumor for analysis. Thus, in total, 35 of 43 (81%) and 10 of 13 (77%) subjects with Ollier disease and Maffucci syndrome, respectively, had *IDH1* or *IDH2* mutations (Figure 1a, Table 1 and Supplementary Table 1). The frequency of mutations in tumors is shown in Figure 1b.

Other subtypes of enchondromatosis syndromes are known to be caused by mutations in *PTPN11* (metachondromatosis)[21,22] and *ACP5* (spondyloenchondrodysplasia)[23,24] and by *PTHLH* duplication (symmetrical enchondromatosis)[25]. Mutations in *PTH1R*, which encodes a protein involved in enchondral bone formation, are found in ~8% of individuals with Ollier disease but not in those with Maffucci syndrome[5–7]. Previously, an absence of *PTPN11* mutations was shown in the current cohort of individuals[22]. In the current study, we did not detect *PTH1R* mutations in a screen of 35 subjects with Ollier disease or Maffucci syndrome.

Table 1 Results of *IDH1* and *IDH2* mutation analysis

	Total	Gender (M:F) (median age in years)	Number with *IDH1* mutations (%)	Number with *IDH1* R132C (*IDH1* CGT>TGT) (%)	Number with R132H (*IDH1* CGT>CAT) (%)	Number with *IDH2* mutation (%)	Total with *IDH1* or *IDH2* mutation (%)
Ollier disease							
Number of subjects	43	21:21[a] (24)	34 (79)			1 (2)	35 (81)
Enchondroma	25		22 (88)	15(68)	7 (32)	0	22 (88)
Chondrosarcoma grade I	23		20 (87)	18 (90)	2 (10)	0	20 (87)
Chondrosarcoma grade II	8		5 (63)	5 (100)	0	1 (12)	6 (75)
Chondrosarcoma grade III	2		1 (50)	1 (100)	0	1 (50)	2 (100)
Total number of tumors	58		48 (83)	39 (81)	9 (19)	2 (3)	50 (86)
Maffucci syndrome							
Number of subjects	13	5:8 (15)	10 (77)			0	
Enchondroma	5		4 (80)	4 (100)	0	0	
Chondrosarcoma grade I	1		1 (100)	1 (100)	0	0	

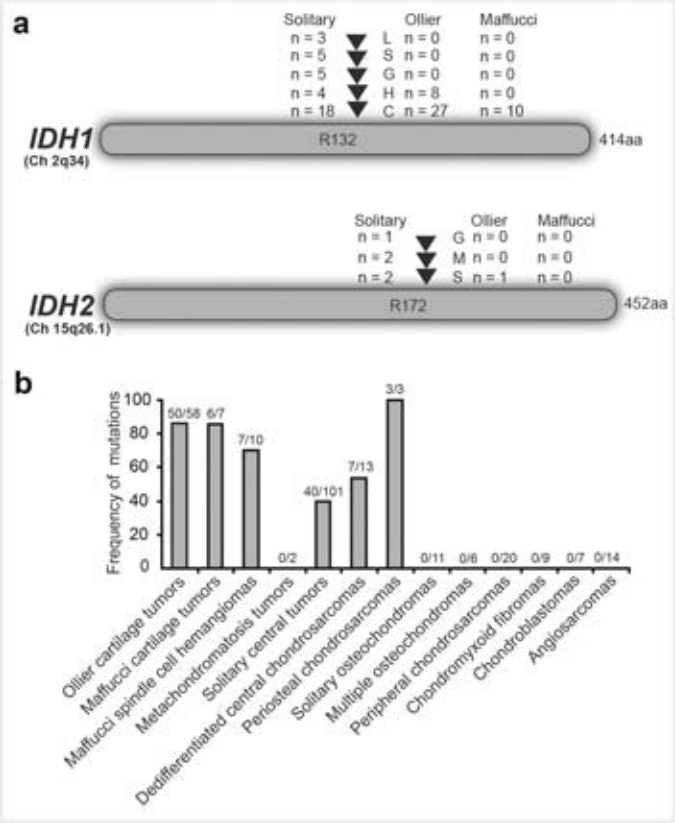
Table 1 (Continue)

	Total	Gender (M:F) (median age in years)	Number with *IDH1* mutations (%)	Number with *IDH1* R132C (*IDH1* CGT>TGT) (%)	Number with R132H (*IDH1* CGT>CAT) (%)	Number with *IDH2* mutation (%)	Total with *IDH1* or *IDH2* mutation (%)
Chondrosarcoma grade II	1		1(100)	1(100)	0	0	
Spindle cell hemangioma	10		7 (70)	7(100)	0	0	
Total number of tumors	17		13 (76)	13 (100)	0	0	
Solitary tumors							
Enchondroma	9		3 (33)	2 (67)	1 (33)	2 (22)	5 (56)
Central chondrosarcoma grade I	20		7[b] (35)	2 (29)	2 (29)	0	7 (35)
Central chondrosarcoma grade II	57		18[b] (32)	9 (50)	1 (6)	3 (5)	21 (37)
Central chondrosarcoma grade III	15		7[b] (47)	5 (71)	0	0	7 (47)
Dedifferentiated chondrosarcoma	13		6[b] (46)	3 (50)	1 (17)	1 (8)	7 (54)
Periosteal chondrosarcoma	3		3 (100)	3 (100)	0	0	3 (100)

[a]Unknown gender for one subject. [b]Other types of mutations present beyond those encoding R132C or R132H.

Analysis performed using a custom-made Agilent tiling array (Supplementary Table 2) did not show evidence of loss or gain of *IDH1, IDH2, PTHLH, PTPN11, PTH1R, EXT1, EXT2* or *ACP5*. Thus, even though individuals with enchondromatosis syndromes have overlapping clinical features, they seem to be genetically discrete entities, with the exception of Ollier disease and Maffucci syndrome, which we have now shown to both contain *IDH1* or *IDH2* mutations.

Figure 1 Frequency of *IDH1* and *IDH2* alterations



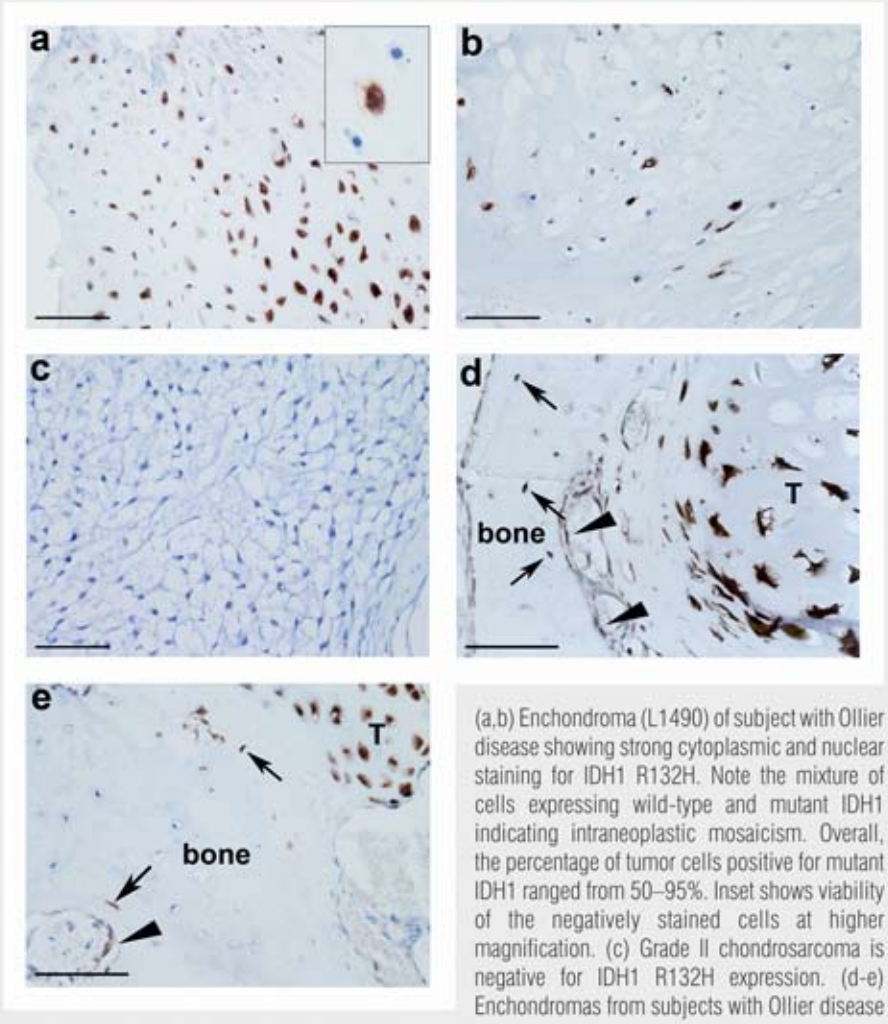
(a) Distribution of the different Arg132 alterations in *IDH1* and Arg172 alterations in *IDH2* among the subjects with Ollier disease, Maffucci syndrome and solitary tumors. (b) Frequency of somatic heterozygous *IDH* (*IDH1* and *IDH2*) mutations in tumors of subjects with Ollier disease or Maffucci syndrome in comparison to different subtypes of solitary cartilaginous tumors and angiosarcomas.

As Ollier disease and Maffucci syndrome are not inherited and enchondromas are often unilateral, we hypothesized that mutations may occur in a somatic mosaic fashion. Fourteen of 16 subjects (88%) possessed identical mutations, including rare variants, in more than one tumor (Supplementary Table 1). We additionally used immunohistochemistry to determine the distribution of the IDH1 R132H mutant protein. Of 68 tumors from subjects with Ollier disease, 17 (25%) showed mutant protein expression, whereas 51 (75%) were negative (Figure 2 and Table 2). Within tumors that were positive for IDH1 R132H staining, we observed a mixture of cells that did and did not express the mutant protein (cells were of the same histologic type and therefore did not include entrapped or supporting elements), a pattern we refer to as intraneoplastic mosaicism (Figure 2a,b). Within these tumors, the percentage of tumor cells staining positive for IDH1 R132H ranged from 50% to 95%. Intraneoplastic mosaicism has also been described for other benign bone tumors. In fibrous dysplasia, experimental evidence showed that both normal cells and those with mutations in *GNAS* were needed to develop fibrous dysplasia–like lesions[26]. Also, in osteochondromas, which are benign cartilaginous tumors arising at the surface of the bone that are caused by mutations in *EXT1* or *EXT2*, a mixture of cells with wild-type *EXT* and cells with mutations in *EXT* was observed[27–30]. The EXT protein is involved in heparan sulfate biosynthesis, and it is hypothesized that cells with mutations in *EXT* that are therefore deficient in heparan sulfate need heparan sulfate from neighboring cells for cellular signaling and survival[31,32].

We additionally studied the surrounding normal tissue of Ollier disease–derived and solitary tumors expressing mutant IDH1 R132H protein and observed a very low frequency (on average $<1\%$) of mutant protein in osteoblasts, osteocytes, adipocytes and fibroblasts (Figure 2d,e). We were able to perform the hydrolysis probe assay on DNA isolated from one normal bone of a subject with Ollier disease, and we did not detect any changes at the *IDH1* locus. Mutant IDH1 R132H protein was absent in 12 bones resected for reasons other than chondrosarcoma removal as well as in normal growth plates and articular cartilage (Table 2). Therefore, our current data support a model of somatic mosaicism, similar to that described for polyostotic fibrous dysplasia in which somatic mosaic mutations in *GNAS* have a causative role[33,34]. Unfortunately, the nature of the samples (decalcified, paraffin-embedded bone) and the occurrence of mutations in single, scattered cells did not allow verification of this theory using other techniques. However, the antibody recognizing IDH1 R132H was shown to be highly reliable for glioma diagnosis[35] and correlated well with sequence analysis in our series.

Twelve tumors were negative for *IDH1* or *IDH2* hotspot mutations. For 5 of these, all exons were sequenced and no mutations were identified. This finding was not surprising, as only *IDH1* mutations affecting Arg132 and *IDH2* mutations affecting Arg140 or Arg172 have been identified in other *IDH*-associated tumors. It is possible that, because of intralesional mosaicism, only a small fraction of tumor cells contains the *IDH1* or *IDH2* hotspot mutations, which may be below the detection level of the techniques used to identify them. Alternatively, mutations in other genes such as *TET2*, in which mutations are mutually exclusive to those in *IDH1* or *IDH2* in acute myeloid leukemia (AML)[36], might be involved[18,37].

Figure 2 Immunostaining for mutant IDH1 R132H protein



(a,b) Enchondroma (L1490) of subject with Ollier disease showing strong cytoplasmic and nuclear staining for IDH1 R132H. Note the mixture of cells expressing wild-type and mutant IDH1 indicating intraneoplastic mosaicism. Overall, the percentage of tumor cells positive for mutant IDH1 ranged from 50–95%. Inset shows viability of the negatively stained cells at higher magnification. (c) Grade II chondrosarcoma is negative for IDH1 R132H expression. (d-e) Enchondromas from subjects with Ollier disease showing occasional cells positive for mutant IDH1 in the surrounding normal bone. Some positively stained osteocytes (arrows) and osteoblasts (arrowheads) are seen. T, tumor tissue (scale bars, 5 μm).

Table 2 Immunohistochemical detection of mutant IDH1 R132H protein

	Total number of tumors	IDH1 R132H–positive tumors/total tumors analyzed (%)
Ollier disease		
Enchondroma	46	14/43[a] (32)
Chondrosarcoma grade I	22	3/17[a] (18)
Chondrosarcoma grade II	10	0/8[a]
Maffucci syndrome		
Enchondroma	9	0/9
Spindle cell hemangioma	14	0/14
Solitary tumors		
Enchondroma	19	4/19 (21)
Central chondrosarcoma grade I	42	4/38[a] (10)
Central chondrosarcoma grade II	36	1/32[a] (3)
Central chondrosarcoma grade III	14	0/11[a]
Central dedifferentiated chondrosarcoma	26	1/24[a] (4)
Periosteal chondrosarcoma	6	1/6 (17)

Table 2 (Continue)

	Total number of tumors	IDH1 R132H–positive tumors/total tumors analyzed (%)
Solitary osteochondroma	20	0/17[a]
Multiple osteochondroma	7	0/7
Peripheral chondrosarcoma	45	0/35[a]
Peripheral dedifferentiated chondrosarcoma	16	0/16
Conventional hemangioma	3	0/3
Hemangioendothelioma	2	0/2
High grade angiosarcoma of bone	44	0/44
High grade angiosarcoma of soft tissue	22	0/22
Controls		
Normal growth plate	3	0/3
Articular cartilage	3	0/3
Normal bone	12	0/12

[a]Not all tumors included were evaluated due to loss of tissue on the tissue microarrays.

Recently, point mutations in *IDH1* or *IDH2* were reported in 56% of solitary central and periosteal cartilaginous tumors[18], and the data within our control group are in concordance with these findings. In total, 40 of 101 (40%) solitary central tumors, 7 of 13 (54%) dedifferentiated chondrosarcomas and 3 of 3 (100%) periosteal chondrosarcomas had *IDH1* or *IDH2* mutations (Figure 1b and Table 1). In six additional tumors, the mutant allele seemed to be present below the detection level of Sanger sequencing. *IDH1* or *IDH2* mutations were absent in other subtypes of cartilaginous tumors, in angiosarcomas (Figure 1b) and in DNA isolated from subjects' blood. Immunostaining for IDH1 R132H protein on tissue microarrays (TMAs) containing cartilaginous and vascular tumor samples confirmed that the expression of mutant IDH1 was restricted to central, dedifferentiated and periosteal cartilage tumors, whereas all other tumors lacked mutant expression (Table 2). Of note, four of eight solitary chondrosarcoma cell lines carried different types of mutations in *IDH1* or *IDH2* (Table 3). To the best of our knowledge, no representative cell lines with *IDH1* or *IDH2* mutations were previously available. *IDH1* or *IDH2* mutations were more frequently found in solitary central tumors located in the hands and feet (11 of 14 tumors) compared to those located in long and flat bones (28 of 84 tumors) ($P = 0.006$, Pearson's $\chi 2$ test), which was also reported previously[18]. This correlation was absent in Ollier disease (20 of 22 tumors from the hands and feet compared to 28 of 34 tumors from long or flat bones, $P = 0.5$, Pearson's $\chi 2$ test). Whereas in gliomas, mutations in *IDH1* or *IDH2* predict a favorable outcome[38], we found no significant prognostic value of these mutations in solitary central cartilaginous tumors using multivariate analysis (Cox regression, P value $= 0.3$).

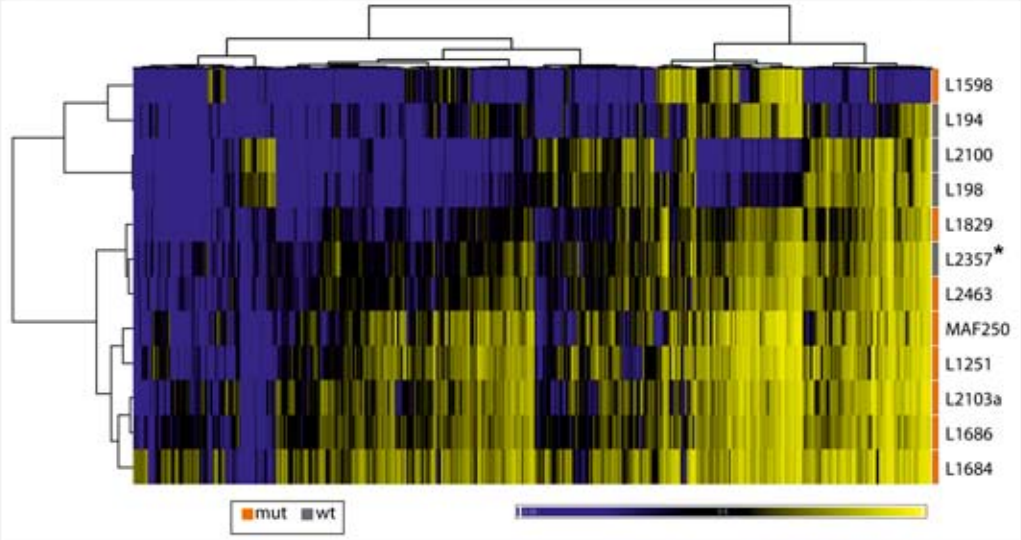
Table 3 *IDH1* or *IDH2* alterations in solitary central chondrosarcoma cell lines and primary tissue culture

Cell line	Tumor type	Tumor grade	Passage	*IDH1* alteration	*IDH2* alteration	Reference
SW1353	Solitary central	CSII	12	WT	R172S	ATCC
JJ012	Solitary central	CSII	15	R132G	WT	51
CH2879	Solitary central	CSIII	16	G105G	WT	52
OUMS27	Solitary central	CSIII	18	WT	WT	53
L835	Solitary central	CSIII	38	R132C	WT	Established by the authors
C3842	Ollier disease	CSII	32	WT	WT	54
L2975	Dedifferentiated CS		31	WT	R172W[a]	Established by the authors
NDCS1	Dedifferentiated CS		12	WT	WT	55

CS, chondrosarcoma; WT, wild type. [a]L2975 had a homozygous mutation in *IDH2* encoding R172W.

IDH1 and *IDH2* mutations have also been reported at lower frequencies in various other cancers, such as AML (8%)[39,40], prostate cancer (2.7%)[40,41], paragangliomas (0.7%)[40,42] and thyroid carcinoma (16%)[43]. The high mutation frequency in enchondromas and the fact that these mutations occur early suggest a causal rather than a bystander role for *IDH1* and *IDH2* mutations in tumorigenesis in Ollier disease and Maffucci syndrome. In gliomas, mutations in *IDH1* and *IDH2* lead to a gain of function, causing the production of 2-hydroxyglutarate (2HG), a structural analog of α-ketoglutarate (α-KG), which thereby reduces α-KG production[44]. In AML, it was demonstrated that mutant IDH expression results in DNA hypermethylation and impairment of hematopoietic differentiation[36], and in gliomas, the presence of an *IDH1* mutation was strongly associated with hypermethylation[45]. Therefore, we used Illumina HumanMethylation27 BeadChips to examine possible differences in methylation between enchondromas with (N = 8) and without (N = 4) *IDH1* mutations, as determined by Sanger sequencing. Unsupervised clustering of the 2,000 most variable CpG methylation sites gave two subgroups (Figure 3).

Figure 3 CpG island methylator phenotype in enchondromas with *IDH1* mutations



Heatmap depicting unsupervised clustering analysis of the 2,000 most variable CpG sites of enchondromas with (orange, N = 8) and without (gray, N = 4) *IDH1* mutation. The level of DNA methylation (beta value) for each probe (columns) in each sample (rows) is represented by color, ranging from 0 (0% methylation, blue) to 1 (100% methylation, yellow). The asterisk indicates sample L2357 in which the mutated allele of *IDH1* encoding R132G was detected in a subpopulation of cells. However, the mutation escaped detection by Sanger sequencing, and therefore the sample is labeled wild type.

One of these subgroups showed an overall higher methylation at the examined CpG sites, a phenotype that is similar to the CpG island methylator phenotype (CIMP) described in colon carcinoma and glioblastoma[45,46]. All but one enchondromas with an *IDH1* mutation were positive for this CIMP. Supervised clustering analysis indicated that 797 CpG sites were differentially methylated by more than 20% (with $P < 0.05$) between enchondromas with and without *IDH1* mutations. Of note, 710 of these differentially methylated CpG sites (89.1%) were methylated in the enchondromas with *IDH1* mutations (Supplementary Table 3). These results are in line with the hypothesis that *IDH1* mutations induce methylation and thus contribute to CIMP occurence[36].

To assess the effect of *IDH1* or *IDH2* mutation on mRNA expression levels in cartilaginous tumors, we performed whole-genome gene expression analysis using Illumina Human-6 v3 arrays. High-quality mRNA was available for only three tumors in which no mutation was found (N = 1) or in which mutations occurred at a frequency below the threshold of detection with Sanger sequencing (thus possibly carrying a low percentage of cells with mutations) (N = 2). Comparison of mRNA expression in these tumors with that from 18 tumors with clearly detectable *IDH1* or *IDH2* mutation using LIMMA analysis revealed 36 differentially expressed probes encoding 33 genes (Supplementary Table 4). Of these 33 genes, 32 were downregulated in the tumor samples with an *IDH1* or *IDH2* mutation. There was no overlap between the affected genes identified by methylation or expression analysis.

One of the most differentially methylated genes was *DLX5*, for which there was a trend of downregulation in the expression data comparing enchondromas from subjects with Ollier disease and controls. However, this difference was not significant (adjusted *P* value = 0.3, Supplementary Figure 2). The controls consisted of two growth plates and four articular or rib cartilage samples. *DLX5* encodes a homeodomain transcription factor that is a cell-autonomous positive regulator of chondrocyte maturation during endochondral ossification, promoting the conversion of immature proliferating chondrocytes into hypertrophic chondrocytes[47,48] The Dlx5 protein also induces expression of Runx2 and osterix, promoting osteogenic differentiation[49,50]. Future studies should reveal whether downregulation of *DLX5* through methylation as a consequence of *IDH1* mutation delays hypertrophic differentiation of chondrocytes and inhibits subsequent osteogenic differentiation, thereby leaving clusters of proliferating chondrocytes behind.

In summary, we report a large multi-institutional series demonstrating somatic heterozygous point mutations in *IDH1* or, rarely, in *IDH2* in tumor tissues of 81% of subjects with Ollier disease and 77% of those with Maffucci syndrome, and we provide evidence for intraneoplastic and somatic mosaicism. Future studies using deep-sequencing approaches should reveal whether the percentage of individuals carrying somatic mosaic mutations in *IDH1* or *IDH2* is even higher than that detected in our series or whether other genes are involved. We show the *IDH1* mutation to be associated with hypermethylation and downregulation of several genes. Future studies will examine whether there is a causal effect, and it will be of great interest to assess how this dysregulation leads to enchondroma and spindle cell hemangioma formation. Finally, this is the first report of four chondrosarcoma cell lines carrying *IDH1* or *IDH2* mutations, thereby providing good *in vitro* models for functional studies to dissect the role of *IDH1* and *IDH2* in Ollier disease and Maffucci syndrome, as well as allowing studies of their function in tumorigenesis in general.

Methods

Subjects and clinical specimens

Fresh-frozen tumor tissues (N = 60) of 44 subjects with multiple cartilage tumors (36 individuals with Ollier disease and 8 with Maffucci syndrome) (Table 1 and Supplementary Table 1) were collected from the EuroBoNeT consortium[8] and the Laboratory of Human Molecular Genetics at the de Duve Institute, Université catholique de Lovain. In addition, paraffin-embedded tumor tissues (N = 15) from 12 subjects were obtained from the archives of the Children's Hospital Boston. Samples were handled according to the ethical guidelines of the host institutions. All samples were coded and the ethical guidelines described in the "Code for Proper Secondary Use of Human Tissue in The Netherlands" (Dutch Federation of Medical Scientific Societies) were followed in all procedures. Chondrosarcoma samples were graded as described[56]. DNA derived from normal saliva, blood or muscle was available from 12 individuals with Ollier disease. The ages of the subjects were documented at the time of operation. Demographic and survival data were obtained from patient records at the host institutions. Written informed consent was obtained for all study participants from whom normal DNA was included. For subjects from whom we only used tumor tissue, the Code for Proper Secondary Use of Human Tissue in the Netherlands guidelines were followed.

For comparison with other cartilage tumors, we included DNA from solitary enchondromas (N = 9), solitary central chondrosarcomas (N = 92), central dedifferentiated chondrosarcomas (N = 13), periosteal chondrosarcomas (N = 3) and 37 peripheral cartilaginous tumors (solitary osteochondroma (N = 11), peripheral chondrosarcomas (N = 20) and multiple osteochondromas (N = 6)), as well as from chondromyxoid fibromas (N = 9), chondroblastomas (N = 7) and osteochondroma-like lesions of metachondromatosis (N = 2). Matching blood-derived DNA was also available from 24 subjects as controls. Additionally, we included DNA from angiosarcomas (N = 14), because individuals with Maffucci syndrome have central cartilage tumors combined with vascular tumors. The angiosarcomas, chondromyxoid fibromas and chondroblastomas were analyzed for *IDH1* mutations only. Thus, in total, we analyzed 261 tumors from 242 subjects.

DNA extraction and mutation analysis

Genomic DNA from frozen tumors containing at least 80% tumor cells, as estimated on haematoxylin and eosin–stained frozen sections, and from blood and saliva was isolated as described previously[8]. DNA from paraffin-embedded tissue was isolated after microdissection as described[8]. For cell lines and primary tissue culture, DNA was isolated from cell pellets using the Wizard Genomic DNA Purification kit (Promega), according to the manufacturer's instructions.

PCR amplification was performed on exon 4 of IDH1 for all the samples. Exon 4 of IDH2 was amplified in samples without *IDH1* mutation, and exon 8 of *GNAS* was studied in samples without *IDH1* or *IDH2* mutation. To correlate with possible *PTH1R* mutations, we also amplified exon 4 of *PTH1R* for mutations encoding G121E and A122T substitutions, exon 5 for mutations encoding R150C and exon 9 for mutations encoding R255H using DNA from 35 subjects with Ollier disease or Maffucci syndrome.

PCR was carried out in a reaction volume of 25 µl, with 10 ng of DNA, 12.5 µl of iQ SYBR green Supermix (Bio-Rad) and 10 pmol M13-tailed primers (sequences provided in Supplementary Table 5). PCR was performed in a CFX 96 Real-Time PCR detection system (Bio-Rad), with an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C. After a final elongation step of 10 min at 72 °C, a melt curve was obtained to evaluate the quality of the PCR products. PCR products were purified using the Qiagen MinElute 96 UF PCR Purification system and eluted in 25 µl of sterile water. PCR amplicons were sequenced by a commercial entity using standard forward and reverse M13 primers (Macrogen). The sequence trace files were analyzed with Mutation Surveyor DNA Variant Analysis software (version 3.97, SoftGenetics).

To validate the mutations in *IDH1* encoding R132C and R132H, we designed hydrolysis probe assays (probe sequences provided in Supplementary Table 6), using the Custom Taqman Assay Design Tool (Applied Biosystems). Assays were performed on 144 samples, including tumors derived from subjects with Ollier disease and Maffucci syndrome, as well as solitary cartilaginous tumors, chondrosarcoma cell lines and blood from subjects with Ollier disease. Assays were also performed on negative controls (healthy donor DNA) and no template controls. qPCR was carried out in a reaction volume of 10 µl as described previously[57] in a CFX38 Real-Time PCR Detection System (Bio-Rad), with an initial denaturation step of 10 min at 95 °C followed by 40 cycles of 10 s at 92 °C and 30 s at 60 °C. The quantification cycle (Cq) was used for quality assessment and samples with Cq >35 for the wild-type allele were considered as DNA negative. The threshold for the mutant alleles of *IDHI* encoding R132C (c.394C>T) or R132H (c.395G>A) was set after subtracting the highest background signal from the negative controls.

There was sufficient DNA left from 5 of 12 tumors without mutation to perform sequence analysis for all exons of *IDH1* and *IDH2*. One tumor with an *IDH1* mutation was also sequenced. PCR was performed as described above for exon 4, and primer sequences are listed in Supplementary Table 5.

Tiling resolution targeted oligonucleotide arrays

Custom-designed Agilent tiling oligonucleotide array–comparative genomic hybridization analysis consisting of 15,000 probes with a tiling coverage of genes involved in the different types of enchondromatosis syndromes (*IDH1, IDH2, ACP5, PTH1R, PTPN11, EXT1, EXT2* and *PTHLH*) (Supplementary Table 2) was performed to detect possible small intragenic losses and gains in these genes. In total, 16 enchondromas and chondrosarcomas from subjects with Ollier disease or Maffucci syndrome, with (N = 14) and without (N = 2) *IDH1* or *IDH2* mutations were selected. Labeling and hybridization of genomic DNA from freshly frozen tumors and data processing were performed as described[58].

Immunohistochemistry

To examine the protein expression of the IDH1 R132H mutant, immunohistochemistry was performed as described[8] using antibody recognizing IDH1 R132H from Dianova (1:200 dilution in 5% non-fat milk, citrate antigen retrieval and blocking for 30 min with 5% non-fat milk). We used 403 tumors (Table 2) on 19 TMAs, for which details were previously published[8,59-61].

Additional samples from subjects with Ollier disease or Maffucci syndrome were collected through the European Musculo-Skeletal Oncology Society (EMSOS), and clinical details for these individuals are described separately[5]. Glioma tissue with a known *IDH1* mutation was used as a positive control, and primary antibody was omitted as a negative control. Only strong cytoplasmic staining combined with nuclear staining was considered a positive result[35]. To study possible mosaicism in the tumors and in surrounding normal tissues, we selected resection specimens from tumors expressing the mutant IDH1 R132H protein (N = 7) and stained multiple tissue blocks from different areas. All except nine tumors from subjects with Ollier disease that were used for mutation analysis were also included in the TMAs, and results were confirmed.

Statistical analysis for clinical correlation

Follow-up data were available from 83 subjects with solitary tumors (range 2–335 months, mean 115.23). To investigate the relation of *IDH1* or *IDH2* mutation with the clinical features of the subjects, multivariate survival analysis (Cox regression) and cross-tabulations (Pearson's $\chi 2$ test) were performed using SPSS version 16.0. Statistical analysis was not performed for subjects with Ollier disease, because nearly all subjects with available follow-up data had *IDH1* or *IDH2* mutations. All the *P* values reported are two-sided, and $P < 0.05$ was considered to indicate statistical significance.

DNA methylation profiling

A total of 12 samples, which included 8 enchondromas with *IDH1* mutation (4 Ollier enchondromas, 2 Maffucci enchondromas and 2 solitary enchondromas) and 4 enchondromas (1 Ollier enchondroma and 3 solitary enchondromas) without *IDH1* or *IDH2* mutations, were analyzed. Of the 4 enchondromas without *IDH1* mutation, one had cells with mutated *IDH1* encoding the R132G alteration present in a subpopulation, which was below the threshold of detection by Sanger sequencing. Bisulfite treatment was performed using the EZ DNA Methylation kit (Zymo Research). Bisulfite-converted DNA was then hybridized to Illumina HumanMethylation27 BeadChips by following the manufacturer's instructions. Infinium unsupervised clustering analysis was performed using the Ward's clustering algorithm based on Euclidian distance. The 2,000 most variable CpG sites (excluding those on the X and Y chromosomes) were used in the clustering analysis.

Genome-wide gene expression analysis

A total of 21 tumors, including 6 enchondromas and 10 chondrosarcomas (6 grade I and 4 grade II) from subjects with Ollier disease and Maffucci syndrome, as well as 1 solitary enchondroma, 4 solitary chondrosarcomas, grade II, and 6 controls (2 growth plates and 4 normal cartilage), were used. We determined differential expression between tumors with *IDH1* or *IDH2* mutation (N = 18) compared to tumors without detectable *IDH1* or *IDH2* mutation (N = 3) using Sanger sequencing. Two of these samples showed sub-threshold peaks for mutations in *IDH1* encoding R132G and R132C, suggesting that the mutation was present in a minor subpopulation of tumor cells. Expression analysis using Illumina Human-6 v3.0 Expression BeadChips were performed as described previously[8,62,63]. LIMMA analysis[64] was used to determine differential expression between the groups. Probes with Benjamini and Hochberg false discovery rate–adjusted *P* values <0.05 and a log fold change >0.1 were considered to be significantly differentially expressed.

Accession numbers

MIAME-compliant data from the tiling, expression and methylation arrays have been deposited in the GEO database (GSE30844). Sequence data for IDHI and IDH2 has been deposited in GenBank (NM_005896.2 and NM_002168.2).

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Author Contributions

The study was designed, written and reviewed by T.C.P. and J.V.M.G.B. Mutation analysis was designed and performed by T.C.P., M.A.J.H.v.R., J.V.M.G.B., K.S., T.v.W. and R.v.E. Immunohistochemistry was conducted and evaluated by T.C.P., M.A.J.H.v.R. and J.V.M.G.B. T.C.P., S.L.J.V., J.G.v.O. and D.M. contributed tissue microarrays. Expression profiling was designed and performed by A.-M.C.-J., T.C.P., J.V.M.G.B. and J.O. and analyzed by J.O. and M.L.K. Methylation profiling was designed by A.-M.C.-J., J.V.M.G.B. and L.S., performed by P.d.A., and the results analyzed by P.d.A. and P.J.F. K.H.N., S.D., L.S., B.T., B.L.-A., M.S.-J., R.S., N.L., L.-G.K., C.G., M.V., L.M.B. and K.C.K. each contributed frozen or paraffin-embedded tissues for multiple subjects with Ollier disease or Maffucci syndrome and acquired data for these individuals. The manuscript was approved by all authors.Q4Q4Q5Q5

Competing Financial Interests

The authors declare no competing financial interests.

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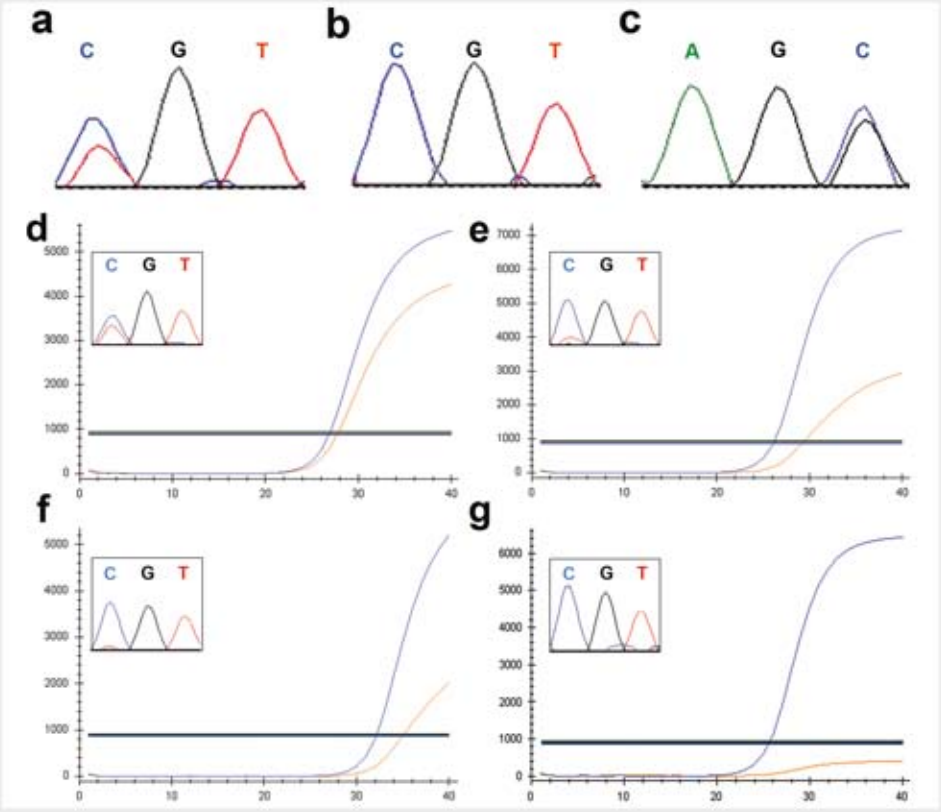
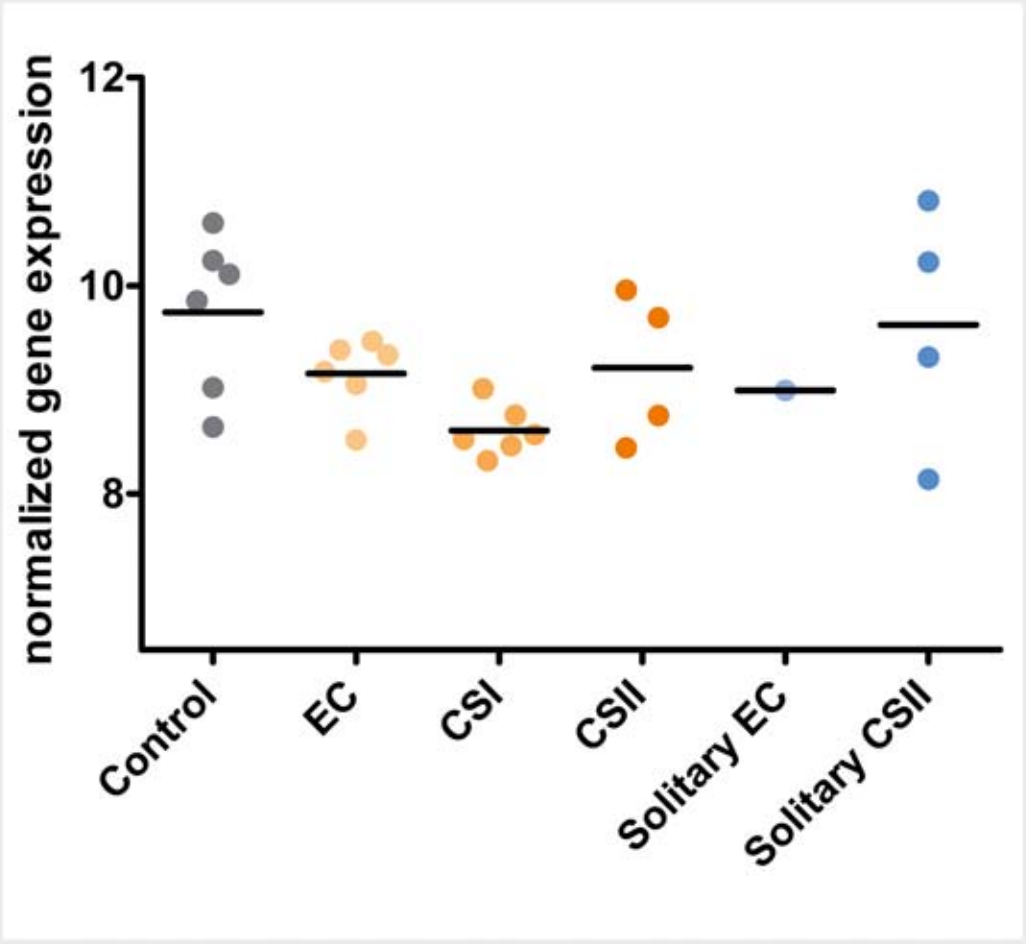
Figure S1 Output of sequencing and hydrolysis probes assay



a, b) Example of Sanger sequencing results showing that R132C *IDH1* mutation was present in enchondroma and absent in corresponding blood DNA of a patient with Ollier disease. c) Example of Sanger sequencing results showing R172S *IDH2* mutation in a single patient with Ollier disease. d-g) Relative Fluorescent Units (RFU) are plotted against the quantification cycle (Cq). The horizontal line at 950 RFU indicates the threshold level for allele calling. All samples show a positive signal for both wild type (blue) and the *IDH1* c.395C>T, p.R132C mutant allele (orange). d) L1684 carrying the R132C *IDH1* mutation on Sanger sequencing (inset) confirmed with hydrolysis probes assay. e) L1980 and f) L204 have minor *IDH1* positive cell populations. The R132C *IDH1* mutant allele is barely visible after Sanger sequencing (inset) but it clearly presents as mutant using hydrolysis probes assay. g) L172 is negative for the mutation, in Sanger sequencing (inset) as well as in hydrolysis probes assay.

Figure S2 Normalized expression levels of *DLX5*



EC: enchondroma, CS : chondrosarcoma

Table S1 Clinical information of patients with Ollier disease and Maffucci syndrome

Patient ID	Sample ID	*IDH1* mutation	*IDH2* mutation	Gender	Age	Disease	Tumor	Tumor location
01	L1083	R132C		male	48	Ollier	CSI	metacarpal
	L2218	R132C		male	49	Ollier	CSI	digit V
02	L172[3,6]	no mutation	no mutation	male	40	Ollier	CSII	scapula
03	L271	R132C		female	26	Ollier	CSI	distal femur
	L286 T	R132C		female	23	Ollier	CSII	femur
04[1]	L149 T3	R132C		male	34	Ollier	CSI	unknown
	L204 II[2,3]	R132C		male	26	Ollier	CSI	femur
	L253 T3	R132C		male	26	Ollier	CSI	tibia
05	L206	R132C		female	25	Ollier	EC	hand
06	L816-1	no mutation	R172S	male	68	Ollier	CSIII	humerus
	L813 T	no mutation	R172S	male	68	Ollier	CSII	femur
07[1]	L898[6]	no mutation	no mutation	male	18	Ollier	CSI	femur
08[1]	L1251	R132H		male	15	Ollier	EC	hand
	L2220	R132H		male	14	Ollier	EC	digit
09	L1974	R132C		male	48	Ollier	CSII	scapula
010[1]	L1975	R132C		male	31	Ollier	CSII	femur
011	L1976	R132C		male	41	Ollier	CSII	tibia
012[1]	L1977	R132C		male	41	Ollier	CSI	tibia
	L1978	R132C		male	38	Ollier	EC	foot
	L1979	R132C		male	41	Ollier	CSI	tibia
	L3363	R132C		male	36	Ollier	EC	toe
013	L1980[2]	R132C		female	63	Ollier	CSII	knee
014	L1685	R132C		female	23	Ollier	CSI	pubic bone

Table S1 (Continue)

Patient ID	Sample ID	*IDH1* mutation	*IDH2* mutation	Gender	Age	Disease	Tumor	Tumor location
O15	L1686	R132C		male	18	Ollier	EC	phalanx
	L1687	R132C		male	18	Ollier	CSI	phalanx
O16	L2386	R132H		female	13	Ollier	CSI	digit III
O17[1]	L2463	R132C		female	12	Ollier	EC	tibia
O18	L1629	R132C		male	36	Ollier	EC	unknown
	L1630	R132C		male	36	Ollier	CSI	iliac bone
O19	L2095	R132C		female	23	Ollier	EC	distal tibia
O20[5]	L2590[2]	R132H		female	36	Ollier	CSI	metatarsal I
	L2761	no mutation	no mutation	female	37	Ollier	CSI	tibia
O21	L2098[3]	no mutation	no mutation	female	15	Ollier	CSII	humerus
O22	L2099	R132C		female	49	Ollier	CSI	prox humerus
O23	L2100	no mutation	no mutation	male	27	Ollier	EC	femur
O24	L2103a	R132C		male	39	Ollier	EC	phalanx
	L2103b	R132C		male	39	Ollier	CSI	distal phalanx
O25	L2104a	R132C		male	36	Ollier	CSIII	prox tibia
O26[1]	L2221	R132C		female	42	Ollier	CSI	distal femur
O27[1]	L1513	R132C		female	23	Ollier	CSI	distal femur
O28[1]	L1490	R132H		female	12	Ollier	EC	phalanx
O29	L2640	R132C		female	34	Ollier	EC	phalanx
	L2641	R132C		female	34	Ollier	EC	thumb
O30	L2205	R132C		male	6	Ollier	EC	illum
O31	L1683	R132C		unknown	29	Ollier	CSI	metacarpal
O32[1]	L2280	R132C		female	24	Ollier	CSI	left acromion

Table S1 (Continue)

Patient ID	Sample ID	*IDH1* mutation	*IDH2* mutation	Gender	Age	Disease	Tumor	Tumor location
O33	L2513[3]	no mutation	no mutation	male	33	Ollier	CSI	pelvis
O34	L2746	R132C		female	58	Ollier	CSI	digit II
O35[1]	L3325A	R132H		male	6	Ollier	EC	hand
O36[1]	L3362A	R132C		female	6	Ollier	EC	proximal tibia
	L3362C	R132C		female	6	Ollier	EC	left distal femur
O37	OLR30[4]	R132C		male	8	Ollier	EC	right leg
O38	S-03-3802[2,4]	R132H		female	16	Ollier	EC	hand
O39	S-05-4941[4]	R132H		male	13	Ollier	EC	hand
O40	S-05-6625[4]	R132C		female	11	Ollier	EC	hand
O41	S-08-3234[4]	R132H		female	4	Ollier	EC	hand
O42	S-08-7943[4]	no mutation	no mutation	male	12	Ollier	EC	hand
O43	S-08-9181[4]	no mutation	no mutation	female	12	Ollier	EC	hand
M1	L1684	R132C		female	37	Maffucci	EC	phalanx
M2	L2097b	R132C		female	19	Maffucci	EC	toe
	L2097a	R132C		female	19	Maffucci	EC	prox tibia
M3	L2102	R132C		male	29	Maffucci	CSII	distal femur
M4	MAF100[3]	R132C		male	birth	Maffucci	CSI	hand
M5	MAF200	R132C		female	4	Maffucci	SCH	right hand
M6	MAF210[3]	no mutation	no mutation	female	-	Maffucci	SCH	-
M7	MAF230	R132C		female	2	Maffucci	SCH	right foot
M8	MAF250[3]	no mutation	no mutation	female	3	Maffucci	EC	right hand
M9	S08-0010959 4A[4]	no mutation	no mutation	male	15	Maffucci	SCH	digit
	S08-0010959 5A[4]	no mutation	no mutation	male	15	Maffucci	SCH	forearm

Table S1 (Continue)

M10	S08-0007382[2,4]	R132C		male	9	Maffucci	SCH	hand
M11	S05-0006227[2,4]	R132C		male	10	Maffucci	SCH	digit
M12	S97-0002538 1A[4]	R132C		female	30	Maffucci	SCH	foot
	S97-0002538 3A[4]	R132C		female	30	Maffucci	SCH	first web space
M13	S03-0001121[4]	R132C		female	23	Maffucci	EC	digit
	S97-0004447 4B[2,4]	R132C		female	17	Maffucci	SCH	lower back

All patients were diagnosed as having Ollier disease or Maffucci syndrome based on the radiographical features and/or presence of more than two cartilaginous tumors (Ollier disease) in combination with hemangioma (Maffucci syndrome). [1] indicates DNA from normal tissue was also tested, [2] indicates cases negative in Sanger sequencing but positive in hydrolysis probes assay, [3] indicates cases used for sequencing of all exons of IDH1 and IDH2, [4] indicates DNA was isolated from paraffin embedded tissue, [5]indicates patient with multiple tumors in which one tumor showed clearly mutation in the sequencing and another tumor was negative for the mutation. DNA was unavailable to perform hydrolysis probe assay. [6]Two chondrosarcomas of patients with Ollier disease were negative at the mutation analysis, while other tumors of the same patients demonstrated positive cells at R132H IDH1 immunohistochemistry, suggesting that the percentage of patients carrying mutations in IDH1 or IDH2 is even higher than we report in this study. EC: enchondroma, CS: chondrosarcoma, SCH: spindle cell hemangioma.

Table S2 Tiling Array design

Gene	Number of probes
IDH1	312
IDH2	208
PTHLH	338
PTPN11	413
PTH1R	104
EXT1	833
EXT2	334
ACP5	93

Table S3(L) Different methylated sites

Index	TargetID	ProbeID_A	ProbeID_B	L1684. AVG_Beta	L1829. AVG_Beta	L1598. AVG_Beta	MAF250. AVG_Beta	L2103a. AVG_Beta	L1251. AVG_Beta	L1686. AVG_Beta
22413	cg22442090	2070324	2070008	0.1848371	0.2185057	0	0	0.5814463	0	0.3329082
8399	cg08450982	3800139	2350523	0.1100114	0.2611653	0.09177592	0	0.1041835	0.03501094	0.1062992
6684	cg06690548	4480167	3360008	0.170406	0.8494078	0.2249152	0.02788536	0.2761823	0.1059524	0.316043
7884	cg07922606	7320719	7320398	0.2281141	0.3192771	0.1360397	0.07273802	0.2583545	0.166755	0.1644749
6407	cg06434428	7160086	4670435	0.1261764	0.3005146	0.1435832	0.01117799	0.2098659	0.03615886	0.1997608
7782	cg07823492	1430132	1430491	0.2043384	0.366	0.2075359	0.08771437	0.2065943	0.1085657	0.1974618
21663	cg21663431	830441	5910075	0.155234	0.4232825	0.291686	0	0.1709022	0.04581732	0.1790756
1336	cg01324261	1240243	1780161	0.1456849	0.256	0.1069736	0.07170542	0.1258803	0.07535322	0.0931624
21433	cg21453309	1050390	4900377	0.08465447	0.2001105	0.07835243	0.006024096	0.07591522	0.04675159	0.07536232
4175	cg04126335	4120110	2350491	0.07277453	0.08045325	0.02733485	0	0	0.03635243	0.0203749
17852	cg17826679	7200400	2900390	0.09147287	0.2878049	0.1851064	0.004569839	0.1399118	0.05752961	0.1170739
26566	cg26608667	3390538	6660161	0.2009503	0.2532403	0.1254081	0.184076	0.2259414	0.2004545	0.1914157
12016	cg12069042	2030600	2490600	0.1988473	0.4683586	0.190639	0.1105858	0.2567744	0.1410658	0.2619452
8523	cg08578641	6250376	5570446	0.09503632	0.2448716	0.1318403	0.01808682	0.0778626	0.04301759	0.08563194
22547	cg22580512	7560017	1450017	0.07238949	0.1943258	0.1190033	0.01297968	0.07011494	0.05758963	0.06808074
635	cg00616135	2940546	2940437	0.0608931	0.2368263	0.09176788	0	0.07741386	0.1147471	0.06829489
21396	cg21416237	2230022	5360022	0.2673218	0.4347915	0.283985	0.04835473	0.2963827	0.2016468	0.2902088
597	cg00573606	7560440	1450438	0.1443299	0.1787749	0.1125917	0.125	0.114158	0.1400481	0.130849
24384	cg24433189	4860484	6270520	0.08097929	0.1744723	0.07089874	0.002761954	0.08934221	0.019631	0.05668151
9754	cg09837648	780474	5340474	0.3633727	0.7797911	0.3566205	0.319695	0.5624712	0.354095	0.4788733
14470	cg14467840	2850021	6760370	0.2900697	0.5274104	0.2131003	0.03192365	0.3517469	0.1923225	0.3182451
19878	cg19884658	10364	2680280	0.008781978	0.1063014	0	0.009250399	0.05360395	0	0.02420091
21088	cg21120063	4860500	6270315	0.1170727	0.2642019	0.1594053	0.0177548	0.1404155	0.09257835	0.1353896
17261	cg17233506	4070075	6900433	0.06682252	0.1921083	0.04269211	0.03136435	0.06766749	0.04909448	0.06471622
4146	cg04099420	4810170	5720474	0.06028369	0.1963658	0.07692308	0.1207865	0.01805416	0	0.1229682

Table S3(R) Different methylated sites

L2463. AVG_Beta	L2100. AVG_Beta	L198. AVG_Beta	L194. AVG_Beta	L2357. AVG_Beta	Gene. Symbol	ttest	Meth change	FC mut v wt	FC mut v normal	present in TCGA GBM set
0.2210636	0.7407407	0.6489918	0.4687225	0.5575524	*GIMAP5*	0.004	-0.412	0.028	0.150	
0.09696969	0.8642069	0.7077352	0.05559368	0.2513404	*NUMBL*	0.020	-0.369	-0.039	0.112	
0.4333574	0.8141414	0.778991	0.4648357	0.6074498	*SLC7A11*	0.027	-0.366	-0.033	0.053	390
0.2048392	0.7948602	0.8080082	0.2226377	0.4000449	*HIST1H3E*	0.006	-0.363	#N/A	#N/A	464
0.1782178	0.8043478	0.7143432	0.1621918	0.3712297	*HAPLN1*	0.009	-0.362	0.786	0.576	
0.36	0.8855434	0.820531	0.1263489	0.387401	*HOXB1*	0.028	-0.338	-0.035	0.012	
0.1839364	0.7947037	0.7532467	0.1120901	0.3646728	*SLC44A2*	0.030	-0.325	1,404	0.711	
0.08252427	0.7004992	0.6179664	0.1820128	0.2545721	*SCRG1*	0.006	-0.319	1,764	-0.361	
0.1275912	0.7261189	0.6586753	0.05085223	0.1736886	*FAM101A*	0.023	-0.315	-0.079	0.106	
0.05769231	0.7164502	0.5984252	0.02212052	0.07015858	*ITGA10*	0.026	-0.315	1,916	-0.420	
0.1122292	0.7545383	0.6392597	0.07336745	0.2717073	*SLC44A2*	0.022	-0.310	1,404	0.711	
0.5584695	0.7240678	0.7241541	0.1895579	0.5725678	*MGC11257*	0.017	-0.310	0.498	0.257	
0.4331599	0.7592163	0.758442	0.2721696	0.4795041	*PLXNB1*	0.014	-0.310	0.568	-0.301	
0.1280915	0.7596288	0.6122671	0.07479157	0.2008523	*DNAI1*	0.023	-0.309	0.030	-0.084	
0.07233369	0.7394772	0.6367751	0.05336951	0.1303279	*NCOR2*	0.029	-0.307	-0.282	-0.036	
0.1190832	0.714386	0.6241342	0.09505542	0.1650683	*LACTB*	0.021	-0.304	-0.706	0.034	
0.2993279	0.8938702	0.783901	0.2034191	0.3889681	*FKBP10*	0.033	-0.302	0.247	0.497	
0.1179525	0.8153057	0.6043462	0.1489976	0.1714141	*COL6A3*	0.021	-0.302	-0.679	1,504	
0.06192122	0.7015101	0.579423	0.04418118	0.1459529	*SSTR5*	0.023	-0.298	0.056	-0.024	
0.7733009	0.9437935	0.9032156	0.6508114	0.6881803	*PLXNB1*	0.021	-0.298	0.568	-0.301	
0.549842	0.8075926	0.7791875	0.2329037	0.6083576	*S100A1*	0.039	-0.298	1,191	-0.133	
0.1129032	0.5730909	0.5045181	0	0.2651962	*KLHL21*	0.008	-0.296	-0.009	-0.342	1091
0.1823814	0.6842263	0.5601755	0.1337752	0.3406341	*UNQ830*	0.009	-0.291	2,885	0.309	
0.07642701	0.6301561	0.6038228	0.0558343	0.1407421	*HOXB1*	0.022	-0.284	-0.035	0.012	
0.1092095	0.6932516	0.5830303	0.08073654	0.1232877	*RIPK1*	0.028	-0.282	-0.181	-0.052	

Table S3(L) (Continue)

Index	TargetID	ProbeID_A	ProbeID_B	L1684. AVG_Beta	L1829. AVG_Beta	L1598. AVG_Beta	MAF250. AVG_Beta	L2103a. AVG_Beta	L1251. AVG_Beta	L1686. AVG_Beta
20794	cg20847746	6650356	2750356	0.1929653	0.3885039	0.231499	0.07259647	0.2080692	0.1544944	0.1847996
3567	cg03547924	3190041	3190037	0.02684124	0.1699452	0	0	0	0	0
6847	cg06848073	7150059	7380719	0.124525	0.231997	0.07859922	0.02826087	0.142126	0.06252853	0.1259131
9966	cg10052840	7050681	7050435	0.1203767	0.3130285	0.1096991	0.1614029	0.2367575	0.1123471	0.1593329
9959	cg10045881	1010270	6280041	0.1494812	0.3503103	0.05745342	0.02035351	0.1898605	0.1281764	0.168134
13743	cg13795840	6980129	4280187	0.1100332	0.2652093	0.06920471	0.1749946	0.167054	0.1975574	0.3542843
12975	cg13047596	150082	6450301	0.5389208	0.7248635	0.4978155	0.1450187	0.671567	0.3976912	0.5655454
15475	cg15475323	2100747	2940692	0.1287273	0.08169014	0	0	0	0	0.04441454
1430	cg01420388	1190561	1710524	0.2410425	0.3628534	0.2367991	0.06038838	0.2407344	0.1060032	0.2303674
15705	cg15679095	4760008	1300192	0.1538889	0.295082	0.159322	0.130039	0.1450151	0.103276	0.1687284
3408	cg03386869	1170044	6250615	0.1460108	0.3103026	0.1372227	0.3778547	0.197478	0.2128764	0.1907164
19932	cg19948393	1740091	4760091	0.2464419	0.4473319	0.4261934	0.1096902	0.6353386	0.1225178	0.4469705
16256	cg16191009	2570731	5550731	0.1770948	0.2913216	0.4751006	0.2666419	0.2000456	0.1570743	0.2180338
9574	cg09632136	3420343	5130220	0.02897416	0.1118048	0.02324037	0	0.07220497	0.0457097	0.02737851
25148	cg25152942	3400520	1340242	0.07860489	0.1571952	0.05012531	0	0.04748284	0.00886918	0.05239521
876	cg00850538	6550022	6220451	0.2188414	0.2772898	0.1610817	0.01903367	0.1445154	0.1327635	0.1514966
10849	cg10942056	7050553	6130553	0.1651434	0.2745672	0.1806854	0.1381476	0.1513778	0.1097837	0.1929419
5950	cg05955301	5560358	4920010	0.1525364	0.296394	0.1417554	0.09851447	0.1450467	0.1120531	0.1575179
25348	cg25370441	2570301	5550301	0.07834359	0.1119465	0.04806346	0.1100413	0.3050847	0.06473517	0.2055716
23528	cg23579062	4860037	6270056	0.2410155	0.3861935	0.3878559	0.03389142	0.2333427	0.1234737	0.1953359
9822	cg09893305	3310121	540706	0.1382676	0.1964353	0.08	0.05917553	0.1050864	0.04451583	0.06615315
8090	cg08124030	3990278	3170762	0.2457971	0.2063718	0.05837563	0.0362117	0.05248807	0.07717042	0.05683356
2997	cg02989257	450240	6250450	0.2070794	0.3285059	0.2397534	0.02399166	0.2522295	0.1088371	0.2612613
3562	cg03544320	110487	6100066	0	0	0.07678617	0	0	0	0.02673267
26750	cg26782833	3360343	5220431	0.1487889	0.1715006	0	0	0.1806495	0.08852802	0.1208927

Table S3(R) (Continue)

L2463. AVG_Beta	L2100. AVG_Beta	L198. AVG_Beta	L194. AVG_Beta	L2357. AVG_Beta	Gene. Symbol	ttest	Meth change	FC mut v wt	FC mut v normal	present in TCGA GBM set
0.3505025	0.6944162	0.6570533	0.2225519	0.4454353	*S100A1*	0.011	-0.282	1,191	-0.133	
0.001570681	0.5249376	0.6315151	0	0.06845966	*GDF5*	0.030	-0.281	-0.126	-0.015	
0.1495913	0.655163	0.5972655	0.06163643	0.2781244	*FBXO44*	0.018	-0.280	0.021	-0.116	
0.3349209	0.7983117	0.5258636	0.3274615	0.2133758	*SEMA6B*	0.019	-0.273	0.050	0.023	
0.327693	0.5857295	0.540077	0.1012312	0.5483743	*CHI3L2*	0.020	-0.270	0.354	0.794	572
0.1755696	0.7014493	0.6546378	0.07059923	0.4035294	*C19orf24*	0.031	-0.268	-0.017	0.072	
0.6243036	0.9550463	0.9281293	0.5156818	0.7552499	*UNQ830*	0.043	-0.268	2,885	0.309	
0.04527448	0.5131129	0.5662505	0	0.139446	*FLJ36070*	0.020	-0.267	-0.085	0.039	
0.2469696	0.7782214	0.5771658	0.1644304	0.4109969	*FBXO2*	0.023	-0.267	1,632	0.267	76
0.2614504	0.7508929	0.6610615	0.1117647	0.2444969	*KIAA0240*	0.037	-0.265	0.263	0.034	
0.170475	0.7734748	0.7174081	0.1164253	0.3232704	*ITGBL1*	0.043	-0.265	-0.024	0.149	
0.2643803	0.6773944	0.5769199	0.7040836	0.4487841	*ANKRD33*	0.026	-0.264	0.087	1,089	
0.2490185	0.6727494	0.593568	0.4537455	0.3487101	*CPNE9*	0.004	-0.263	-0.008	0.070	
0.0221843	0.6050724	0.4538116	0.09945256	0.05256327	*NNMT*	0.018	-0.261	-0.062	0.602	
0.06869689	0.6067747	0.4844106	0.01828411	0.1466346	*MIA*	0.024	-0.256	1,372	0.860	
0.1840491	0.6743674	0.6218376	0.1169231	0.2503329	*CRIM1*	0.029	-0.255	-0.061	0.199	
0.2329791	0.7069409	0.6260623	0.1986153	0.2034768	*DISP1*	0.023	-0.253	0.299	0.204	
0.1770082	0.6899129	0.6175333	0.1202698	0.2225815	*PRELP*	0.030	-0.252	0.380	-0.987	
0.06458333	0.6749688	0.5190797	0.04010184	0.2499024	*FLJ20184*	0.040	-0.247	0.002	0.014	
0.2228435	0.7401806	0.6223531	0.1797537	0.3574482	*DNAI1*	0.040	-0.247	0.030	-0.084	
0.1334746	0.6442155	0.4569626	0.09595714	0.1984501	*HAPLN1*	0.018	-0.246	0.786	0.576	
0	0.4266539	0.638139	0.08256881	0.1989589	*TM4SF1*	0.026	-0.245	-0.190	0.468	
0.337653	0.722585	0.6732684	0.1561091	0.3039578	*COL16A1*	0.047	-0.244	1,119	1,727	
0.001634958	0.4430544	0.3302785	0.2155054	0.03828045	*CRMP1*	0.002	-0.244	0.037	0.025	194
0.1074561	0.5517241	0.5608496	0.1124418	0.1504986	*MGC4268*	0.023	-0.242	0.086	0.166	

For more detail please see *Nat Genet.* 2011, doi:10.1038/ng.1004.

Table S4 Differentially expressed genes between tumors with and without *IDH1* or *IDH2* mutations at Sanger sequencing

Probe ID	Target ID	logFC	adj.PVal
6280168	*SERPINA3*	3.24	0.04267
4220431	*EXT1*	-0.73	0.03714
4210750	*STARD7*	-0.73	0.04397
2690541	*C18ORF10*	-0.57	0.04310
5050608	*TIMM23*	-0.49	0.03737
7320386	*TTL*	-0.42	0.04267
780021	*OPN3*	-0.37	0.04293
7380709	*YWHAB*	-0.35	0.02844
7040600	*ARSB*	-0.35	0.04267
2060112	*CCNYL1*	-0.29	0.02035
4480341	*DHCR24*	-0.29	0.03737
6270148	*AK5*	-0.29	0.02711
1410398	*CCNYL1*	-0.29	0.04152
6380193	*DLX3*	-0.27	0.02844
70270	*MGC39900*	-0.27	0.03099
4200070	*MGC39900*	-0.27	0.03313
6100390	*CD276*	-0.24	0.04267
6480333	*TCIRG1*	-0.23	0.04901

Table S4 (Continue)

Probe ID	Target ID	logFC	adj.PVal
2810022	*C10RF163*	-0.21	0.03174
5290358	*CPT1A*	-0.21	0.04293
3840750	*15E1.2*	-0.20	0.04267
4570242	*LARGE*	-0.19	0.03455
1050278	*SRD5A1*	-0.19	0.04293
6840753	*SPTLC2*	-0.18	0.03737
1570064	*KIAA1522*	-0.18	0.03737
6900309	*ARSB*	-0.18	0.04901
3440451	*ADAMTS7*	-0.17	0.04293
2450202	*KIF3C*	-0.17	0.00149
4920382	*VAC14*	-0.16	0.03737
360463	*SRR*	-0.16	0.03737
4180376	*PI4KII*	-0.14	0.04267
1450451	*DOPEY2*	-0.11	0.04293
1580397	*ISCA2*	-0.11	0.02844
2000020	*CAMKK2*	-0.11	0.03737
3170102	*C12ORF49*	-0.11	0.02844
10440	*MARS2*	-0.10	0.03778

Table S5 Primers used for Sanger sequencing

Gene	Direction	Exon	Tissue type	Primer sequence (5' to 3')
IDH1	Forward	4	Frozen	TGTAAAACGACGGCCAGTCCATCACTGCAGTTGTAGGTT
IDH1	Reverse	4	Frozen	CAGGAAACAGCTATGACCCACATACAAGTTGGAAATTTCTGG
IDH1	Forward	4	Paraffin	TGTAAAACGACGGCCAGTCGGTCTTCAGAGAAGCCATT
IDH1	Reverse	4	Paraffin	CAGGAAACAGCTATGACCGCCAACATGACTTACTTGATCC
IDH1	Forward	2	Frozen	TGTAAAACGACGGCCAGTGGGCTGTCTGGCAGGTACTA
IDH1	Reverse	2	Frozen	CAGGAAACAGCTATGACCTGTTGGAATTCGTTGTTGGA
IDH1	Forward	3	Frozen	TGTAAAACGACGGCCAGTACCGCGTGTGAAACATAACA
IDH1	Reverse	3	Frozen	CAGGAAACAGCTATGACCGTTTGCTACACGGAGGGGTA
IDH1	Forward	5	Frozen	TGTAAAACGACGGCCAGTTCCTTACAATTCCTGCTAGGG
IDH1	Reverse	5	Frozen	CAGGAAACAGCTATGACCTTGTGCCTTTATTCATGCCA
IDH1	Forward	6	Frozen	TGTAAAACGACGGCCAGTTGGTGGGTGATTTTAGCCTT
IDH1	Reverse	6	Frozen	CAGGAAACAGCTATGACCTGGTTTTGTTTCACTCCTGCT
IDH1	Forward	7	Frozen	TGTAAAACGACGGCCAGTTGTTTGGGACAAGCAGATGA
IDH1	Reverse	7	Frozen	CAGGAAACAGCTATGACCCAAAACTCCCCTTCCCAAAT
IDH1	Forward	8	Frozen	TGTAAAACGACGGCCAGTTGCTCTTCATGCAGTTGGAC
IDH1	Reverse	8	Frozen	CAGGAAACAGCTATGACCTGCACACAAAACACTGAGCA
IDH1	Forward	9	Frozen	TGTAAAACGACGGCCAGTCCATGCCATGAAAATGTGTT
IDH1	Reverse	9	Frozen	CAGGAAACAGCTATGACCGATGCTCTGAGCCCAGTGAG
IDH1	Forward	10	Frozen	TGTAAAACGACGGCCAGTGGACTTTACCACTACCTGCTACC
IDH1	Reverse	10	Frozen	CAGGAAACAGCTATGACCTGGCCTGAGCTAGTTTGATCT
IDH2	Forward	4	Frozen	TGTAAAACGACGGCCAGTTTGTTGCTTGGGGTTCAAAT
IDH2	Reverse	4	Frozen	CAGGAAACAGCTATGACCCTGCAGAGACAAGAGGATGG
IDH2	Forward	4	Paraffin	TGTAAAACGACGGCCAGTAACATCCACGCCTAGTCC
IDH2	Reverse	4	Paraffin	CAGGAAACAGCTATGACCCAGTGGATCCCCTCTCCAC
IDH2	Forward	1	Frozen	TGTAAAACGACGGCCAGTCTCGTTCGCTCTCCAGCTT

Table S5 (Continue)

Gene	Direction	Exon	Tissue type	Primer sequence (5' to 3')
IDH2	Reverse	1	Frozen	CAGGAAACAGCTATGACCGCCACCGTCCCTCAAGTC
IDH2	Forward	2	Frozen	TGTAAAACGACGGCCAGTATGATGCGCTGTGTGTCC
IDH2	Reverse	2	Frozen	CAGGAAACAGCTATGACCGGGACAGAACAATCCCTGG
IDH2	Forward	3	Frozen	TGTAAAACGACGGCCAGTGTCCCTGAGTCACTGGGGT
IDH2	Reverse	3	Frozen	CAGGAAACAGCTATGACCCCTGTGACCCTCCCTGG
IDH2	Forward	5	Frozen	TGTAAAACGACGGCCAGTAGCTCCTCGCCTAGCCAT
IDH2	Reverse	5	Frozen	CAGGAAACAGCTATGACCTGAAGAGACAAGCTGGGAGA
IDH2	Forward	6	Frozen	TGTAAAACGACGGCCAGTCCAGGCTAGGGCACCAC
IDH2	Reverse	6	Frozen	CAGGAAACAGCTATGACCGGGAAGAAAGGCCACAGAGT
IDH2	Forward	7	Frozen	TGTAAAACGACGGCCAGTCCTCTCCCCATAACAGACCTT
IDH2	Reverse	7	Frozen	CAGGAAACAGCTATGACCAGAAGACCAACAGTCCACCC
IDH2	Forward	8	Frozen	TGTAAAACGACGGCCAGTAGGCCCTGAGAGAAAGGCT
IDH2	Reverse	8	Frozen	CAGGAAACAGCTATGACCGGTAGAGGGGCATTGTGAGG
IDH2	Forward	9	Frozen	TGTAAAACGACGGCCAGTGCTCTTGATCTCCCTGCAAC
IDH2	Reverse	9	Frozen	CAGGAAACAGCTATGACCGGACCCAGAGCCTGTCCT
IDH2	Forward	10	Frozen	TGTAAAACGACGGCCAGTGCACAGATGGGGTCTCATTC
IDH2	Reverse	10	Frozen	CAGGAAACAGCTATGACCAGGGTCTGCCTACCACCC
PTH1R	Forward	4	Frozen	CCTGTCTGCCGGAATGG
PTH1R	Reverse	4	Frozen	TGATTGAAGTCATAAATGTAGTCCG
PTH1R	Forward	5	Frozen	TTGGAGCTAGGGGTTCAGTG
PTH1R	Reverse	5	Frozen	GTAGTTGGCCCACGTCCTGT
PTH1R	Forward	9	Frozen	ATCCACATGCACCTGTTCCT
PTH1R	Reverse	9	Frozen	GGCAGAGGGGTACTCACGTA
GNAS	Forward	8	Frozen	TGTAAAACGACGGCCAGTTCGGTTGGCTTTGGTGAGATCCAT
GNAS	Reverse	8	Frozen	CAGGAAACAGCTATGACCTGACTTTGTCCACCTGGAACTTGGT

Table S6 Probes used in hydrolysis probe assays for *IDH1*

Name	Direction	Sequence	Dye	Remark
R132C *IDH1*	Forward	CTTGTGAGTGGATGGGTAAAACCTA	-	-
R132H *IDH1*	Forward	CTTGTGAGTGGATGGGTAAAACCTA	-	-
R132C *IDH1*	Reverse	CACATTATTGCCAACATGACTTACTTGAT	-	-
R132H *IDH1*	Reverse	CCAACATGACTTACTTGATCCCCATA	-	-
R132C *IDH1*_V	-	AAGCATGACGACCTATG	VIC	Reporter 1
R132H *IDH1*_V	-	CATCATAGGTCGTCATGC	VIC	Reporter 1
R132C *IDH1*_M	-	AAGCATGACAACCTATG	FAM	Reporter 2
R132H *IDH1*_M	-	ATCATAGGTCATCATGC	FAM	Reporter 2

Chapter 7

Summary and Concluding Remarks

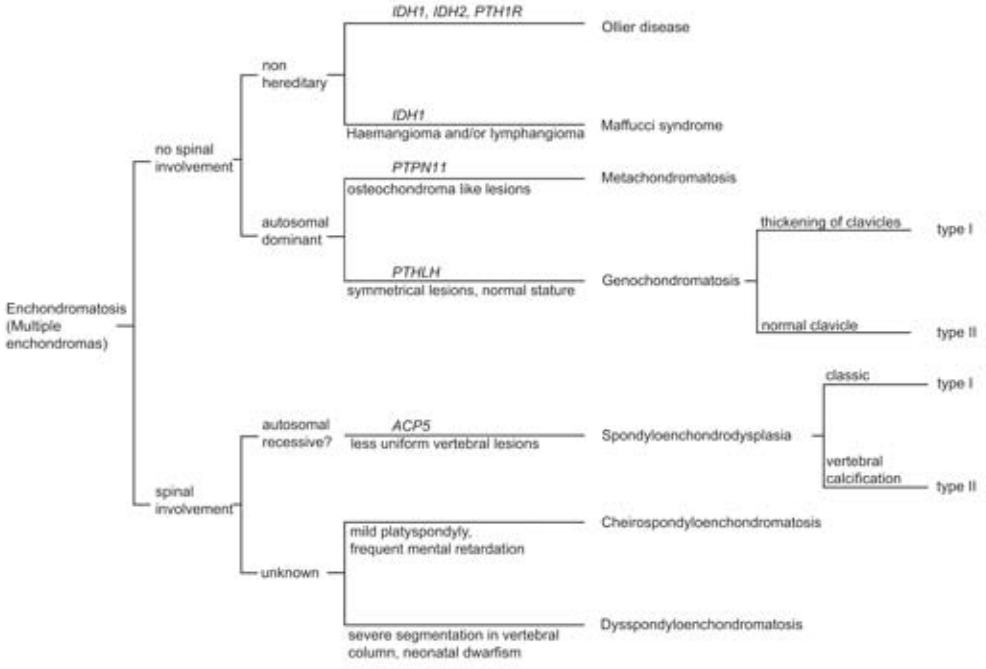
Chapter 1 gives a general introduction of this thesis, describes the aim and research questions. Enchondromatosis is characterized by the presence of multiple enchondromas with a marked unilateral predominance. It includes various subtypes with Ollier and Maffucci syndrome as the most common ones. At present, seven subtypes of enchondromatosis syndromes are recognized including Ollier disease, Maffucci syndrome, metachondromatosis, genochondromatosis, spondyloenchondromatosis, cheiro-spondyloenchondromatosis and dysspondyloenchondromatosis as delineated in **Chapter 2**.

Due to the rarity of these diseases, studies on the genetic cause of Ollier disease are sparse and hampered by low numbers. A small subset of patients with Ollier disease has been reported to have four different types of point mutations in *PTH1R* (1-3). Therefore, the genetic defect involved in the majority of the patients with Ollier disease and Maffucci syndrome was not known. During the course of our studies mutations were found in several other subtypes of enchondromatosis syndromes (summarized in Figure 1). *PTPN11* was found to be mutated in patients with metachondromatosis syndrome (4;5). *PTH1R* nor *PTPN11* were mutated in our patient series with Ollier disease and Maffucci syndrome. *PTPN11* is a protein tyrosine phosphatase and an important intracellular signaling molecule linking several growth factor receptors to the Ras/MAPK and other signaling pathways. Duplication of 12p11.23 to 12p11.22 containing *PTHLH* was found in one patient with symmetrical enchondromatosis (genochondromatosis features as reviewed in **Chapter 2**). Like PTH1R, PTHLH is also involved in growth plate signaling. Mutations in *ACP5* were found in patients with spondyloenchondrodysplasia (6;7). *ACP5* encodes for tartrate-resistant phosphatase (TRAP) and TRAP is required for processing and/or degradation of osteopontin. Osteopontin is a bone matrix protein that mediates osteoclast substrate adhesion, migration and its dephosphorylation by TRAP reduces the affinity of osteoclast binding to different substrates (7). Over the past years by means of either genomewide (*PTPN11, ACP5, PTHLH*) or hypothesis driven approach (*PTH1R, IDH1, IDH2*) the genetic background of the different subtypes of enchondromatosis has been explored in detail (Figure 1).

The aim of the research described in this thesis was to identify the genetic cause of Ollier disease and Maffucci syndrome through molecular analyses. To achieve this goal, we collected a large series of patient materials with the collective efforts of the EuroBoNet and EMSOS networks and used a genome-wide as well as a hypothesis driven approach.

In **Chapter 3**, we have applied genome-wide analysis and reported very few genetic alterations for enchondromas of Ollier diseases. Nonrecurrent copy number alterations were found at *FAM86D, PRKG1* and *ANKS1B* in enchondromas. LOH with copy number loss of chromosome 6 was found in enchondromas from two unrelated patients with Ollier disease and one of these patients also showed loss of chromosome 3. There were no common genomic alterations found for all enchondromas. Using an integration approach of SNP and expression array, we screened for *POU5F1* (loss and downregulation) and *NIPBL* (gain and upregulation). Strikingly, none of these candidate regions were affected in more than two patients with Ollier diseases suggesting these changes to be a random secondary event in enchondroma development. Chondrosarcomas from patients with Ollier disease were proven to be genetically unstable. We also found that LOH and copy number alterations were associated with chondrosarcomas and it mainly involved chromosomes 3, 5, 6 and 9.

Figure 1 Enchondromatosis sub-types



Different genes are involved in different subtypes of enchondromatosis syndrome, with the exception of Ollier disease and Maffucci syndrome for which we have now shown that both can be caused by mutations in *IDH1*.

In **Chapter 4**, we have used the same approach to study tumors related to Maffucci syndrome. Like in chapter 3, we found neither LOH nor common copy number alterations shared by all enchondromas related to Maffucci syndrome, with the exception of some copy number variations. Similar to solitary and Ollier related chondrosarcomas, the chondrosarcomas of Maffucci syndrome were found to have multiple genomic imbalances. Our results are in line with the multistep genetic progression model. Expression profiling using Illumina BeadArray-v3 chip revealed that cartilaginous tumors in Maffucci patients are more similar to tumors in Ollier patients than to solitary cartilage tumors.

Based on findings of the genome-wide approach, we hypothesized that point mutation(s) in a single gene or other copy number neutral genomic changes might play a role in enchondromagenesis. We therefore applied a hypothesis driven approach and studied *NDST1, PTH1R, IDH1, IDH2* and *GNAS* as possible candidate genes.

In **Chapter 5**, we explored possible parallels with multiple osteochondromas therefore we studied *EXT1*, heparan sulfate and *NDST1* in patients with Ollier disease as well as Maffucci syndrome. We found normal protein expression of EXT1 and heparan sulfate and NDST1. Mutation analysis of *NDST1* did not reveal any point mutations that altered the function of *NDST1*.

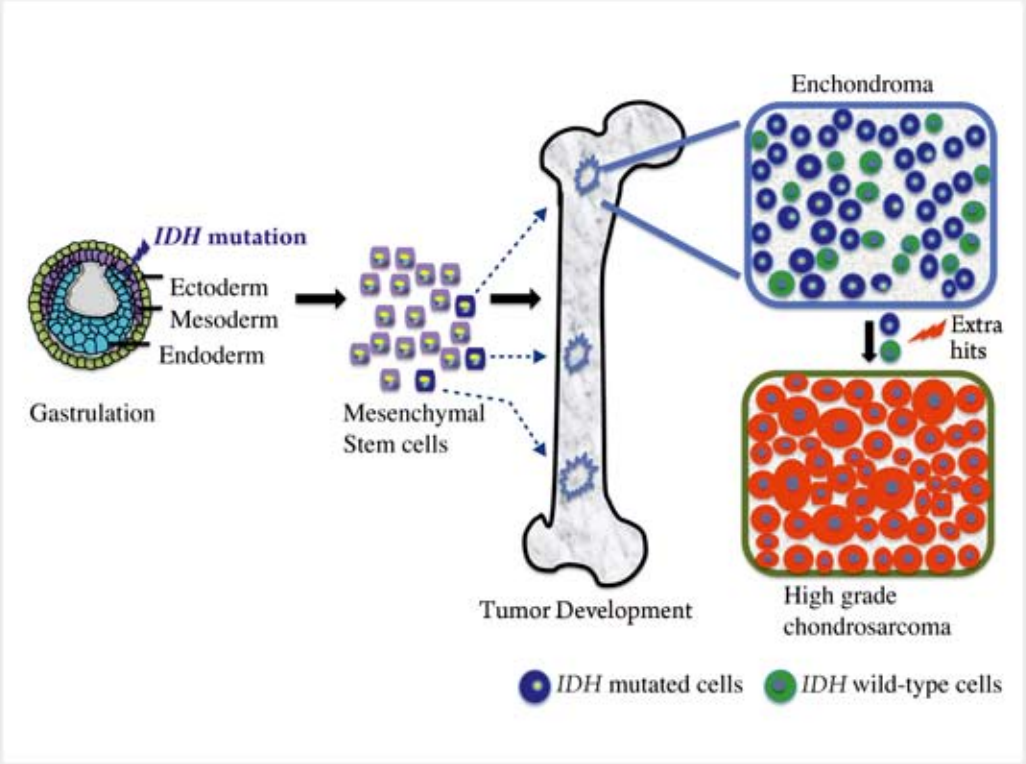
In **Chapter 6**, we have considered *PTH1R, IDH1, IDH2* and *GNAS* as candidate genes. *PTH1R* is involved in growth plate signaling and mutations were reported in a subset (8%) of patients with Ollier disease (1-3). We screened 35 patients with Ollier disease in which mutations were absent, decreasing the overall mutation rate for *PTH1R* to only ~5%.

Based on the increased incidence of gliomas and juvenile ovarian granulosa cell tumors in Ollier disease and Maffucci syndrome, we screened the *IDH1, IDH2* and *GNAS* genes for mutations. *GNAS* mutations were absent in tumors as well as in control DNA from patients. We reported somatic heterozygous *IDH1* (R132C and R132H) or *IDH2* (R172S) mutations in 87% of enchondromas and 70% of spindle cell hemangiomas. With the elucidation of *IDH1* and *IDH2* mutations in patients with Ollier disease (81%) and Maffucci syndrome (77%), we demonstrate that the different enchondromatosis subtypes are caused by mutations in different genes with the exception of Ollier disease and Maffucci syndrome. Using a highly sensitive hydrolysis probes assay, the presence of a subpopulation of cells with an *IDH1* mutation was confirmed. These findings were also supported by using the R132H IDH1 mutation specific antibody, where we observed presence of tumor cells with and without *IDH1* mutation (intraneoplastic mosaicism, Figure 2), within the tumor. Parallel to our study, Amary et al. reported an additional 40 individuals with Ollier disease and Maffucci syndrome, of which 35 individuals carried *IDH1* or *IDH2* mutations in at least one of their tumors (8).

These results are in line with the hypothesis that the mutation in Ollier disease or Maffucci syndrome should occur early during embryonic development resulting in somatic mosaicism. Using a R132H IDH1 mutation specific antibody, we also showed mosaic distribution of osteocytes, osteoblasts, adipocytes and fibroblasts in surrounding area of the tumor expressing mutant protein with very low frequency.

Moreover, fourteen of sixteen patients (88%) possessed identical mutations, including a rare variant (R172S *IDH2*), in more than one tumor. These observations support the model that *IDH1* or *IDH2* mutations occur in the mesoderm (from which the skeleton will be formed) during early gastrulation. Thus, a small percentage of mesenchymal stem cells in the body of these patients contain mutations in *IDH1* or *IDH2* and may give rise to the formation of enchondromas at different locations in the skeleton, with a unilateral predominance (Figure 2). Indeed, Amary and colleagues detected 9-16% mosaic mutation levels in bone marrow derived DNA from two unrelated subjects with Ollier disease (8). The facts that Ollier disease and Maffucci syndrome are not familial and that a low level of mosaic somatic can be detected in patients may indicate that mutations at 132 *IDH1* and 172 *IDH2* positions could be embryonically lethal.

Figure 2 Model for enchondroma and secondary central chondrosarcoma development in Ollier disease and Maffucci syndrome



Based on the results from chapter 6 we expect *IDH* mutations to occur early in mesenchymal stem cells in the mesoderm (from which the skeleton will be formed) causing somatic mosaicism underlying multiple enchondromas development. Our results suggest enchondromas consist of *IDH1* or *IDH2* mutated cells intermingled with wild-type cells (intraneoplastic mosaicism). For secondary central chondrosarcoma an additional hit is postulated to occur in either the *IDH1* or *IDH2* mutated or in wild-type cells.

Amary et al. showed that *IDH* mutations in solitary tumors and tumors related to Ollier disease and Maffucci syndrome are associated with increased levels of 2HG in the tumor (8). The increased 2HG production may result in altered methylation pattern through the genome, as was previously shown for gliomas and acute myeloid leukemia (9;10).

Indeed, in our study we showed that *IDH* mutations are associated with a CpG Island methylator Phenotype in enchondromas. We further hypothesized that due to hypermethylation of several genes the differentiation of chondrocytes into bone is inhibited, resulting in islands of proliferating chondrocytes within the skeleton. There might be a close interaction between mutated and wild-type tumor cells and a paracrine effect of 2HG may prevent the wild-type cells to differentiate towards bone cells.

Enchondromas related to Ollier disease and Maffucci syndrome or solitary enchondromas can progress towards secondary central chondrosarcomas. It was recently shown that the risk of malignant transformation in Ollier disease and Maffucci syndrome is variable and depends on the location of enchondromas; enchondroma restricted to the small bones of hands and/or feet bear a risk of 14% while those in the long bones progress to malignancy in 44-50% (12), which is much higher as compared to solitary tumors in these locations. This might be due to the fact that there are multiple tumors present in Ollier disease and Maffucci syndrome having an additive effect on the probability of malignant transformation. In general, solitary enchondromas are diagnosed accidentally. The malignant transformation rate of solitary enchondroma might therefore be underestimated because in case of presence of symptoms (mainly pain), a lesion is more likely to be diagnosed as low grade chondrosarcoma while the pre-existing enchondroma remains unidentified. It is hypothesized that extra hits are necessary for the malignant transformation of enchondroma which might occur either in IDH wild-type or IDH mutated chondrocytes (Figure 2). In analogy, for peripheral chondrosarcoma it was recently shown that these predominantly arise in the wild-type cells and not in the EXT mutated cells of a mosaic osteochondroma (11). Since *IDH* mutations are present in the majority of enchondromas as well as chondrosarcomas of syndromic as well as non-syndromic conditions, these mutations do not have any diagnostic value in distinguishing enchondromas from low grade chondrosarcomas.

We and others also evaluated the occurrence of *IDH* mutations in other cartilaginous and vascular tumors, and demonstrated that the occurrence of *IDH* mutations was restricted to enchondromas, central chondrosarcomas, periosteal chondrosarcomas and dedifferentiated central chondrosarcomas.

Mutations were absent in soft tissue chondromas, chondromyxoid fibromas, chondroblastomas, osteochondromas, peripheral chondrosarcomas, synovial chondromatosis, mesenchymal chondrosarcomas and clear cell chondrosarcomas (13) (This thesis).

Concluding Remarks

The purpose of this thesis was to identify the underlying genetic changes in Ollier disease and Maffucci syndrome by studying patient's enchondromas and chondrosarcomas. Ultimately, we identified mutations in two genes (*IDH1* and *IDH2*) that were present in the majority of the tumors from patients with Ollier disease and Maffucci syndrome. A subgroup of patients did not show mutations in IDH1, IDH2 or PTH1R and therefore, other genes (except *ACP5, PTPN11, PTHLH, GNAS, NDST1*) might be involved. Moreover, one can not exclude the possibility of functional links or pathways shared between *IDH1* or *IDH2* with *EXT1, EXT2, PTH1R, PTPN11, PTHLH* and *ACP5* (8). A high-resolution, genome-wide, approach like next generation sequencing may facilitate the identification of additional genes. Mutations of other members involved in metabolic pathway (such as D- and L-2- hydroxyglutarate dehydrogenases, malate dehydrogenase) which leads to an increased 2HG production can be anticipated (8). There is also a possibility of presence of mutations in *TET2* (9).

Functional studies to dissect the role of *IDH1* and *IDH2* in Ollier disease and Maffucci syndrome using chondrosarcoma cell-lines with, and without *IDH1* or *IDH2* mutations might shed light onto the pathogenesis of Ollier disease and Maffucci syndrome. Amary et al. confirmed that indeed tumors with *IDH1* mutation produce 2HG (8). We have shown that *IDH1* mutations are associated with hypermethylation and consequently downregulation of several genes. Future studies should clarify whether 2HG is causative for the hypermethylation and how this leads to enchondroma formation. *DLX5* was the most differentially methylated gene between enchondromas with, and without *IDH1* mutations. Future studies could reveal whether down regulation of *DLX5* through methylation as a consequence of *IDH1* mutation delays hypertrophic differentiation of chondrocytes and inhibits subsequent osteogenic differentiation, thereby leaving clusters of proliferating chondrocytes behind.

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Hoofdstuk 7 Nederlandse Samenvatting

Hoofdstuk 1 geeft een algemene inleiding van dit proefschrift, beschrijft het doel en de onderzoeksvragen. Enchondromatosis wordt gekenmerkt door de aanwezigheid van multipele enchondromen in het skelet, waarbij de ene zijde van het lichaam vaak ernstiger is aangedaan dan de andere zijde. Het omvat verschillende subtypes waarvan de ziekte van Ollier en het syndroom van Maffucci het meest voorkomen. Momenteel worden zeven subtypes van enchondromatosis erkend waaronder de ziekte van Ollier, het syndroom van Maffucci, metachondromatosis, genochondromatosis, spondyloenchondromatosis, cheiro-spondyloenchondromatosis en dysspondyloenchondromatosis. In **hoofdstuk 2** worden deze subtypes in detail beschreven.

Wegens de zeldzaamheid van deze aandoeningen is er tot nu toe weinig onderzoek verricht naar de genetische oorzaak van de ziekte van Ollier. Voor een klein deel van de patiënten met de ziekte van Ollier zijn vier verschillende puntmutaties beschreven in *PTH1R* (1-3). Gedurende het beloop van ons onderzoek werden ook mutaties beschreven in enkele andere subtypes van enchondromatosis syndroom (samengevat in hoofdstuk 7 figuur 1). Mutaties in het *PTPN11*-gen werden gevonden in patiënten met metachondromatosis (4; 5). Zowel *PTH1R* als *PTPN11* bleken niet afwijkend te zijn in onze reeks patiënten met de ziekte van Ollier en het syndroom van Maffucci. *PTPN11* is een eiwit tyrosine phosphatase en een belangrijke intracellulair signaal transductie molecuul. In één patiënt met symmetrische enchondromatosis (genochondromatosis zoals beschreven in Hoofdstuk 2) werd amplificatie gevonden van chromosoom 12p11.23 tot 12p11.22, het gebied waar het *PTHLH*-gen gelocaliseerd is. Net als PTH1R, is PTHLH ook betrokken bij de signaaltransductie cascades in de normale groeischijf. In patiënten met spondyloenchondrodysplasia werden afwijkingen in het *ACP5*-gen aangetoond (6; 7). *ACP5* codeert voor tartraat resistent phophatase (TRAP) en is nodig oor verwerking en/of degradatie van osteopontin, een eiwit in de bot matrix (7). Kortom, door middel van een genoom-wijde (*PTPN11, ACP5, PTHLH*) of hypothese gerichte benadering (*PTH1R, IDH1, IDH2*) werd recent de genetische achtergrond van de verschillende subtypes van enchondromatosis in detail onderzocht (zie hoofdstuk 7 figuur 1).

Het doel van het onderzoek zoals dat in dit proefschrift wordt beschreven was de genetische oorzaak van de ziekte van Ollier en het syndroom van Maffucci op te helderen door middel van een moleculaire analyse. Om dit doel te bereiken, verzamelden wij materiaal van een grote reeks patiënten, gebruik makend van de onderzoeksnetwerken EuroBoNeT en EMSOS. We gebruikten zowel een genoom-wijde als een hypothese gedreven benadering.

In **hoofdstuk 3** beschrijven wij een genoom-wijde analyse bij de ziekte van Ollier. Het bleek dat de enchondromen van patiënten met de ziekte van Ollier maar weinig genetische veranderingen bevatten. Deze genetische veranderingen betroffen met name *FAM86D, PRKG1* en *ANKS1B*, maar dit werd niet in andere enchondromen terug gevonden. Wij vonden verlies van heterozygotie gecombineerd met verlies van een copie van chromosoom 6 in enchondromen van twee niet verwante patiënten met de ziekte van Ollier, en één van deze patiënten toonde ook verlies van chromosoom 3. Er waren echter geen gemeenschappelijke genomische veranderingen die in alle enchondromen werden gevonden.

Door het combineren van de informatie over het aantal kopieen van genen met gen expressie data, identificeerden wij *POU5F1* (verlies en downregulatie) en *NIPBL* (amplificatie en upregulatie). Deze twee kandidaat genen bleken echter niet in meer dan twee patiënten met de ziekte van Ollier aangedaan te zijn. Dit suggereert dat deze veranderingen door toeval zijn ontstaan. Chondrosarcomen van patiënten met de ziekte van Ollier bleken genetisch instabiel te zijn. Daarnaast toonden wij aan dat verlies van heterozygotie en veranderingen in het aantal kopieen met name werd gevonden in chondrosarcomen, en dat dit hoofdzakelijk de chromosomen 3, 5, 6 en 9 betrof.

In **hoofdstuk 4** hebben wij dezelfde benadering gebruikt voor het syndroom van Maffucci. Net als in hoofdstuk 3 vonden wij noch LOH noch gemeenschappelijke veranderingen in het aantal kopieen in alle enchondromen. Net als in solitaire en Ollier gerelateerde chondrosarcomen, waren de chondrosarcomen bij het syndroom van Maffucci genetisch instabiel. Deze resultaten ondersteunen het multistep genetisch progressiemodel. Het genexpressie profiel dat door middel van Illumina beadArray-V3 chip werd bepaald liet zien dat de kraakbeen tumoren bij Maffucci syndroom meer overeenkomen met kraakbeentumoren bij de ziekte van Ollier, dan met solitaire kraakbeentumoren.

Gebaseerd op de bevindingen van de genoom-wijde benadering, stelden wij de hypothese op dat puntmutaties in één enkel gen, of een andere verandering die het aantal kopieen onveranderd laat, ten grondslag ligt aan het ontstaan van een enchondroom. Wij pasten daarom een hypothese gedreven benadering toe en bestudeerden *NDST1*, *PTH1R, IDH1, IDH2* en *GNAS* als mogelijke kandidaatgenen.

In **hoofdstuk 5** exploreerden wij mogelijke parallellen met multipele osteochondromen. *EXT1*, heparansulfaat en *NDST1* werden bestudeerd in patiënten met de ziekte van Ollier en het syndroom van Maffucci. Wij vonden normale eiwit expressie van *EXT1*, heparansulfaat en *NDST1*. Mutatie analyse van het *NDST1* gen leverde geen puntmutaties op.

In **hoofdstuk 6** hebben wij *PTH1R, IDH1, IDH2* en *GNAS* als kandidaatgenen onderzocht. *PTH1R* is betrokken bij de signaaltransductiecascades in de groeischijf en mutaties zijn beschreven in een subgroep (8%) van de patiënten met de ziekte van Ollier (1-3). Wij onderzochten 35 patiënten met de ziekte van Ollier en vonden geen mutaties. Daarmee daalt het totale percentage *PTH1R* mutaties in patiënten met de ziekte van Ollier naar slechts ~5%.

Gebaseerd op de verhoogde incidentie van gliomen en juveniele granulosaceltumoren van het ovarium in patiënten met de ziekte van Ollier en het syndroom van Maffucci, onderzochten wij de genen *IDH1, IDH2* en *GNAS*. *GNAS* mutaties waren afwezig in zowel de tumoren als normaal DNA van patiënten. Wel vonden wij somatische, heterozygote *IDH1* (R132C en R132H) of *IDH2* (R172S) mutaties in 87% van de enchondromen en 70% van de spoelcel hemangiomen. Met de opheldering van *IDH1* en *IDH2* mutaties in patiënten met de ziekte van Ollier (81%) en het syndroom van Maffucci (77%), tonen wij aan dat de verschillende enchondromatosis subtypes veroorzaakt worden door veranderingen in verschillende genen, met uitzondering van de ziekte van Ollier en het syndroom van Maffucci welke door hetzelfde gendefect worden veroorzaakt. Met behulp van een sensitieve allel specifieke PCR werd een subpopulatie van cellen aangetoond met een *IDH1* mutatie. Deze bevindingen werden ook ondersteund door het gebruik van immuunhistochemie met een IDH1 R132H specifiek antilichaam, waarmee de aanwezigheid van tumorcellen met en zonder IDH1 mutatie binnen de tumor werd aangetoond (intraneoplastisch mosaicisme, zie hoofdstuk 7 figuur 2).

Parallel aan onze studie, publiceerden Amary et al. 40 extra individuen met de ziekte van Ollier en het syndroom van Maffucci, waarvan 35 individuen *IDH1* of *IDH2* mutaties toonden in tenminste één van hun tumoren (8).

Deze resultaten onderschrijven de hypothese dat de mutatie in de ziekte van Ollier of het syndroom van Maffucci vroeg tijdens de embryonale ontwikkeling zou moeten optreden, resulterend in somatisch mosaicisme. Met behulp van het IDH1 R132H specifieke antilichaam toonden wij aan dat ook enkele normale osteocyten, osteoblasten, adipocyten en fibroblasten uit het weefsel rondom de tumor mutant eiwit tot expressie brachten. Daarnaast hadden veertien van de zestien patiënten (88%) identieke veranderingen in meer dan één tumor, waarbij ook een zeldzame variant (R172S *IDH2*) werd gezien. Deze bevindingen steunen de hypothese dat *IDH1* of *IDH2* mutaties in de mesoderm ontstaan (waaruit het skelet zal worden gevormd) tijdens de vroege gastrulatie fase. Het gevolg is dat een klein percentage van de mesenchymale stamcellen in het lichaam van deze patiënten mutaties bevatten in *IDH1* of *IDH2*. Dit zou kunnen leiden tot de vorming van enchondromen op verschillende plaatsen in het skelet, waarbij een zijde van het lichaam ernstiger is aangedaan dan de andere zijde (zie hoofdstuk 7 figuur 2). Amary en collega's beschrijven een mozaiek waarin 9-16% gemuteerde cellen worden gevonden in DNA geisoleerd uit beenmerg van 2 onverwante patiënten met de ziekte van Ollier (8). Het feit dat de ziekte van Ollier en het syndroom van Maffucci niet familiair zijn, en dat een somatisch mozaiek wordt gevonden in patiënten, zou erop kunnen duiden dat embryos met veranderingen ter plaatse van de 132 *IDH1* en 172 *IDH2* posities niet levensvatbaar zijn.

Amary et al. toonde aan dat mutaties in *IDH* geassocieerd zijn met verhoogde niveaus van de oncometaboliet 2 hydroxyglutaraat (2HG) in solitaire maar ook in Ollier en Maffucci gerelateerde tumoren (8). De verhoogde 2HG productie kan leiden tot veranderingen in methylering van het genoom, zoals reeds eerder voor gliomen en acute myeloide leukemie werd aangetoond (9; 10). In onze studie bevestigden wij dat ook in enchondromen het voorkomen van een *IDH* mutatie is geassocieerd met hypermethylatie van het genoom. Wij speculeren daarom dat door de hypermethylatie van verscheidene genen de differentiatie van chondrocyten naar bot geremd wordt, hetgeen zou kunnen leiden tot de ophoping van clusters prolifererende chondrocyten in het bot. Er zou een nauwe interactie kunnen zijn tussen *IDH* gemuteerde en wild-type cellen, waarbij 2HG via een paracrien effect de wild-type cellen zou kunnen verhinderen om verder te differentieren naar bot.

Enchondromen kunnen progressie vertonen naar een secundair centraal chondrosarcoom, zowel bij de ziekte van Ollier en het syndroom van Maffucci als wanneer zij solitair voorkomen. Binnen de context van de ziekte van Ollier en het syndroom van Maffucci varieert het risico op maligne transformatie naar centraal chondrosarcoom afhankelijk van de localisatie van de enchondromen. Enchondromen in de handen en/of voeten hebben een kans van 14%, terwijl enchondromen in de lange pijpbeenderen en/of het axiale skelet een risico van 44-50% op maligne transformatie in zich dragen (12). Dit is veel hoger dan het risico in solitaire tumoren. Dit verschil zou toe te schrijven kunnen zijn aan het feit dat er meerdere tumoren aanwezig zijn bij de ziekte van Ollier en het syndroom van Maffucci, welke een additief effect op het risico kunnen hebben. In het algemeen worden solitaire enchondromen vaak per toeval ontdekt.

Het percentage solitaire enchondromen dat progressie vertoont naar chondrosarcoom zou onderschat kunnen worden, omdat een laesie eerder als een laaggradig chondrosarcoom zal worden gediagnostiseerd wanneer er klachten zijn (hoofdzakelijk pijn), waarbij een eventueel onderliggend enchondroom onopgemerkt kan blijven. Het ligt in de lijn der verwachting dat er extra genetische veranderingen nodig zijn voor maligne transformatie. Deze genetische veranderingen kunnen zowel ontstaan in IDH wild-type cellen als in IDH gemuteerde chondrocyten (zie hoofdstuk 7 figuur 2). Ter vergelijking: in perifere chondrosarcomen toonde men onlangs aan dat deze extra genetische veranderingen met name optraden in de wild-type cellen en niet in de EXT gemuteerde cellen van een mozaisch osteochondroom (11). Aangezien *IDH* mutaties in de meerderheid van de enchondromen alsmede de chondrosarcomen aanwezig zijn, kunnen deze mutaties niet worden gebruikt om het lastige onderscheid tussen enchondromen en laaggradige chondrosarcomen te maken.

Wij en andere onderzoekers evalueerden ook het voorkomen van *IDH* mutaties in andere kraakbeen- en vasculaire tumoren, en toonden aan dat *IDH* mutaties alleen voorkomen in enchondromen, centrale, periostale en gededitferentieerde chondrosarcomen. Mutaties zijn afwezig in weke delen chondromen, chondromyxoid fibromen, chondroblastomen, osteochondromen, perifere chondrosarcomen, synoviale chondromatosis, mesenchymale chondrosarcomen en heldercellige chondrosarcomen (13).

Conclusies

Het doel van het onderzoek beschreven in dit proefschrift was de onderliggende genetische veranderingen in de ziekte van Ollier en het syndroom van Maffucci te identificeren, door middel van het bestuderen van enchondromen en chondrosarcomen van deze patiënten. Uiteindelijk hebben wij in de meerderheid van de tumoren van patiënten met de ziekte van Ollier en het syndroom van Maffucci mutaties geïdentificeerd in twee genen: *IDH1* en *IDH2*. Een kleine restgroep van patiënten toonde geen afwijkingen in *IDH1, IDH2* of *PTH1R* en daarom zouden hier andere genen (met uitzondering van *ACP5, PTPN11, PTHLH, GNAS, NDST1*) betrokken kunnen zijn. Bovendien kan men vooralsnog een eventuele functionele relatie tussen *IDH1* of *IDH2* en *EXT1, EXT2, PTH1R, PTPN11, PTHLH* of *ACP5* (8) niet uitsluiten. Een genoom-wijde benadering door middel van next generation sequencing zou de identificatie van extra genen kunnen vergemakkelijken. Mogelijk is er betrokkenheid van andere genen betrokken bij het metabolisme van de cel (zoals van D- en L-2 hydroxyglutaraat dehydroxygenases, malaat dehydroxygenases) welke eveneens tot een verhoging van 2HG kunnen leiden (8).

Om de rol van *IDH1* en *IDH2* in het ontstaan van de ziekte van Ollier en het syndroom van Maffucci verder te ontrafelen kunnen de chondrosarcoom cellijnen met en zonder mutaties in *IDH1* of *IDH2* worden gebruikt voor functionele studies. Deze studies zouden meer licht kunnen werpen op de pathogenese van de ziekte van Ollier en het syndroom van Maffucci. Amary et al. bevestigde reeds dat kraakbeentumoren met *IDH1* mutaties inderdaad 2HG produceren (8). Wij hebben aangetoond dat *IDH1* mutaties geassocieerd zijn met hypermethylering en als gevolg hiervan downregulatie van verscheidene genen. Toekomstige studies zouden moeten verduidelijken of er een causaal verband is tussen verhoogd 2HG en hypermethylatie, en hoe dit tot de vorming van enchondromen leidt. *DLX5* was het gen waarbij het verschil in methylering het grootst was tussen enchondromen met en zonder *IDH1* mutatie. ëNog onderzocht zal moeten worden of downregulatie van *DLX5* door hypermethylatie als gevolg van de *IDH1* mutatie de hypertrofe differentiatie van chondrocyten en de verdere osteogene differentiatie remt, met als gevolg het achterblijven van clusters kraakbeen in het bot.

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Curriculum Vitae

The author of this thesis was born on April 3rd, 1985 in Amreli, Gujarat, India. She attended secondary school at Kamani Forward High School, Amreli, India, where she got her diploma in 2002. She subsequently obtained a Bachelors degree in Biotechnology at the Shri M&N Virani Science College, Saurashtra University, Rajkot, India in 2005. She followed a Master study of Molecular Biology at Skövde University, Skövde, Sweden, where she obtained her Master's degree in 2007. During her Master study she performed a 6-months research project on "Transient over-expression and biochemical analysis of Arabidopsis and tomato papain-like cystein proteases" at the Max Planck Institute For Plant Breeding Research, Cologne, Germany under the supervision of Dr. Renier van der Hoorn, for which she received a scholarship from Max Planck Institute.

From September 2007 until December 2011 she performed the research described in this thesis at the Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands under the supervision of Dr. Judith V.M.G. Bovee and Prof. Dr. Pancras C.W. Hogendoorn. During her PhD program, she has visited the Children's Hospital, Boston, USA (Dr Kyle Kurek, Prof. Dr. Matthew Warman) and Semmelweis University, Budapest, Hungary (Dr. Tibor Krenács). For the research described in this thesis she received two awards: she was a semifinalist for the Trainee Research Award, American Society of Human Genetics (ASHG) at the 12th International Congress of Human Genetics (ICHG), Montreal, Canada, 2011 and she received a travel award from the Leiden University Fund (LUF).

She will work as a postdoctoral researcher at Washington University in St. Louis, Missouri, United States of America on skin functions under the supervision of Dr. Cristina de Guzman Strong.