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Mutation Screening of *EXT1* and *EXT2* by Denaturing High-Performance Liquid Chromatography, Direct Sequencing Analysis, Fluorescence *in Situ* Hybridization, and a New Multiplex Ligation-Dependent Probe Amplification Probe Set in Patients with Multiple Osteochondromas

Ivy Jennes,* Mark M. Entius,† Els Van Hul,* Alessandro Parra,‡ Luca Sangiorgi,‡ and Wim Wuyts*

From the Department of Medical Genetics, University and University Hospital of Antwerp, Antwerp, Belgium; MRC-Holland,† Amsterdam, The Netherlands; and the Medical Genetics Unit,‡ Rizzoli Orthopaedic Institute, Bologna, Italy*

Multiple osteochondromas (MO) is an autosomal-dominant skeletal disorder characterized by the formation of multiple cartilage-capped protuberances. MO is genetically heterogeneous and is associated with mutations in the *EXT1* and *EXT2* genes. In this study we describe extensive mutation screening in a set of 63 patients with clinical and radiographical diagnosis of MO. Denaturing high-performance liquid chromatography analysis revealed mutations in 43 patients. Additional deletion analysis by fluorescence *in situ* hybridization and a newly developed multiplex ligation-dependent probe amplification probe set identified one patient with an intragenic *EXT1* translocation, three patients with a partial *EXT1* deletion, and one patient with a partial *EXT2* deletion. Thirty-six patients harbored an *EXT1* mutation (57%), and 12 had an *EXT2* mutation (19%). We show that our optimized denaturing high-performance liquid chromatography/sequencing/multiplex ligation-dependent probe amplification protocol represents a reliable and highly sensitive diagnostic strategy for mutation screening in MO patients. Clinical analysis showed no clear genotype-phenotype correlation in our cohort of MO patients. *(J Mol Diagn 2008, 10:85–92; DOI: 10.2353/jmoldx.2008.070086)*

Multiple osteochondromas (MO) is an autosomal-dominant skeletal disorder characterized by the formation of multiple cartilage-capped protuberances, or osteochondromas. Osteochondromas are the result of excessive chondrocyte proliferation and bone growth at the juxta-epiphyseal regions of long tubular bones.[1] In theory, they can arise in every bone with an endochondral origin, but they mainly occur in distal femur, proximal humerus, and proximal tibia. The great variability in size and number of osteochondromas reflects the clinical heterogeneity and variable severity of MO.[2] This disorder has an estimated prevalence of ~1/50,000, making it one of the most frequent skeletal dysplasias.[3] Osteochondromas are rarely present at birth, but in more than 80% of the patients they develop gradually during the first decade of life and increase in size until closure of the growth plates at the end of puberty.[1]

Although osteochondromas are benign, they can cause several secondary complications. By exerting pressure on neighboring tissues, osteochondromas cause pain, nerve compression, and disturbance of the blood circulation as a result of blood vessel compression. Additionally, complications of abnormal skeletal growth are observed in MO patients with shortening of the long bones, restricted range of joint movement, limb length inequalities, and short stature. Especially deformities of the forearm are characteristic. Surgery may be required to correct the most severe deformities. The most serious complication of MO, however, is malignant transformation of osteochondromas resulting in peripheral secondary chondrosarcomas, which occurs in 0.5 to 2% of cases.[2–4]

MO is genetically heterogeneous and is associated with mutations in the *EXT1*[5] and *EXT2* genes.[6,7] In ~10 to

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Address reprint requests to Wim Wuyts, Department of Medical Genetics, Universiteitsplein 1, 2610 Antwerp, Belgium. E-mail: wim.wuyts@ua.ac.be.

20% of the patients no *EXT1* or *EXT2* mutation can be detected. Both EXT genes belong to the larger EXT gene family, which also comprises three homologues EXT-like genes (*EXTL1*, *EXTL2*, and *EXTL3*). *EXT1* and *EXT2* are ubiquitously expressed and encode proteins that function as glycosyltransferases in the biosynthesis of heparan sulfate. Both proteins interact in the Golgi apparatus to form a hetero-oligomeric complex that catalyzes the transfer of *N*-acetyl-glucosamine (Glcnac) and D-glucuronic acid (GlcA) to the elongating heparan sulfate glycosaminoglycans chains.[8,9]

Both *EXT1* and *EXT2* are presumed to be tumor suppressor genes based on mutation and loss of heterozygosity studies. Indeed, loss of heterozygosity has been demonstrated in the *EXT1* 8q24 region in both sporadic and hereditary osteochondromas[10–13] and chondrosarcomas.[10,14] DNA copy number aberrations of this region have also been detected in non-MO related tumors such as colorectal carcinoma.[15] Moreover, somatic homozygous *EXT1* deletions have been found in nonhereditary osteochondromas.[16] Loss of heterozygosity of the *EXT2* 11p11 region has been described previously in chondrosarcomas.[14]

In this study we describe extensive mutation screening in a large set of MO patients. To improve the identification of intragenic *EXT1* and *EXT2* deletions, a new multiplex ligation-dependent probe amplification (MLPA) probe set was designed and validated.

Materials and Methods

Patients

In this study we investigated patients from 63 families with MO. Diagnosis was based on the presence of MOs confirmed by radiographical examination (X-rays). Ethylenediaminetetraacetic acid or heparin blood samples were obtained from patients and available relatives for DNA mutation screening of the *EXT1* and *EXT2* genes. Genomic DNA was isolated from peripheral blood according to standard procedures. Eleven additional MO patients, in whom no point mutation was found in previous mutation screening studies, were also included for MLPA analysis.

DHPLC, Sequencing Analysis, and Fluorescence in Situ *Hybridization (FISH) Analysis*

Polymerase chain reaction (PCR) amplification of the *EXT1* and *EXT2* coding exons and DHPLC analysis on a WAVE-3500HT fragment analysis system (Transgenomic, Crewe, UK) were performed as previously described.[17] If a fragment showed an aberrant chromatograph in DHPLC analysis it was PCR reamplified and the sequence was determined using ABI v1.1 chemistry with sequencing analysis on an ABI3130xl genetic analyzer (Applied Biosystems, Foster City, CA). DNA mutation and nucleotide numbering for *EXT1* and *EXT2* were based on the cDNA reference sequences (GenBank accession numbers NT_023811.12 and NT_009237.13, respectively) with base one corresponding to the first base of the initiation codon. FISH analysis was performed with *EXT1* probes 46F10 and 65G5[5] and *EXT2* probes A1151 and D0694.[7]

MLPA

For MLPA analysis a new MLPA probe set with 13 *EXT1* and 16 *EXT2* probes was developed (Table 1). All probes were designed in such a way that they are located in the *EXT1* and *EXT2* exon sequences. For each exon one probe was developed, with exception of the large *EXT1* exons 1 and 11 for which two probes were designed. Also probes against *EXT2* noncoding exons 1, 1a, and 1b were included. Additionally, 15 probes located outside the *EXT1* and *EXT2* regions were included as reference probes (Table 1). For these reference probes we selected regions that have not been reported to be implicated in osteochondroma or chondrosarcoma development. The MLPA reactions were basically performed as described by Schouten and colleagues[18] with an annealing temperature for all exons of 60°C. Fragment data were quantitatively analyzed using capillary electrophoresis on an ABI3130xl genetic analyzer (Applied Biosystems). Thresholds for deletion and duplication were set at 0.80 and 1.35, respectively.

RNA Analysis

RNA was isolated from EBV lymphoblastoid cell lines using QIAamp RNA blood mini kit (Qiagen, Hilden, Germany). cDNA was subsequently prepared with SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA).

Long-Range PCR

Long-range PCR for the characterization of the *EXT2* exon 2 deletion in family 150 was performed with primers 5′-TCAGAGTTGCTGTTTCTCCTTGAG-3′ and 5′-AACCCATCATAAGGACAGCCC-3′ located in exon 1b and exon 3, respectively.

Results

DHPLC, Sequencing Analysis, and FISH Analysis

Genomic DNA from 63 families, clinically diagnosed with MO, was analyzed for mutations in the *EXT1* and *EXT2* genes by DHPLC analysis of all coding exons from both genes. This point mutation analysis resulted in the identification of a mutation in 43 families (Table 2). In total, 32 families harbored an *EXT1* mutation and in 11 families an *EXT2* mutation was identified. Additionally, FISH analysis revealed a partial deletion of *EXT1* with probe 46F10 deleted in two families and a translocation between chromosomes 4 and 8 involving *EXT1* in one family. No mutation was detected in 17 families.

Table 1. Overview of MLPA-P215 Probes

	Amplicon size (bp)	Upstream hybridizing sequence	Downstream hybridizing sequence
EXT probes			
EXT1-ex 1_1	292	5′-GGACACATGCAGGCCAAAAAACGCTA-3′	5′-TTTCATCCTGCTCTCAGCTGGCTCTTGTCTCGCCCTTTTGTTTTCATG-3′
EXT1-ex 1_2	328	5′-CCAAAGCCAGCATCAGTACTGAAAACT-3′	5′-TCCGACCCAACTTTGATGTTTCTATTCCCCTCTTTTCTAAGGACATG-3′
EXT1-ex 2	247	5′-GGAAATGCTGCACAATGCCACTT-3′	5′-TCTGTCTGGTTCCTCGTGGTCGCAGGCTTGGGTCCTTCAGACATG-3′
EXT1-ex 3	310	5′-CTATGACGGCAGCTTGGTTCCAATTA-3′	5′-ATCACTTCAGAGAATGGCAACTCCCATCCATTGCTGAGCATCACCATG-3′
EXT1-ex 4	382	5′-CCTAGCACTTAGACAGCAGACACAATTCT-3′	5′-TGTGGGAGGCTTATTTTTCTTCAGTTGAGAAGATTGTATTAACTACACATG-3′
EXT1-ex 5	208	5′-CCTGGAGGATTGTTCGTACTACCACAGTAT-3′	5′-TCATCTTATCTGGGAGATTTTCCTTACTACTATGCTAATTTAGGTAAGTCATG-3′
EXT1-ex 6	148	5′-GTGGTCTCTCAGTCCCAGCCAGTGT-3′	5′-TGAAGCTTCTCGTGGCTGCAGCCAAGTCCCAGTACTGTGCCATG-3′
EXT1-ex 7	160	5′-GTTCTATGGAATTGTGACAAGCCCCT-3′	5′-ACCAGCCAAACACCGCTGGCCTGCCACTGCTGTGCCATG-3′
EXT1-ex 8	178	5′-TGAGCAGCCGTTTTCTGCCCTACGACA-3′	5′-ACATCATCACAGACGCCGTGCTCAGCCTTGACGAGGACACCATG-3′
EXT1-ex 9	214	5′-GAGGAGCGGTGGGGATACACATCAA-3′	5′-AGTGGACGAACGACTACTCCATGGTGTTGACAGGAGCTGCTATTTACCATG-3′
EXT1-ex 10	355	5′-CAGCCTGAAGAACATGGTGGACCAAT-3′	5′-TGGCCAATTGTGAGGACATTCTCATGAACTTCCTGGTGTCTCATG-3′
EXT1-ex 11_1	265	5′-TCAGCGACAGAGCTGCATGAATACGTT-3′	5′-TGCCAGCTGGTTTGGCTACATGCCGCTGATCCACTCTCAGACATG-3′
EXT1-ex 11_2	346	5′-GCTGCTCTCTCTTCCCAGTGCAGA-3′	5′-TCCACTCATCAGCAGAGCCAGATTGTGCCAACTATCCCATG-3′
EXT2-ex 1	154	5′-TGAGCGCGCCTGCCTGGGAAA-3′	5′-ACACTGCAGCGGTGCTCGGACTCCTCCTGTCCAGCAGCATG-3′
EXT2-ex 1a	220	5′-GAAGGGGGATGTCCTGCGCCTCAG-3′	5′-GGTCCGGTGGTGGCCTGCGGCATCCCTTGCGCATG-3′
EXT2-ex 1b	283	5′-GAGCTACTCAGAGTTGCTGTTTCTCCT-3′	5′-TGAGATGCTTTTGGTAAGTATATTTTAAAATAATTTTTCCATGTTATCTGAGCATG-3′
EXT2-ex 2	247	5′-GGCCCCATTCTATCGAGTCCTCA-3′	5′-AATGACTGGAATGTAGAGAAGCGCAGCATCCGTGATGTGCCGGTCATG-3′
EXT2-ex 3	184	5′-TGGGATCGAGGTACGAATCACCTGTTGTT-3′	5′-CAACATGTTGCCTGGAGGTCCCCCAGATTATAACACAGCCCCATG-3′
EXT2-ex 4	337	5′-CTGTTGGCTGGTGGCGGCTTTTCT-3′	5′-ACGTGGACTTACCGGCAAGGCTACGATGTCAGCACATG-3′
EXT2-ex 5	409	5′-CCAACCTCTCAGAGGGTGTCCTT-3′	5′-TCTGTCCGTAAGCGCTGCCACAAGCACCAGGTCTTCGATTACCCCATG-3′
EXT2-ex 6	202	5′-TCTACTTTCTGTGTGGTTCTTCGTGGA-3′	5′-GCTCGGCTGGGCCAGGCAGTATTGAGCGATGTGTTACAAGCCATG-3′
EXT2-ex 7	190	5′-CTGTTGTCTCCAGAGCATCTGTGGTT-3′	5′-GTACCAGAAGAAAAGATGTCAGATGTGTACAGTATTTTGCACATG-3′
EXT2-ex 8	166	5′-TGCAGATTATCAATGACCGGATCTATCCAT-3′	5′-ATGCTGCCATCTCCTATGAAGAATGGAATGACCCTCCTGCTGTCATG-3′
EXT2-ex 9	418	5′-CGCTGATCCCACCACAGTCTCAA-3′	5′-GGGTTCACCGCCATAGTCCTCACCTACGACCGAGTAGAGAGCCTCATG-3′
EXT2-ex 10	364	5′-CTGGCCCAAAATCCGGGTTCCATTAA-3′	5′-AAGTTGTGAGGACTGCTGAAAACAAGTTAAGTAACCGTTTCTTCCCTCATG-3′
EXT2-ex 11	136	5′-TGGCGGGAATTTCCTGACCGGTT-3′	5′-GGTGGGTTACCCGGGTCGTCTGCATCTCTGGGACCATGCATG-3′
EXT2-ex 12	142	5′-TGGGTAGATGCTCATATGAACTGTGAAGA-3′	5′-TATTGCCATGAACTTCCTGGTGGCCAACGTCACGGGAAAAGCAGCATG-3′
EXT2-ex 13	229	5′-GAAATTCAAGTGTCCTGAGTGCACAGCCAT-3′	5′-AGATGGGCTTTCACTAGACCAAACACACATGGTGGAGAGGCATG-3′
EXT2-ex 14	436	5′-CAGAGTGCATCAACAAGTTTGCTTCAGTCT-3′	5′-TCGGGACCATGCCTCTCAAGGTGGTGGAACACCGAGCTGACATG-3′
Reference probes			
SLC7A1	130	5′-GAGACGCGGCTGTCTCGCTGCCTGAACACT-3′	5′-TTTGATCTGGTGGCCCTCGGGGTGGGCAGCA-3′
VIPR2	172	5′-CGCATTCACCCAGAATGCCGATTTCAT-3′	5′-CTGGAAATACAGGAGGAAGAAACAAAATGTGCAGAGCTTCT-3′
KIF21A	196	5′-TCTCAGGTCTTCCTAGGGAAAGATAAGGCT-3′	5′-TTTACTTTTGACTATGTATTTGACATTGACTCCCAGCAAGAGC-3′
KCNH2	238	5′-GGACCTGCTCACCGCCCTGTACT-3′	5′-TCATCTCCCGGGGCTCCATCGAGATCCTGCGG-3′
IL1A	256	5′-GATGCCTGAGATACCCAAAACCAT-3′	5′-CACAGGTAGTGAGACCAACCTCCTCTTCTTC-3′
GJB6	301	5′-GGATTGGGGGACGCTGCACACTT-3′	5′-TCATCGGGGGTGTCAACAAACACTCCACCAGCATC-3′
GBAS	321	5′-GGTGTTGCCAAAGATTCACGAAGATA-3′	5′-AACACTACCCTTGTACTTTGGTGGGGACTTGGAACACGTGGT-3′
LRRK2	373	5′-GCTGAACAATGTCCAGGAAGGAAAACAGAT-3′	5′-AGAAACGCTGGTCCAAATCCTGGAGGATCTGCTGGTG-3′
NIPBL	391	5′-GGTGATGATGATGAAATTCCTCAGGAACT-3′	5′-GCTCTTAGGAAAACATCAGCTTAATGAACTTGGCAGTGAATCTG-3′
STCH	441	5′-CCAGAAAATACTGGTACCCATTCAGCAAGTA-3′	5′-TTGAAAGAAGGCCACCTGGAAAAGACTGAGATTGATGAGGT-3′
GNAS	454	5′-CTGTGAACACCCCACGTGTCTTTCTTT-3′	5′-TTCTCCCAGCTTCCTGGACAAGATCGACGTGATC-3′
PKP2	463	5′-CCGACATCAGTGGCTCAGACAGT-3′	5′-TGTCCAGAAGGAAAGTGGCCTGCAGCACACCC-3′
MCCC1	472	5′-CCAGGAAGAAACAGTGACAGGCA-3′	5′-ACCAAGAGGGGGAGGATGGAGAAAACCCTTGCCTTAGTCAGAGCAGG-3′
CASR	481	5′-CCAGTGCCTGTAACAAGTGCCCAGATGACT-3′	5′-TCTGGTCCAATGAGAACCACACCTCCTGCATTGCCAAGGA-3′

MLPA Assay

To create a new MLPA assay, 13 *EXT1* and 16 *EXT2* MLPA probes covering all exons of *EXT1* and *EXT2*, respectively, were designed, and 15 reference probes were included. This MLPA probe set was validated using 10 EXT-negative (Figure 1) and 22 EXT-positive samples, consisting of 13 positive controls for mutations in *EXT1* and 9 for *EXT2* (Table 3). All control patients harbored a (partial) *EXT1* or *EXT2* deletion or duplication. The mutations in these positive control samples were previously identified using FISH with intragenic *EXT1* and *EXT2* probes, two-color MLPA,[19] real-time PCR or DHPLC, and sequencing analysis. No false-positive results were obtained for EXT-negative samples, and all mutations in *EXT1*- or *EXT2*-positive control samples were identified accurately. The MLPA probe set will be referred to as MLPA-P215 hereafter.

After validation, MLPA-P215 was used to screen 17 samples from the set of 63 families that failed to show a variant chromatograph profile with DHPLC analysis and in which no genetic aberration could be found using FISH, as well as 11 samples from former mutation screenings failing to identify a pathogenic mutation. In the set of 17 families, family 200 harbored an *EXT1* exon 8 deletion, and in family 150 an *EXT2* exon 2 deletion was found (Figure 1, Table 2). The presence of the *EXT1* exon 8 deletion was confirmed by RNA analysis showing an aberrant mRNA lacking the complete exon 8 in the proband of this family. The *EXT2* exon 2 deletion was confirmed by long-range PCR with primers located in the exon 2 flanking exons. All affected patients in this family showed a secondary amplification product of ~3 kb, in addition to the expected 5.6-kb PCR amplification product. Sequencing analysis revealed that the deletion actually comprises 2748 bp, with the deletion breakpoints located 1470 bp upstream of the ATG start codon in exon 2 and 742 bp downstream of exon 2. In the set of 11 families from the former screening, one patient with a deletion from *EXT1* exon 2-11 was identified, as well as one patient with an *EXT2* exon 8 deletion. The presence of the latter single exon deletion was confirmed by RNA analysis showing an aberrant EXT2 mRNA lacking only the complete exon 8 sequence.

Clinical Analysis

Detailed clinical data were available for 28 of the EXT1 and 6 of the EXT2 patients, for 5 patients without a mu-

Table 2. Overview of Families in Which an *EXT1* or *EXT2* Mutation Was Identified

Gene	Exon/intron	Mutation at DNA level	Protein change	Mutation type	First reference	Detection technique	Family number
EXT1							
	Exon 1	c.247dupC		Frame shift	This study	DHPLC	155
	Exon 1	c.538-539delAG		Frame shift	This study	DHPLC	183
	Exon 1	c.560-562delGTAinsC		Frame shift	This study	DHPLC	188
	Exon 1	c.644-664del		Frame shift	This study	DHPLC	179
	Exon 1	c.786C>G	p.Y262X	Nonsense	This study	DHPLC	158
	Exon 1	c.793delG		Frame shift	This study	DHPLC	164
	Intron 1	c.962-2G>A		Splice site	This study	DHPLC	199
	Exon 2	c.992C>A	p.A331D	Missense	This study	DHPLC	204
	Exon 2	c.992C>A	p.A331D	Missense	This study	DHPLC	220
	Exon 2	c.1019G>T	p.R340L	Missense	[20]	DHPLC	166
	Exon 2	c.1019G>T	p.R340L	Missense	[20]	DHPLC	171
	Exon 2	c.1019G>T	p.R340L	Missense	[20]	DHPLC	197
	Intron 2	c.1056 + 1G>A		Splice site	[21]	DHPLC	187
	Intron 4	c.1284 + 1G>T		Splice site	[22]	DHPLC	145
	Exon 5	c.1362dupA		Frame shift	This study	DHPLC	144
	Exon 5	c.1374T>G	p.Y458X	Nonsense	This study	DHPLC	207
	Intron 5	c.1417 + 1G>A		Splice site	[23]	DHPLC	181
	Exon 6	c.1431delC		Frame shift	This study	DHPLC	161
	Exon 6	c.1468dupC		Frame shift	This study	DHPLC	184
	Exon 6	c.1469delT		Frame shift	[5]	DHPLC	160
	Exon 6	c.1469delT		Frame shift	[5]	DHPLC	180
	Exon 6	c.1477C>T	p.Q493X	Nonsense	This study	DHPLC	176
	Intron 6	c.1536-1G>C		Splice site	This study	DHPLC	165
	Exon 7	c.1557T>A	p.C519X	Nonsense	This study	DHPLC	174
	Intron 7	c.1632-2A>G		Splice site	[22]	DHPLC	146
	Exon 8	c.1659C>A	p.Y553X	Nonsense	This study	DHPLC	186
	Exon 8	c.1685T>C	p.L562P	Missense	This study	DHPLC	191
	Exon 8	c.1685T>C	p.L562P	Missense	This study	DHPLC	178
	Exon 8	Deletion exon 8		Deletion	This study	MLPA	200
	Intron 8	c.1722 + 1G>T		Splice site	This study	DHPLC	152
	Intron 8	c.1722-2A>C		Splice site	This study	DHPLC	159
	Exon 9	c.1810G>T	p.E604X	Nonsense	This study	DHPLC	194
	Exon 10	c.2051delG		Frame shift	This study	DHPLC	182
		Deletion exon 2-11		Deletion	This study	FISH/MLPA	147
		Deletion exon 2-11		Deletion	This study	FISH/MLPA	163
		t(4;8)(q25;q24.1)		Translocation	This study	FISH	156
	Exon 3	c.1065C>T		Polymorphism			
	Exon 6	c.1431C>T		Polymorphism			
	Exon 9	c.1761G>A		Polymorphism			
EXT2							
	Exon 2	c.178A>T	p.K60X	Nonsense	This study	DHPLC	170
	Exon 2	c.269-271GTT>AAA	p.C90X	Nonsense	This study	DHPLC	193
	Exon 2	c.451-461dup		Frame shift	This study	DHPLC	185
	Exon 2	c.484delC		Frame shift	This study	DHPLC	177
	Exon 2	Deletion EXT2 exon 2		Deletion	This study	MLPA	150
	Exon 4	c.668G>C	p.R223P	Missense	[24]	DHPLC	202
	Intron 4	c.743 + 1G>C		Splice site	This study	DHPLC	169
	Exon 7	c.1139dupT		Frame shift	This study	DHPLC	172
	Exon 7	c.1151-1154delAGAT		Frame shift	This study	DHPLC	203
	Intron 7	c.1173 + 1G>T		Splice site	[25]	DHPLC	151
	Exon 8	c.1191delA		Frame shift	This study	DHPLC	175
	Exon 8	c.1250G>C	p.R417P	Missense	This study	DHPLC	198
	Intron 6	c.1080-18T>A		Polymorphism			
	Intron 7	c.1174-18G>T		Polymorphism			
	Intron 11	c.1807-51C>T		Polymorphism			

tation identified as well as for 6 relatives from EXT1 patients, in whom the same mutation was identified as in the index patient of their family. The number of affected sites was found to be more than 10 in 73% of the EXT1 patients, 80% of the patients with an *EXT2* mutation, and 75% in the mutation-negative patients. The average number of sites affected was 6.8 sites in EXT1 patients, 9.0 for EXT2 patients, and 7.4 in mutation-negative patients. Deformities were present in 80% of both EXT1 and EXT2 patients and in all mutation-negative patients. Twenty-seven percent of the EXT1 patients reported to have secondary complications, whereas in EXT2 patients this group accounted for 67%. No complications were reported in the mutation-negative group. Stature evaluation showed that 85% of the EXT1 patients fell below the 50th percentile with 61% of these smaller than the 10th percentile. Eighty percent of the EXT2 patients were below the 50th percentile, with 75% below the 10th percentile.

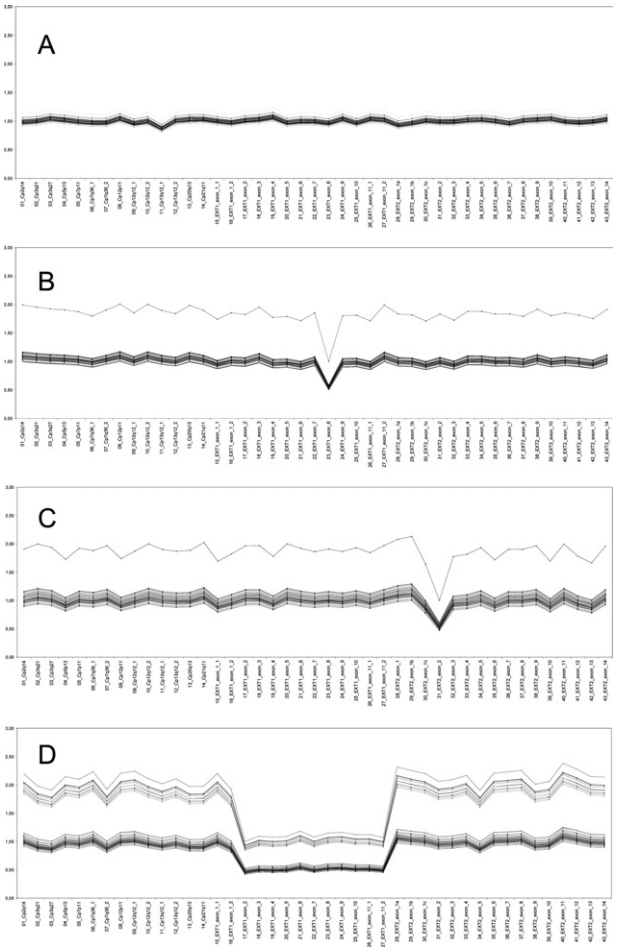

Figure 1. Example of the MLPA curve of *EXT* deletion-negative sample (**A**), *EXT1* exon 8 deletion (family number 200) (**B**), *EXT2* exon 2 deletion (family number 150) (**C**), and *EXT1* exon 2-11 deletion (family number 147) (**D**). Lines represent the series of dosage coefficients (DQ) for each probe ($DQ_{amp\ n/amp\ n+1}$ = [sample (peak area amplicon *n*/peak area amplicon *n* + 1)]/[control (peak area amplicon *n*/peak area amplicon *n* + 1)].

For the mutation-negative group, stature data were available for only two patients, with both below the 50th percentile and one of them below the 10th percentile.

Discussion

In the past, mutation screening of MO patients was based on direct sequencing or less-sensitive screening methods such as single-strand conformation polymorphism. To facilitate and reduce the cost of this mutation screening, optimization of a DHPLC-based protocol for all *EXT1* and *EXT2* coding exons has been described.[17,20] After all, most MO patients have point mutations or small deletions or insertions of a few bp in one of both genes and the *EXT1* and *EXT2* coding regions contain very little polymorphisms, making them very suitable for DHPLC analysis. However, because mutation screening was performed almost exclusively at the sequence level, quantitative (deletions and duplications) and positional (inversions and translocations) changes were not detected by this technique. To complement DHPLC screening, MLPA and/or FISH[16] and RNA analysis can be performed. MLPA is a quick and simple technique for quantitative analysis. It is based on the ligation of two probes that hybridize to adjacent sites of the target sequence. All ligated probes have common end sequences, permitting simultaneous PCR amplification of all target sequences using only one primer pair. The resulting PCR products can be separated according to size and be quantified.[18] In this study, a new MLPA assay (MLPA-P215) was validated and used for screening of MO patients that did not show an aberrant DHPLC profile. We found this new MLPA probe set to produce more reliable and reproducible results compared to a previously developed two-color MLPA for the *EXT1* and *EXT2* genes.[19] This latter MLPA is composed of chemically synthesized oligonucleotide probes that are restricted in length. As a consequence, the various probes differ only minimally in size, complicating the analysis. MLPA-P215, however, was designed with cloned probes, allowing the construction of larger probes and more size difference between the individual probes, resulting in improved results. When MLPA-P215 was validated using 10 negative and 22 positive control samples, no false-positive results were obtained for negative control samples, and all mutations in positive control samples were sensitively identified. These observations lead to a theoretical sensitivity and specificity of both 100% in our validation.

Mutation screening for alterations in *EXT1* and *EXT2* was performed on a set of 63 MO families, resulting in the identification of 48 disease-causing mutations (Figure 2). Forty-three mutations were identified using DHPLC (90%); four deletions and one translocation were detected with FISH/MLPA (10%). No disease-causing genetic alteration was detected in 15 families (24%). This gave an overall mutation detection rate of 76% in 63 families, in accordance with previous studies.[21–23] Thirty-six families (57%) harbored an *EXT1* mutation, whereas only 12 families (19%) had an *EXT2* mutation, giving a mutation frequency ratio of 75% for *EXT1* versus 25% for *EXT2*. The observed higher frequency of *EXT1* mutations is in agreement with previous mutation studies performed in western populations.[20,21,24,25] For *EXT1* we found a frame shift in 11 families, a nonsense mutation in 6 families, and a splice site mutation in 8 families. In seven families a missense mutation was found. Next to these small mutations, three deletions were identified as well as one translocation. Frameshift mutation c.1469delT was found in two families as well as missense mutations c.992C>A (p.A331D) and c.1685T>C (p.L562P). Three families were diagnosed with the missense mutation c.1019G>T (p.R340L). The c.992C>A (p.A331D) and c.1685T>C (p.L562P) mutations have not been described before but there are several arguments in favor of a pathogenic effect. They were found both absent in our control population (>100 chromosomes). Alanine 331 is conserved between the EXT1-EXT2-EXTL1 proteins and between several species (human-mouse-*Xenopus*). Theoretical prediction programs SIFT and Polyphen predict this variant to be pathogenic. Unfortunately only the proband of families 204 and 220 were available for analysis. Also leucine 562 is highly conserved in all members of

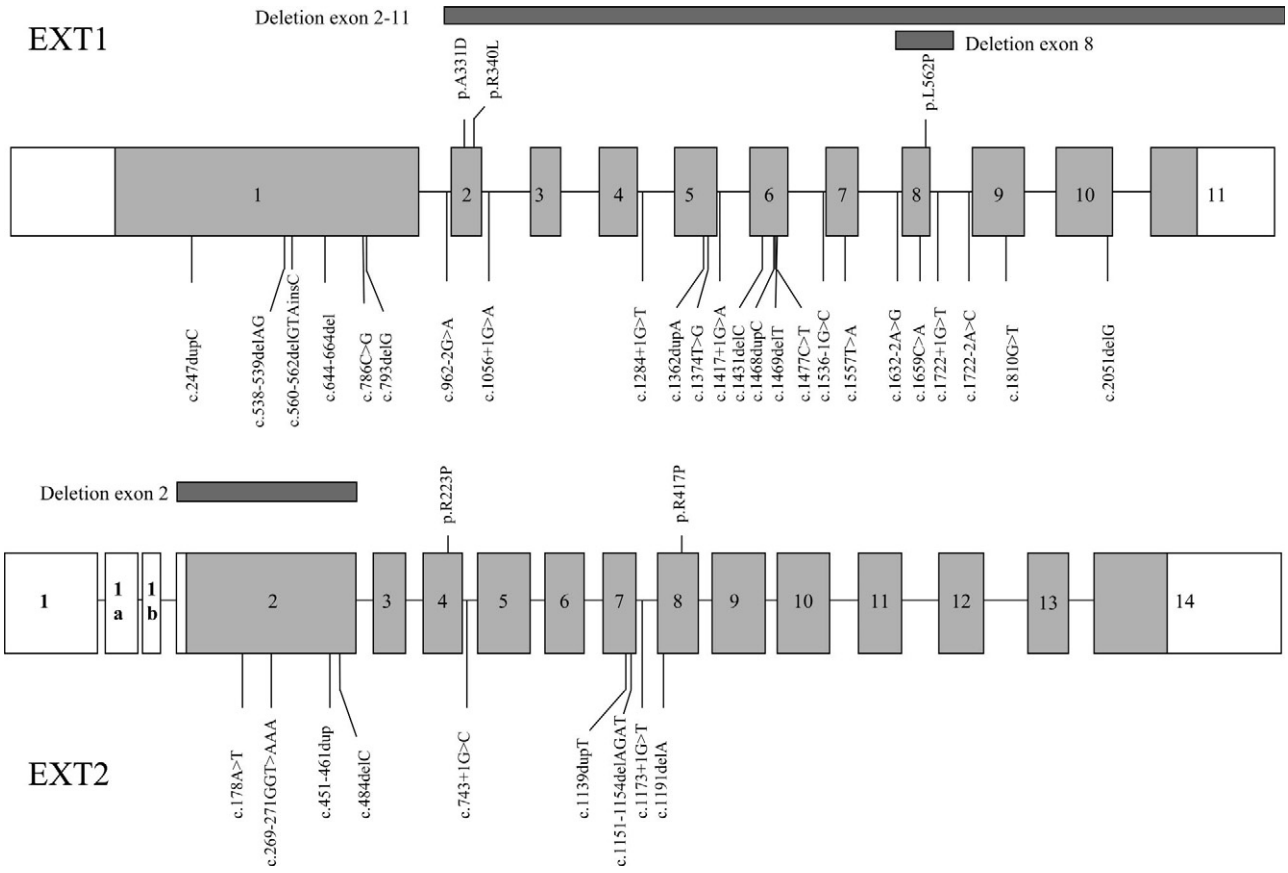

Figure 2. Distribution of mutations detected in the *EXT1* gene (**top**) and *EXT2* gene (**bottom**) in this study.

the EXT/EXTL protein family and between human and *Drosophila*. SIFT and Polyphen also predict the c.1685T>C (p.L562P) variant to be pathogenic. In family 178 both proband and affected father showed the c.1685T>C (p.L562P), which was absent in the nonaffected sibling. In family 191 both parents of the proband patient were reported unaffected, but the c.1685T>C (p.L562P) was also found in the father of the patient. Detailed clinical examination needs to be performed for further clarification and evaluation. The c.1019G>T (p.R340L) missense mutation has previously been shown to result in impaired EXT1 function.[26] In the *EXT2* families

Table 3. Positive Control Samples Used for the Validation of MLPA-P215

Family number	Mutation at DNA level	Detection technique	Reference
87	Deletion *EXT1*	FISH	Unpublished results
Italy_3	Deletion *EXT1*	Real-time PCR	Unpublished results
99	Deletion *EXT1*, exon 1	FISH	Unpublished results
132	Deletion *EXT1*, exon 1	2-color MLPA	Unpublished results
Italy_6	Deletion *EXT1*, exon 1	Real-time PCR	Unpublished results
Italy_5	Deletion *EXT1*, exon 8	DHPLC and sequencing analysis	Unpublished results
Italy_7	Deletion *EXT1*, exon 8	DHPLC and sequencing analysis	Unpublished results
Italy_4	Deletion *EXT1*, exon 2-5	Real-time PCR	Unpublished results
89	Deletion *EXT1*, exon 2-11	FISH	Unpublished results
128	Deletion *EXT1*, exon 2-11	2-color MLPA	Unpublished results
147	Deletion *EXT1*, exon 2-11	FISH	Unpublished results
Italy_1	Duplication *EXT1*, exon 4	Real-time PCR	Unpublished results
Italy_2	Duplication *EXT1*, exon 4	Real-time PCR	Unpublished results
8	Deletion *EXT2*	FISH	[26]
Defect 11_1	Deletion *EXT2*	FISH	[27]
Defect 11_2	Deletion *EXT2*	FISH	[27]
Defect 11_3	Deletion *EXT2*	FISH	[27]
Defect 11_4	Deletion *EXT2*	FISH	[27]
Italy_8	Deletion *EXT2*	Real-time PCR	Unpublished results
Italy_9	Deletion *EXT2* exon 1-5	Real-time PCR	Unpublished results
Italy_10	Deletion *EXT2* exon 1-5	Real-time PCR	Unpublished results
125	Deletion *EXT2* exon 1-10	2-color MLPA	Unpublished results

five frame shifts, two nonsense, two splice site, and two missense mutations were found. In one family an *EXT2* exon 2 deletion was present. The two missense mutations identified were c.668G>C (p.R223P, family 202) and c.1250G>C (p.R417P, family 198). Both were absent in our control population (>100 chromosomes) and are conserved between *EXT1* and *EXT2* and between several species from human to *Drosophila*. SIFT and Polyphen predict both variants to be pathogenic. c.668G>C (p.R223P) has been described before in MO patients[27] and was also found to segregate with MO in family 202 (two affected and three unaffected individuals). For family 198, only the proband was available for analysis. In total, ~70% of all identified genetic aberrations were truncating mutations, whereas missense mutations represented ~20% and deletions and translocations were responsible for MO in ~10% of cases. Additionally, our finding of four deletions in a cohort of 63 families (6%) confirmed the suggestion of White and colleagues[19] that in a series of MO patients one can expect to find a deletion of one or more exons in ~5 to 8% of cases. We were able to characterize the deletion breakpoint in detail for one family harboring an *EXT2* exon 2 deletion. Analysis of the deletion breakpoint regions did not show the presence of low copy repeats nor did these two regions share homology. The mechanism behind the deletion event therefore remains unknown.

Of the 48 mutations identified, 34 mutations (71%) were found only once in our cohort of MO families and had never been reported before. The identification of several novel private mutations confirms the strong allelic heterogeneity of the *EXT1* and *EXT2* genes in MO patients. In addition to the private mutations, we identified some mutations located in previously reported mutation hot spots that might represent functional sites in the *EXT1* and *EXT2* genes. Mutation c.668G>C (p.R223P), found in one family, is located in the *EXT2* region between amino acids 211 and 230, which has been reported to be a MO mutation hot spot.[23,27–29] Two families were diagnosed with deletion 1469delT in exon 6 from *EXT1*, which was already mentioned to be located in a mutation hot spot by Francannet and colleagues.[25] Finally, missense mutation c.1019G>T (p.R340L) in *EXT1* exon 2 was found in three families and is known to be a recurrent missense mutation in a region that harbors key elements for EXT1 function.[8,23,26,28,30]

In 24% of cases no mutation could be identified with the performed multistep mutation screening. Several plausible reasons exist as to why disease-causing mutations could not be detected, including mutations that may occur in introns, regulatory regions, or promoter regions of *EXT1* and *EXT2*. A recent study however, suggests that the latter is not a frequent cause of MO because no promoter mutations were found in any of the EXT1- and EXT2-negative MO patients of a large British-Caucasian cohort.[21] Also, the existence of causative mutations located in another MO-causing gene cannot be excluded. To explain the relatively high percentage of patients without an identified mutation, one has to consider that it is also uncertain whether in all cases the phenotype of the patients fully matches the clinical picture of MO. MO can for example be confused with other skeletal disorders affecting multiple bones, such as metachondromatosis.[22] Multidisciplinary re-evaluation of the radiographical and histological material by bone tumor experts is advised in these cases to exclude misdiagnosis. Interestingly, for the majority of mutation-negative patients (11 of the 15) there was no familial history reported. It is possible that these patients harbor MO causing somatic mutations, which cannot be detected in peripheral blood.

To study the genotype-phenotype correlation, the phenotype was reviewed for all 63 families in whom a mutation was identified. Detailed clinical descriptions were available for 34 EXT1, 6 EXT2 patients, and 5 mutation-negative patients. Previous studies suggested a more severe phenotype associated with *EXT1* mutations,[17,25,31] although this could not always be confirmed.[32] We also could not confirm this observation in our cohort with none of the differences in evaluated parameters (number of osteochondromas, $P = 1.000$; deformities, $P = 1.000$; presence of secondary complications, $P = 0.149$; and stature ≤ P10, $P = 1.000$) being statistically significant. Also when making an evaluation between EXT1 patients, EXT2 patients and patients without an identified mutation, no significant differences could be found for the same parameters (number of osteochondromas,; $P = 1.000$; deformities, $P = 0.813$; presence of secondary complications, $P = 0.067$; and stature ≤ 10th percentile, $P = 1.000$). Possibly, the population of especially EXT2 patients with a clinical overview was too small to make relevant conclusions about the genotype-phenotype correlation. The same parameters were evaluated for patients with truncating mutations versus patients with missense mutations. No significant difference could be identified (number of osteochondromas, $P = 1.000$; deformities, $P = 0.068$; presence of secondary complications, $P = 0197$; and stature ≤ P10, $P = 1.000$). Most of the patients in our study were index patients, and only a small number of relatives were included in the clinical study. This may have created a bias toward more severely affected patients in all groups because they tend to go for genetic testing more rapidly. We are aiming to expand our clinical data by including more relatives and pooling larger cohorts of patients to evaluate this further. In conclusion we provided a sensitive molecular screening strategy with improved deletion analysis for the *EXT1* and *EXT2* genes applicable for MO mutation analysis.

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