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**Tiling resolution array-CGH shows that somatic mosaic deletion of the EXT gene is causative in EXT gene mutation negative multiple osteochondromas patients**

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Key Words:	somatic mosaicism, array-CGH, multiple osteochondroma, bone tumors, EXT1, EXT2, bone, multiple exostosis, FISH

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For Peer Review

## Tiling resolution array-CGH shows that somatic mosaic deletion of the *EXT* gene is causative in *EXT* gene mutation negative multiple osteochondromas patients



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# These authors contributed equally to this study.

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Short Title: Somatic mosaicism in multiple osteochondroma

Communicated by <Please don't enter>

### ABSTRACT:

Multiple osteochondromas (MO) is a hereditary skeletal disorder characterized by the presence of cartilage capped bony outgrowths at bone surface. Causative mutations in *EXT1* or *EXT2* genes have been described in 85-90 % of MO cases. However, in about 10-15 % of the MO cases, genomic alterations can not be detected, implying the potential role of other alterations.

We have designed a custom-made Agilent oligonucleotide-based microarray, containing 44,000 probes, with tiling coverage of *EXT1/2* genes and addition of 68 genes involved in heparan sulfate biosynthesis and other related pathways.

Out of the 17 patient samples with previously undetected mutations, a low level of deletion of the *EXT1* gene in about 10-15% of the blood cells was detected in two patients and mosaic deletion of the *EXT2* was detected in one patient.

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Here we show that for the first time somatic mosaicism with large genomic deletions as the underlying mechanism in MO formation was identified. We propose that the existence of mosaic mutations and not alterations of other heparan sulfate biosynthesis related genes play a significant role in the development of MO in patients who are tested negative for mutations in Exostosins. ©2010 Wiley-Liss, Inc.

**KEY WORDS:** Somatic mosaicism, multiple osteochondroma, EXT1, EXT2, array-CGH, FISH, bone tumors, bone

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**INTRODUCTION**

Multiple osteochondromas (MO) (previously known as hereditary multiple exostosis) is characterized by cartilage capped bony outgrowths that typically occur at diaphyseal region of a bone. MO is an autosomal dominant disorder caused by mutations of the *EXT1* (MIM ID \*608177) or *EXT2* genes (MIM ID \*608210) (Fletcher et al., 2002; Ahn et al., 1995; Stickens et al., 1996; Wuyts et al., 1996). Similarly, mutations of the *EXT1* gene were detected in the cartilage cap of sporadic osteochondroma cases (Bernard et al., 2001). Loss of the wild-type allele in hereditary cases (Bovee et al., 1999) and homozygous loss of both alleles in sporadic cases (Hameetman et al., 2007) indicates that inactivation of both *EXT* alleles is required for osteochondroma formation, confirming its tumor suppressor action resulting in a loss of polarization of chondrocytes (de Andrea et al., 2010). The complexity of osteochondroma formation, however, is not yet completely clarified by the bi-allelic inactivation as mosaic distribution of cells with retained chromosome 8 regions (presumably normal cells) was observed in the cartilage cap of both human osteochondromas and animal model systems (de Andrea et al., 2010; Jones et al., 2010; Bovee et al., 2010). The majority of the hereditary cases (70-75%) are caused by point mutations resulting in truncated proteins (Wuyts and Van Hul, 2000; Signori et al., 2007; Lonie et al., 2006; Pedrini et al., 2005; Jennes et al., 2009). Deletions involving single or multiple exons were found in about 10 % of all hereditary cases using Multiplex Ligation-dependent Probe Amplification (MLPA) (Jennes et al., 2008; Fredman et al., 2004; Vink et al., 2005). However, in about 10-15 % of the MO cases, genomic alterations can not be detected implying the potential role of other alterations such as inversions, translocations or somatic mosaicism (Vink et al., 2005). The involvement of other genes or the identification of the putative *EXT3* gene still awaits investigation. Our goal was to possibly identify a novel gene responsible for the MO phenotype that would be involved in the heparan and chondroitin sulfate synthesis pathways. In order to detect these genomic alterations a custom-made, high density-oligonucleotide microarray was designed. In addition, high density coverage of all exons of the *EXT1* and *EXT2* genes facilitates the detection of partial deletions that could be outside of the single probe per exonic region type of MLPA detection system. Ultimately, this design would allow detection of mosaic deletions of any of the probed regions.

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**MATERIALS AND METHODS**

**Array-design**

A custom-made Agilent oligonucleotide microarray containing 44000 reporter elements was (named further as: EXT-tiling array) designed using the Agilent E-array web tool service (named further as: EXT-tiling array). For oligonucleotide selection we used the probe pool provided by Agilent following recommended selection criteria for probe length, GC content and melting temperature (Tm). In addition to *EXT1*, *EXT2*, *EXTL1*, *EXTL2*, *EXTL3* and several other genes involved in heparan sulfate and chondroitin sulfate biosynthesis pathways were selected. The list of selected gene is shown in Table1. For probe design genomic regions 5000 bps before the first and after last exon of each gene were selected. For each tested gene multiple probes were selected with the highest possible coverage of the coding exonic sequences. In case several genes of interest were clustered together in one chromosomal region adjacent genes were partially or in full length included (see the second and third part of the gene list in Table 1). For small exons exon-intron boundaries were also included for probe design. An average 500 bps interval was used for probe selection within intronic regions, with exceptions of large repeat blocks (such as LINE or SINE elements). For the *EXT1* and *EXT2* genes each probe set was printed in triplicate while for all other genes in duplicate. The reporter probe list and the array design are available upon request.

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**Table 1**

List of genes for the custom-made oligonucleotide array design

GeneSymbol	Number of Probes	Probes on Exons	Fully Covered	Gene name
ADAM12	1177	459	Yes	ADAM metalloproteinase domain 12 (meltrin alpha)
B3GALT6	74	68	Yes	UDP-Gal:betaGal beta 1,3-galactosyltransferase polypeptide 6
B3GAT1	213	152	Yes	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)
B4GALNT1	147	143	Yes	beta-1,4-N-acetyl-galactosaminyl transferase 1
B4GALT7	61	51	Yes	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)
CDCP1	435	313	Yes	CUB domain containing protein 1
CHST11	688	109	Yes	carbohydrate (chondroitin 4) sulfotransferase 11
CHST3	331	243	Yes	carbohydrate (chondroitin 6) sulfotransferase 3
CHSY1	318	160	Yes	chondroitin sulfate synthase 1
CSGALNACT1	713	341	Yes	chondroitin sulfate N-acetylgalactosaminyltransferase 1
DSE	475	181	Yes	dermatan sulfate epimerase
EXT1	724	99	Yes	exostoses (multiple) 1
EXT2	314	85	Yes	exostoses (multiple) 2
EXTL1	59	22	Yes	exostoses (multiple)-like 1
EXTL2	187	147	Yes	exostoses (multiple)-like 2
EXTL3	195	47	Yes	exostoses (multiple)-like 3
GALNAC4S-6ST	320	252	Yes	B cell RAG associated protein
GIPC1	87	70	Yes	GIPC PDZ domain containing familv. member 1
HAPLN4	16	9	Yes	hyaluronan and proteoglycan link protein 4
HS2ST1	668	44	Yes	heparan sulfate 2-O-sulfotransferase 1
HS3ST1	120	7	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
HS3ST2	309	6	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
HS3ST3A1	395	8	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1
HS3ST3B1	156	22	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1
HS3ST4	1342	17	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 4
HS3ST5	26	3	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 5
HS3ST6	22	1	Yes	heparan sulfate (glucosamine) 3-O-sulfotransferase 6
HS6ST1	157	4	Yes	heparan sulfate 6-O-sulfotransferase 1
HS6ST2	1142	26	Yes	heparan sulfate 6-O-sulfotransferase 2
HS6ST3	2591	35	Yes	heparan sulfate 6-O-sulfotransferase 3
IL5RA	261	181	Yes	interleukin 5 receptor, alpha
MED30	73	7	Yes	mediator complex subunit 30
NCAN	101	40	Yes	Neurocan
NDST1	131	50	Yes	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
NDST2	41	29	Yes	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2
NDST3	704	34	Yes	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3
NDST4	1072	32	Yes	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4
NUDT16L1	25	24	Yes	nudix (nucleoside diphosphate linked moiety X)-type motif 16-like 1

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PAPSS1	349	20	Yes	3'-phosphoadenosine 5'-phosphosulfate synthase 1
PAPSS2	335	47	Yes	3'-phosphoadenosine 5'-phosphosulfate synthase 2
PTH	59	45	Yes	parathyroid hormone
PTH2	11	5	Yes	parathyroid hormone 2
PTH2R	365	191	Yes	parathyroid hormone 2 receptor
PTHLH	196	154	Yes	parathyroid hormone-like hormone
PTHRI	203	147	Yes	parathyroid hormone receptor 1
SDC1	198	148	Yes	syndecan 1
SDC2	441	200	Yes	syndecan 2
SDC3	107	87	Yes	syndecan 3
SDC4	166	110	Yes	syndecan 4
SDCBP	126	67	Yes	syndecan binding protein (syntenin)
SDCBP2	90	84	Yes	syndecan binding protein (syntenin) 2
SLC22A7	13	8	Yes	solute carrier family 22 (organic anion transporter), member 7
SLC25A42	118	19	Yes	solute carrier family 25, member 42
SLC26A1	32	22	Yes	solute carrier family 26 (sulfate transporter), member 1
SLC26A10	175	173	Yes	solute carrier family 26, member 10
SLC26A11	86	22	Yes	solute carrier family 26, member 11
SLC26A2	92	39	Yes	solute carrier family 26 (sulfate transporter), member 2
SLC26A3	168	47	Yes	solute carrier family 26, member 3
SLC26A4	226	64	Yes	solute carrier family 26, member 4
SLC26A5	254	42	Yes	solute carrier family 26, member 5 (prestin)
SLC29A1	34	24	Yes	solute carrier family 29 (nucleoside transporters), member 1
SLC30A2	34	16	Yes	solute carrier family 30 (zinc transporter), member 2
SLC30A8	155	36	Yes	solute carrier family 30 (zinc transporter), member 8
SLC35B2	17	11	Yes	solute carrier family 35, member B2
SLC35B3	114	71	Yes	solute carrier family 35, member B3
SOX4	169	138	Yes	SRY (sex determining region Y)-box 4
SULF1	858	477	Yes	sulfatase 1
UST	910	270	Yes	uronyl-2-sulfotransferase
XYLT1	1219	459	Yes	xylosyltransferase I
XYLT2	207	193	Yes	xylosyltransferase II
ARMC6	61	16	Yes	armadillo repeat containing 6
CAMK2G	221	70	Yes	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
CRIP3	6	4	Yes	cysteine-rich protein 3
CXorf22	41	40	Yes	chromosome X open reading frame 22
CXorf27	3	2	Yes	chromosome X open reading frame 27
CXorf59	24	23	Yes	chromosome X open reading frame 59
CYBB	33	32	Yes	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
DMD	240	232	Yes	dystrophin (muscular dystrophy, Duchenne and Becker types)
DYNLT3	4	3	Yes	dynein, light chain, Tctex-type 3
FAM47A	8	7	Yes	family with sequence similarity 47, member A
FAM47B	2	1	Yes	family with sequence similarity 47, member B
FAM47C	3	2	Yes	family with sequence similarity 47, member C
FRS3	16	10	Yes	fibroblast growth factor receptor substrate 3
FUT11	19	17	Yes	fucosyltransferase 11 (alpha (1,3) fucosyltransferase)

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<a href="#">GATAD2A</a>	<a href="#">309</a>	<a href="#">15</a>	<a href="#">Yes</a>	<a href="#">GATA zinc finger domain containing 2A</a>
<a href="#">HSP90AB1</a>	<a href="#">16</a>	<a href="#">12</a>	<a href="#">Yes</a>	<a href="#">heat shock protein 90kDa alpha (cytosolic), class B member 1</a>
<a href="#">KIAA0892</a>	<a href="#">138</a>	<a href="#">62</a>	<a href="#">Yes</a>	<a href="#">KIAA0892</a>
<a href="#">KIAA0913</a>	<a href="#">69</a>	<a href="#">44</a>	<a href="#">Yes</a>	<a href="#">KIAA0913</a>
<a href="#">LOC441376</a>	<a href="#">19</a>	<a href="#">2</a>	<a href="#">Yes</a>	<a href="#">AARD protein</a>
<a href="#">MEF2B</a>	<a href="#">117</a>	<a href="#">23</a>	<a href="#">Yes</a>	<a href="#">myocyte enhancer factor 2B</a>
<a href="#">MID1IP1</a>	<a href="#">13</a>	<a href="#">12</a>	<a href="#">Yes</a>	<a href="#">MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))</a>
<a href="#">NR2C2AP</a>	<a href="#">8</a>	<a href="#">6</a>	<a href="#">Yes</a>	<a href="#">nuclear receptor 2C2-associated protein</a>
<a href="#">OTC</a>	<a href="#">20</a>	<a href="#">19</a>	<a href="#">Yes</a>	<a href="#">ornithine carbamoyltransferase</a>
<a href="#">PAFAH2</a>	<a href="#">106</a>	<a href="#">26</a>	<a href="#">Yes</a>	<a href="#">platelet-activating factor acetylhydrolase 2, 40kDa</a>
<a href="#">PAQR7</a>	<a href="#">29</a>	<a href="#">16</a>	<a href="#">Yes</a>	<a href="#">progesterin and adipoQ receptor family member VII</a>
<a href="#">PLA2G7</a>	<a href="#">34</a>	<a href="#">19</a>	<a href="#">Yes</a>	<a href="#">phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)</a>
<a href="#">RCAN2</a>	<a href="#">254</a>	<a href="#">200</a>	<a href="#">Yes</a>	<a href="#">regulator of calcineurin 2</a>
<a href="#">RFXANK</a>	<a href="#">26</a>	<a href="#">12</a>	<a href="#">Yes</a>	<a href="#">regulatory factor X-associated ankyrin-containing protein</a>
<a href="#">RPGR</a>	<a href="#">45</a>	<a href="#">32</a>	<a href="#">Yes</a>	<a href="#">retinitis pigmentosa GTPase regulator</a>
<a href="#">RPS14</a>	<a href="#">22</a>	<a href="#">15</a>	<a href="#">Yes</a>	<a href="#">ribosomal protein S14</a>
<a href="#">RUNX2</a>	<a href="#">288</a>	<a href="#">88</a>	<a href="#">Yes</a>	<a href="#">runt-related transcription factor 2</a>
<a href="#">SEC24C</a>	<a href="#">83</a>	<a href="#">37</a>	<a href="#">Yes</a>	<a href="#">SEC24 related gene family, member C (S. cerevisiae)</a>
<a href="#">SF4</a>	<a href="#">86</a>	<a href="#">26</a>	<a href="#">Yes</a>	<a href="#">splicing factor 4</a>
<a href="#">SFRS14</a>	<a href="#">135</a>	<a href="#">42</a>	<a href="#">Yes</a>	<a href="#">splicing factor, arginine/serine-rich 14</a>
<a href="#">STMN1</a>	<a href="#">24</a>	<a href="#">15</a>	<a href="#">Yes</a>	<a href="#">stathmin 1/oncoprotein 18</a>
<a href="#">SUPT3H</a>	<a href="#">483</a>	<a href="#">217</a>	<a href="#">Yes</a>	<a href="#">suppressor of Ty 3 homolog (S. cerevisiae)</a>
<a href="#">SYNPO</a>	<a href="#">53</a>	<a href="#">26</a>	<a href="#">Yes</a>	<a href="#">Synaptopodin</a>
<a href="#">SYTL5</a>	<a href="#">30</a>	<a href="#">29</a>	<a href="#">Yes</a>	<a href="#">synaptotagmin-like 5</a>
<a href="#">TDRD6</a>	<a href="#">23</a>	<a href="#">20</a>	<a href="#">Yes</a>	<a href="#">tudor domain containing 6</a>
<a href="#">TM6SF2</a>	<a href="#">34</a>	<a href="#">21</a>	<a href="#">Yes</a>	<a href="#">transmembrane 6 superfamily member 2</a>
<a href="#">TMEM161A</a>	<a href="#">45</a>	<a href="#">11</a>	<a href="#">Yes</a>	<a href="#">transmembrane protein 161A</a>
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<a href="#">TRIM63</a>	<a href="#">54</a>	<a href="#">17</a>	<a href="#">Yes</a>	<a href="#">tripartite motif-containing 63</a>
<a href="#">TSPAN7</a>	<a href="#">20</a>	<a href="#">19</a>	<a href="#">Yes</a>	<a href="#">tetraspanin 7</a>
<a href="#">TSPYL1</a>	<a href="#">147</a>	<a href="#">127</a>	<a href="#">Yes</a>	<a href="#">TSPY-like 1</a>
<a href="#">VEGFA</a>	<a href="#">28</a>	<a href="#">25</a>	<a href="#">Yes</a>	<a href="#">vascular endothelial growth factor A</a>
<a href="#">XK</a>	<a href="#">26</a>	<a href="#">25</a>	<a href="#">Yes</a>	<a href="#">X-linked Kx blood group (McLeod syndrome)</a>
<a href="#">ACCS</a>	<a href="#">6</a>	<a href="#">5</a>	<a href="#">No</a>	<a href="#">1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)</a>
<a href="#">ANKS3</a>	<a href="#">61</a>	<a href="#">56</a>	<a href="#">No</a>	<a href="#">ankyrin repeat and sterile alpha motif domain containing 3</a>
<a href="#">C10orf55</a>	<a href="#">5</a>	<a href="#">4</a>	<a href="#">No</a>	<a href="#">chromosome 10 open reading frame 55</a>
<a href="#">C1orf135</a>	<a href="#">7</a>	<a href="#">0</a>	<a href="#">No</a>	<a href="#">chromosome 1 open reading frame 135</a>
<a href="#">CD74</a>	<a href="#">13</a>	<a href="#">1</a>	<a href="#">No</a>	<a href="#">CD74 molecule, major histocompatibility complex, class II invariant chain</a>
<a href="#">CHCHD1</a>	<a href="#">7</a>	<a href="#">6</a>	<a href="#">No</a>	<a href="#">coiled-coil-helix-coiled-coil-helix domain containing 1</a>
<a href="#">CXorf30</a>	<a href="#">1</a>	<a href="#">0</a>	<a href="#">No</a>	<a href="#">chromosome X open reading frame 30</a>
<a href="#">FLJ32658</a>	<a href="#">18</a>	<a href="#">17</a>	<a href="#">No</a>	<a href="#">hypothetical protein FLJ32658</a>
<a href="#">GLB1L2</a>	<a href="#">65</a>	<a href="#">63</a>	<a href="#">No</a>	<a href="#">galactosidase, beta 1-like 2</a>
<a href="#">IDUA</a>	<a href="#">10</a>	<a href="#">3</a>	<a href="#">No</a>	<a href="#">iduronidase, alpha-L-</a>
<a href="#">INTS9</a>	<a href="#">7</a>	<a href="#">4</a>	<a href="#">No</a>	<a href="#">integrator complex subunit 9</a>
<a href="#">LANCL3</a>	<a href="#">28</a>	<a href="#">27</a>	<a href="#">No</a>	<a href="#">LanC lantibiotic synthetase component C-like 3 (bacterial)</a>





<u>60737</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>42</u>	<u>No</u>
<u>71871</u>	<u>m</u>	<u>MO</u>	<u>-</u>	<u>32</u>	<u>Yes</u>
<u>77729</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>n.a.</u>	<u>n.a.</u>
<u>77771</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>36</u>	<u>No</u>
<u>77964</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>n.a.</u>	<u>Yes</u>
<u>78043</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>24</u>	<u>Yes</u>
<u>78883</u>	<u>m</u>	<u>MO</u>	<u>-</u>	<u>8</u>	<u>No</u>
<u>79030</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>n.a.</u>	<u>No</u>
<u>79410</u>	<u>m</u>	<u>MO</u>	<u>-</u>	<u>35</u>	<u>Yes</u>
<u>82107</u>	<u>f</u>	<u>MO</u>	<u>-</u>	<u>15</u>	<u>Yes</u>
<u>83350</u>	<u>m</u>	<u>MO</u>	<u>-</u>	<u>40</u>	<u>No</u>
<u>84458</u>	<u>m</u>	<u>MO</u>	<u>-</u>	<u>45</u>	<u>No</u>
<u>73585*</u>	<u>m</u>	<u>MO</u>	<u>Del MLPA EXT1 Ex8</u>	<u>13</u>	<u>No</u>
<u>54782*</u>	<u>m</u>	<u>MO</u>	<u>Del MLPA EXT1 Ex2-11</u>	<u>6</u>	<u>Yes</u>
<u>84389*</u>	<u>m</u>	<u>MO</u>	<u>Del MLPA EXT2 Ex8</u>	<u>9</u>	<u>Yes</u>

n.a.: not available

MO: multiple osteochondroma

\*MLPA confirmed EXT deletion samples used as positive control

\*\*Familial: samples with clinically established MO with a positive family history

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**Labeling and hybridization protocols:**

Labeling of the DNA samples was performed using a BioPrime total arrayCGH labeling kit (Invitrogen, Breda, The Netherlands) following the manufacturer's protocol. Array hybridizations, scanning, image and data processing were performed according to standard protocols recommended by Agilent (Agilent, Amstelveen, The Netherlands).

**Data processing:**

Scan-images were loaded into Feature extraction software package (Agilent). The generated raw data files were further processed using Genomic Workbench Standard Edition 5.0. In short, the mean of the background corrected, Lowess normalized log2 ratio values of identical features were calculated. Normalization of the log ratio values was performed using the overall values as well as the build in control reporter probe set values using factory defaults. Furthermore, reporters with <1.5x signal-to-background ratio were excluded from further analysis. If

triplicate probes was available for calculation, reporter probes with >1SD of the mean was removed and the mean of the remaining two reporter elements was calculated. Values were then displayed by Moving average window of 3 points and aberrations were calculated using the ADM-2 algorithm with a threshold value of 11.

**Confirmatory Fluorescence in situ Hybridization (FISH):**

FISH analysis to confirm mosaic *EXT1* deletion in sample 59872 was performed with *EXT1* probes 46F10 and 90D8 (Ahn et al., 1995).

**RESULTS:**

We have designed a custom-made Agilent oligonucleotide-based microarray containing 44000 probes with a tiling coverage of *EXT1/2* genes and additional 68 genes involved in heparan and chondroitin sulfate biosynthesis. This array platform has the potential to detect novel gene alterations involved in cartilaginous matrix formation as well as deletions involving any part of the *EXT1* or *EXT2* genes. The genomic DNA of 17 patient samples with confirmed clinico-pathological appearance of MO and no mutations in *EXT1* and *EXT2* when sequenced and MLPA tested were used in this study. By using the custom-made targeted array approach, deletions with variable sizes involving the *EXT1* and the *EXT2* genes were readily detected in the three control samples tested previously positive (Figure 1A and 1B)(Table 3).

**Table 3:**

Overview of tile-resolution array-CGH results with detected deletions both mosaic and hemizygous of the analyzed mutation and MLPA negative patient samples and positive control, respectively

Sample Nr.	Gender	<i>EXT1</i> ***	<i>EXT2</i> ***	Deletion size (kb)	Familial
59872*	m	chr8:EXT1.(118,808,395-118,808,900) (119,004,413-119,004,064)del(NCBI36, hg18)		196	No
83350*	m	chr8:EXT1.(118,914,729-118,915,174) (119,064,408-119,062,731)del(NCBI36, hg18)		149.7	No
84458*	m		chr11:EXT2.(44,084,506-44,086,611) (44,085,734-44,086,166)del(NCBI36, hg18)	10.6	No
73585**	m	chr8:EXT1.(118,889,693-118,891,168) (118,897,371-118,896,538)del(NCBI36, hg18)		43	No
54782**	m	chr8:EXT1.(118,829,257-118,830,034) (119,089,197-119,087,204)del(NCBI36, hg18)		259.9	Familial

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<u>84389**</u>	<u>m</u>	<u>f</u>	chr11:EXT2.(44,128,628-44,171,712) (44,138,600-44,171,010)del(NCBI36:hg18)	<u>7.7</u>	<u>Familial</u>
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M: male; f: female

\*EXT mutation and MLPA negative samples with mosaic deletion

\*\*MLPA confirmed EXT deletion samples used as positive control in the study

\*\*\*Deletions reported according to HGVS recommendation ( <http://www.hgvs.org/mutnomen/>)

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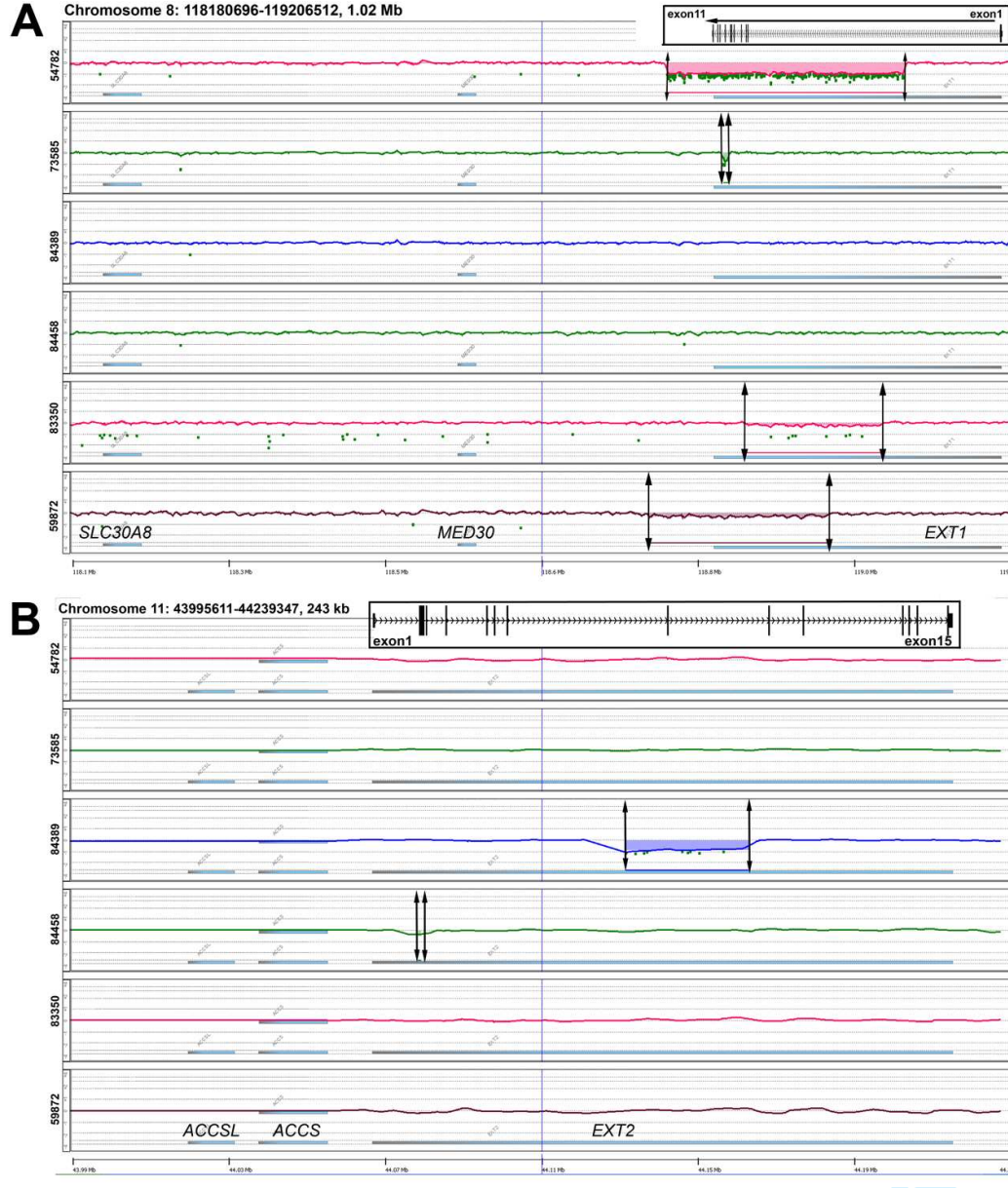
Out of the 17 patient samples, with previously undetected mutations, a low level of deletion of the *EXT1* gene was detected in two patients (Figure 1A) and low lever of deletion of *EXT2* was detected in one patient (Figure 1B).

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For Peer Review

Figure 1



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Based on the level of deletion observed in the array-CGH profile the presence of a mosaic deletion was implicated and estimated to be present in about 10-15% of the blood cells for the patients with both *EXT1* (83350: exon 2 to exon 3; 59872: exon 2 to exon 11) and *EXT2* (84458: exon 2) mosaic deletions (Figure 1A and B). For sample 59872 material was available for FISH confirmation which showed presence of the *EXT1* exon 1 probe 90D8 in all cells but a deletion of the exon 6-11 probe 46F10 in 7 out of 50 cells (14%). The percentage of cells,

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carrying these *EXT* gene deletions were below the detection threshold of MLPA testing and therefore were not detected previously.

No copy number alterations, involving the promoter region of *EXT1* or *EXT2*, were detected in any of the tested samples.

By analyzing all other probes, targeted to members of the cartilaginous matrix synthesis pathway, genomic copy number alterations were observed neither in the translated coding sequences (exons) nor in the promoter regions. However, alterations of other genomic regions were observed involving 9 different genomic regions. One non-coding region of chromosome 6 showed a recurrent 3kb deletion in 4 of the 17 analyzed samples (1 in *EXT*-array negative, 1 mosaic deletion carrier, 2 positive control samples with different deletions involving the *EXT1* gene). This region has been reported in the Database of Genomic Variants (DGV)

(<http://projects.tcag.ca/variation/>). Three genomic regions have been detected with the involvement of intronic parts for *NDST3*, *SUPT3H* and *HS6ST2*. The *NDST3* region has been reported in the DGV database while the other two have not (data not shown). The role of these detected alterations in the development of MO is questionable as they were present both in the *EXT*-deletion carriers positive control samples as well as *EXT* negative patients.

### DISCUSSION

A disease causing germline mutation of either the *EXT1* or *EXT2* gene can be identified in more than 85% of the analyzed *de novo* MO cases and MO families (Wuyts et al., 1998; Wuyts et al., 2005; Lonie et al., 2006; Pedrini et al., 2005; Jennes et al., 2009). Intragenic deletions involving single or multiple exons of the *EXT1* or *EXT2* genes were found in about 10 % of all tested cases (Vink et al., 2005; Jennes et al., 2009). For the remaining mutation or deletion negative cases several possibilities were proposed. Either the involvement of **i.** (an) additional gene(s) **ii.** partial exonic deletions that would not be detected by MLPA probes **iii.** positional changes like inversion, insertion or cryptic translocations causing neither copy number alterations nor sequence substitutions, **iv.** (epi)genetic changes influencing *EXT1/2* promoter function, **v.** presence of somatic mosaic deletions were proposed (Vink et al., 2005).

The aim of this study was to develop a targeted custom-made oligonucleotide-based microarray containing ~44 thousand 60mer oligonucleotide reporters to detect hitherto undetected genomic alterations possibly responsible for the development of MO. For the design we used tiling resolution detection on both *EXT1* and *EXT2* genes including as many probes as possible to cover all exons and introns. This set up facilitates the detection of a partial exonic deletion that could have been unrevealed using the single MLPA probe per exon approach. To facilitate the detection of a candidate *EXT3* gene alterations, additional genomic regions were interrogated by tiling probe resolution containing 68 genes that were selected based on the involvement in heparan or chondroitin sulfate biosynthesis.

By using a highly sensitive, custom-made chip we identified mosaic deletions of the *EXT1* gene in two and of the *EXT2* gene in one out of 17 analyzed MO patients with otherwise negative test results. For the first time somatic mosaicism with large genomic deletions as the underlying mechanism in MO formation was confirmed. As anticipated, none of the mosaic deletion cases were ascertained from the five familial cases (table 1). The presence of somatic and germ-line mosaicism was proved for case 83350, a patient with clinically established MO phenotype without detectable changes who had a daughter with clinically established MO with deletion of exon 2-3 of the *EXT1* gene detected by MLPA and *EXT*-tiling array. Side-by-side comparison these two cases 83350 (father) and 83349 (daughter) showed identical breakpoint regions and the expected differences between the hemizygous and mosaic state (Figure 2).

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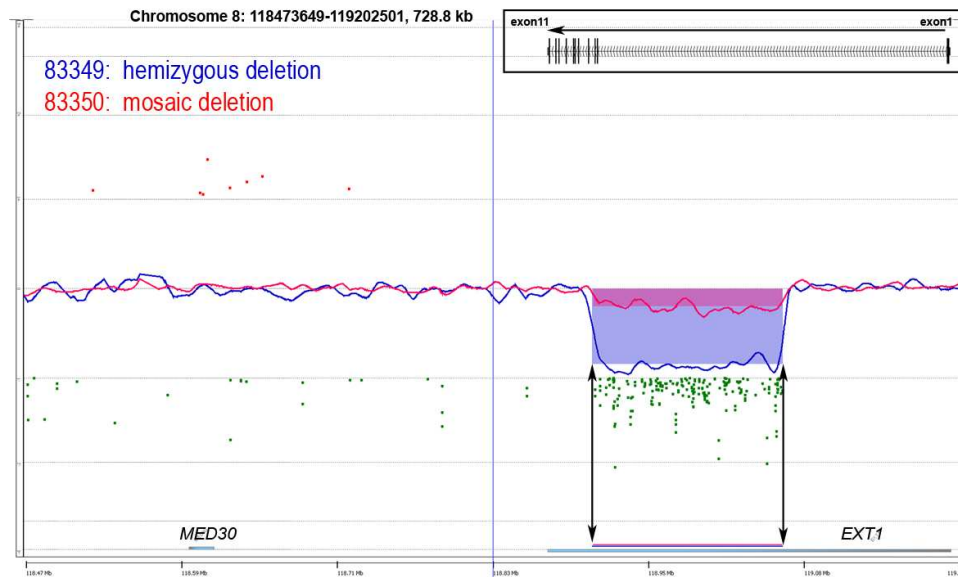
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Figure 2



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Similar mosaic genomic changes somatic or somatic and germline mosaicism have been reported in Duchenne Muscular Dystrophy, Neurofibromatosis 1 and 2 and other skin, blood and neuromuscular disorders, reviewed by Notini et al. and Erickson (Erickson, 2010; Notini et al., 2008). For the first time, we showed that the existence of mosaic mutations (large deletions) may play a significant role in the development of MO in patients who are negative for mutations in Exostosins when analyzed by sequencing and MLPA. In addition, the high probe coverage of the *EXT1* and *EXT2* genes permits a more precise detection of the size of a deletion in cases with similar exonic deletion features as detected by MLPA only (Table 3 and Figure 1).

In theory, partial gene duplication may result in a similar non-sense transcript, however, in our study none of the investigated genomic regions showed duplication of the *EXT1/2* genes or in other genes. Based on this observation, it is unlikely that copy number neutral inversions could play a role in the other cases, especially as genomic inversions are often found to be associated with both by deletions and duplications (Goobie et al., 2008; Male et al., 2002). For the remaining deletion- and mutation-negative cases there are several possible explanations why pathogenic alterations could not be detected. Other genetic or epigenetic alterations without detectable copy number change or sequence alterations, like translocations and insertions that might change the structure of the gene resulting in the loss of function at the protein level, should be also considered. Our tiling array design would not detect small genomic changes (i.e. point mutations) and as sequencing of the other candidate genomic regions was not performed the involvement of the candidate *EXT3* gene could not be ruled out with absolute certainty. However, our results eliminate the necessity of the involvement of a potential third gene responsible for MO. For the remaining sporadic cases, the presence of mosaic point mutation can be predicted based on the fact that in the vast majority (75-80%) of MO cases point mutations are detected and only about 10% of the cases showed the presence of larger deletions involving one or more exons in *EXT1* or *EXT2* genes (Vink et al., 2005; Jones et al., 2010; Lonie et al., 2006). Copy number neutral, structural changes such as inversions involving the *EXT* genes could be expected in mutation negative familial cases. Novel approaches, such as next generation sequencing might provide the required sensitivity to detect mosaic point mutations in these remaining cases.

The high sensitivity of the tiling-oligonucleotide array makes this platform as a versatile tool for studies dealing with genetic mosaicism seen in acquired genomic changes in neoplasia such as solitary osteochondroma or chondrosarcoma cases.

Taken together, we have developed a robust tool for the detection and delineation of mosaic deletions in *EXT1*, *EXT2* and additional genes involved in heparan and chondroitin sulfate biosynthesis. For the first time we prove

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that somatic mosaicism rather than the involvement of a third predicted gene is responsible for the MO development in sporadic and index cases of familial forms.

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#### AUTHORS' CONTRIBUTION:

KS: Designed the study and the array, selected the genes, analyzed the data, interpreted the results and drafted the manuscript

IJ: provided samples, performed EXT mutation studies, performed FISH verification experiments

DJ: Designed the array, validated the data, analyzed the results and partly drafted the manuscript

JVMGB; designed the array, commented on the manuscript

MW selected genes for the array

WW selected genes for the array, provided patient samples, performed EXT mutation studies

PCWH designed the study, reviewed the data and the manuscript

#### FIGURE LEGEND

##### Figure1:

Array-CGH result overview showing the log<sub>2</sub> ratio profile of samples with positive results only.

**A:** An overview of about 1Mb genomic region on chromosome 8 including the *EXT1*, *MED30* and *SLC30A8* gene area. Upper two samples (54782 and 73585) are positive controls with known *EXT1* deletion. Middle two samples (84389 and 84458) with *EXT2* alteration serve as negative control for this view. The bottom two samples (83350 and 59872) show mosaic deletions involving the region exon 2 to exon 3 and exon 2 to exon 11, respectively. For all cases, vertical double headed arrows indicate the borders of the detected alterations.

**B:** An overview of about 240 kb genomic region on chromosome 11 including the *EXT2*, *ACCS* and *ACCSL* gene area. Upper two samples (54782 and 73585) and bottom two samples (83350 and 59872) are with *EXT1* alterations and serving as negative controls for this genome segment. 84389 is a positive control with MLPA detected deletion of exon 8, and 84458 shows the mosaic deletion of exon 2 of the *EXT2* gene. Vertical double headed arrows indicate the borders of the detected alterations. Inset of the right top part in both panels shows the exon localization of the *EXT1* and *EXT2* loci; arrows in inset indicate the direction of transcription, note that *EXT1* gene is transcribed from the minus strand. **C:** Overlay of array-CGH results of a sample with hemizygous deletion (54782, red) and one with a mosaic deletion (59872, blue). Based on the array-profile, the proportion of the deletion containing cell fraction was ~15%. In both cases the deletion involved exon 2-11 of the coding sequence of the *EXT1* gene. The array clearly depicts that the breakpoints were at different position between the two different patients.

##### Figure 2

Side-by-side comparison of mosaic *EXT1* exon 2-3 deletion carrier father (83350, red line) and daughter (83349, blue line) with the inherited paternal deleted allele resulting in a hemizygous deletion with identical breakpoints.

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