Chapter 5

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

T.C. Pansuriya[1], C.E. de Andrea[1], Y.M. Schrage[1], R. Van Eijk[1], and J.V.M.G. Bovée[1]

[1]Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands.

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)

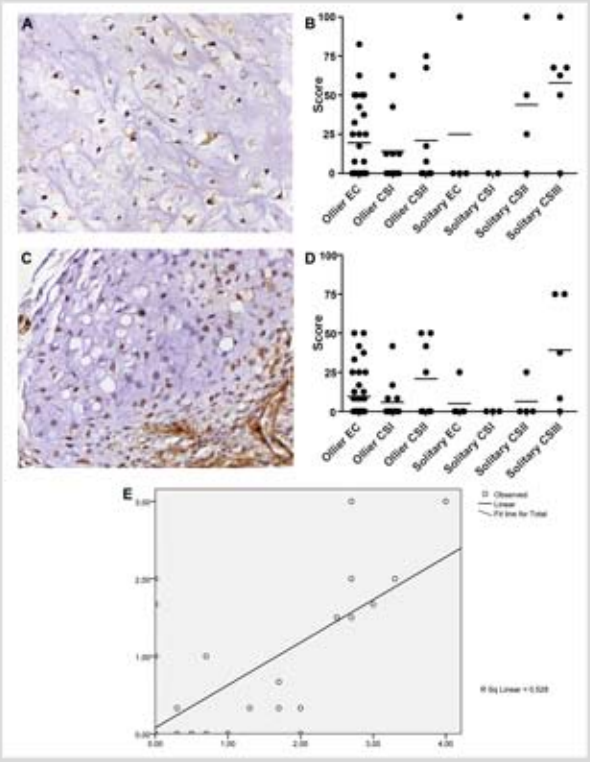
Primer position	Sequence (5' - 3')	Tm °C
*NDST1*_Ex7_F	AGGGTGGCTCAGACACTGAT	73.8
*NDST1*_Ex7_R	TCCATCCCTGTAGCAACCTG	73.8
*NDST1*_Ex8_F	AGTCCACTGACTGGGTTCTG	73.8
*NDST1*_Ex8_R	GGTCAAGAGCAGAGAGACCT	73.8
*NDST1*_Ex9_F	CTGGGTCTCAGGTGTCTACC	74.9
*NDST1*_Ex9_R	TCCCGCTGTGGACAGAAAGG	74.9
*NDST1*_Ex10_F	TGAGTTTTGTCTGTGAGCCG	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.

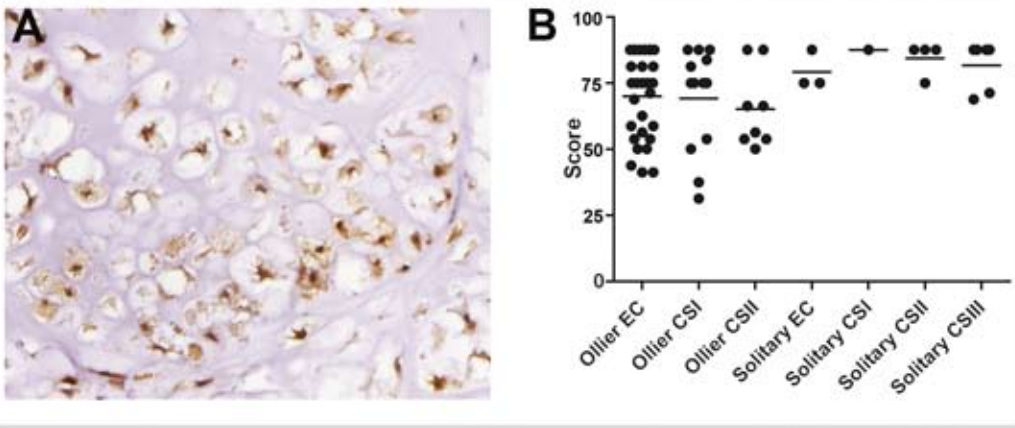
D) Scoring of cytoplasmic expression of HS. On the X-axis different tumor grades of Ollier and solitary tumors are given. Y-axis indicates the percentage of positive cells in each tumor.

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

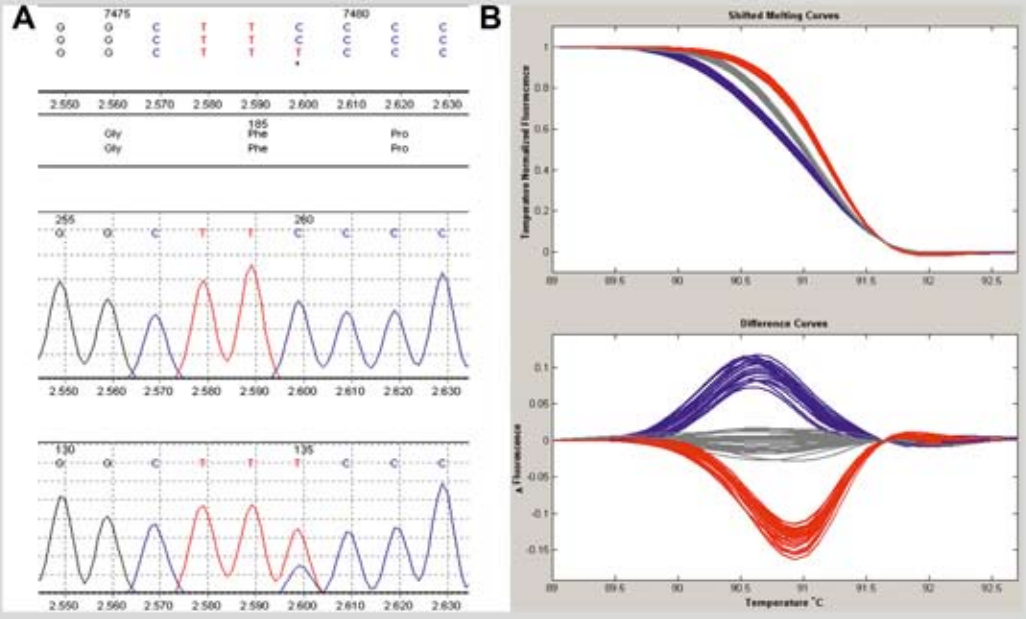
Disease	Tumor Grade	NDST1 expression	EXT1 expression	HS expression
Ollier	EC	28/28 (100%)	15/29 (51%)	15/38 (39%)
	CSI	12/13 (92%)	5/10 (50%)	4/13 (30%)
	CSII	8/8 (100%)	4/8 (50%)	4/8 (50%)
Solitary	EC	5/5 (100%)	1/4 (25%)	1/5 (20%)
	CSI	3/9 (33%)	0/2	0/3
	CSII	9/10 (90%)	3/4 (75%)	1/4 (25%)
	CSIII	9/14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

Acknowledgements

We thank Brendy van den Akker, Maayke van Ruler and Inge H. Briaire-de Bruijn for excellent technical assistance. Our work on Ollier disease is supported by The Netherlands Organization for Scientific Research (917-76- 315), and is performed within EuroBoNet, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumors (018814). We are thankful to S. Verdegaal and A.H.M. Taminiau, LUMC, Netherlands, S. Daugaard, University of Copenhagen, Denmark, L. Sangiorgi, Rizzoli Orthopedic Institute, Bologna, Italy, R. Sciot, University of Leuven, Leuven, Belgium, Tibor Krenács, Semmelweis University, Budapest, Hungary, F. Mertens, Lund University Hospital, Sweden, L. Kindblom, Royal Orthopedic Hospital, United Kingdom, R. Forsyth, Ghent University, Belgium, P. Jutte, UMCG, The Netherlands, W. Wuyts, University of Antwerp, Belgium, P. Mainil-Varlet, Bern University, Switzerland, B. Toker, Istanbul University Medical School, Turkey, B. Liegl-Atzwanger, University Clinic of Orthopaedic Surgery and Medical University of Graz and M. San- Julian, University of Navarra, Spain for providing samples. The continuous support of the Netherlands Committee on Bone Tumors is highly acknowledged.

References

1. Pansuriya TC, Kroon HM, Bovee JVMG. Enchondromatosis: insights on the different subtypes. Int J Clin Exp Pathol 2010;3(6):557-69.
2. Spranger J, Kemperdieck H, Bakowski H, Opitz JM. Two peculiar types of enchondromatosis. Pediatr Radiol 1978 Dec 4;7(4):215-9.
3. Mertens F, Unni KK. Enchondromatosis: Ollier disease and Maffucci syndrome. In: Fletcher CDM, Unni KK, Mertens F, editors. World Health Organization Classification of Tumours. Pathology and genetics of tumours of soft tissue and bone.Lyon: IARC Press; 2002. p. 356-7.
4. Beals RK. Metachondromatosis. Clin Orthop Relat Res 1982;(169):167-70.
5. Hopyan S, Gokgoz N, Poon R, Gensure RC, Yu C, Cole WG, et al. A mutant PTH/PTHrP type I receptor in enchondromatosis. Nat Genet 2002;30(3):306-10.
6. Rozeman LB, Sangiorgi L, Bruijn IH, Mainil-Varlet P, Bertoni F, Cleton Jansen AM, et al. Enchondromatosis (Ollier disease, Maffucci syndrome) is not caused by the PTHR1 mutation p.R150C. Hum Mutat 2004;24(6):466-73.
7. Couvineau A, Wouters V, Bertrand G, Rouyer C, Gerard B, Boon LM, et al. PTHR1 mutations associated with Ollier disease result in receptor loss of function. Hum Mol Genet 2008;17(18):2766-75.
8. Sobreira NL, Cirulli ET, Avramopoulos D, Wohler E, Oswald GL, Stevens EL, et al. Whole-genome sequencing of a single proband together with linkage analysis identifies a Mendelian disease gene. PLoS Genet 2010;6(6):e1000991.
9. Bowen ME, Boyden ED, Holm IA, Campos-Xavier B, Bonafe L, Superti- Furga A, et al. Loss-of-Function Mutations in PTPN11 Cause Metachondromatosis, but Not Ollier Disease or Maffucci Syndrome. PLoS Genet 2011;7(4):e1002050.
10. Khurana J, Abdul-Karim F, Bovée JVMG. Osteochondroma. In: Fletcher CDM, Unni KK, Mertens F, editors. World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone.Lyon (France): IARC Press; 2002. p. 234-6.
11. Dorfman HD, Czerniak B, Kotz R, Vanel D, Park YK, Unni KK. WHO classification of tumours of bone: Introduction. In: Fletcher CDM, Unni KK, Mertens F, editors. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone.Lyon: IARC Press; 2002. p. 226-32.
12. Ahn J, Ludecke H-J, Lindow S, Horton WA, Lee B, Wagner MJ, et al. Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). Nature Genet 1995;11:137-43.
13. Stickens D, Clines G, Burbee D, Ramos P, Thomas S, Hogue D, et al. The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. Nature Genet 1996;14:25-32.
14. Lind T, Tufaro F, McCormick C, Lindahl U, Lidholt K. The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J Biol Chem 1998;273(41):26265-8.
15. McCormick C, Duncan G, Goutsos KT, Tufaro F. The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the golgi apparatus and catalyzes the synthesis of heparan sulfate. Proc Natl Acad Sci USA 2000;97(2):668-73.
16. Schrage YM, Hameetman L, Szuhai K, Cleton-Jansen AM, Taminiau AHM, Hogendoorn PCW, et al. Aberrant heparan sulfate proteoglycan localization, despite normal exostosin, in central chondrosarcoma. Am J Pathol 2009;174(3):979-88.
17. Presto J, Thuveson M, Carlsson P, Busse M, Wilen M, Eriksson I, et al. Heparan sulfate biosynthesis enzymes EXT1 and EXT2 affect NDST1 expression and heparan sulfate sulfation. Proc Natl Acad Sci U S A 2008;105(12):4751-6.
18. Pansuriya TC, Oosting J, Krenacs T, Taminiau AH, Verdegaal SH, Sangiorgi L, et al. Genome-wide analysis of Ollier disease: Is it all in the genes? Orphanet J Rare Dis 2011;6(1):2.
19. Evans HL, Ayala AG, Romsdahl MM. Prognostic factors in chondrosarcoma of bone. A clinicopathologic analysis with emphasis on histologic grading. Cancer 1977;40:818-31.
20. de Andrea CE, Reijnders CM, Kroon HM, de JD, Hogendoorn PCW, Szuhai K, et al. Secondary peripheral chondrosarcoma evolving from osteochondroma as a result of outgrowth of cells with functional EXT. Oncogene 2011. doi: 10.1038/onc.2011.311.

21. Jones KB, Piombo V, Searby C, Kurriger G, Yang B, Grabellus F, et al. A mouse model of osteochondromagenesis from clonal inactivation of Ext1 in chondrocytes. Proc Natl Acad Sci U S A 2010;107(5):2054-9.
22. Willems SM, Schrage YM, Baelde JJ, Briaire-de B, I, Mohseny A, Sciot R, et al. Myxoid tumours of soft tissue: the so-called myxoid extracellular matrix is heterogeneous in composition. Histopathology 2008;52(4):465-74.
23. Gladwin AJ, Dixon J, Loftus SK, Wasmuth JJ, Dixon MJ. Genomic organization of the human heparan sulfate-N-deacetylase/Nsulfotransferase gene: exclusion from a causative role in the pathogenesis of Treacher Collins syndrome. Genomics 1996;15;32(3):471-3.
24. van Eijk R, van Puijenbroek M, Chhatta AR, Gupta N, Vossen RH, Lips EH, et al. Sensitive and Specific KRAS Somatic Mutation Analysis on Whole-Genome Amplified DNA from Archival Tissues. J Mol Diagn 2010;12(1):27-34.
25. Esko JD, Selleck SB. Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu Rev Biochem 2002;71:435-71.
26. Pinhal MA, Smith B, Olson S, Aikawa J, Kimata K, Esko JD. Enzyme interactions in heparan sulfate biosynthesis: uronosyl 5-epimerase and 2-O-sulfotransferase interact in vivo. Proc Natl Acad Sci U S A 2001;98(23):12984-9.
27. Schwartz NB, Roden L, Dorfman A. Biosynthesis of chondroitin sulfate: interaction between xylosyltransferase and galactosyltransferase. Biochem Biophys Res Commun 1974;56(3):717-24.
28. Pansuriya TC, Oostling J, Verdegaal SH, Flanagan AM, Sciot R, Kindblom LG, et al. Maffucci syndrome: A genome-wide analysis using high resolution single nucleotide polymorphism and expression arrays on four cases. Genes Chromosomes Cancer 2011;50(9):673-9.