



Mutations in the *EXT1* and *EXT2* genes in Spanish patients with multiple osteochondromas

SUBJECT AREAS:
GENETIC CAUSE OF DISEASE
BONE CANCER
METABOLIC DISORDERS
BONE DEVELOPMENT

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Multiple osteochondromas is an autosomal dominant skeletal disorder characterized by the formation of multiple cartilage-capped tumours. Two causal genes have been identified, *EXT1* and *EXT2*, which account for 65% and 30% of cases, respectively. We have undertaken a mutation analysis of the *EXT1* and *EXT2* genes in 39 unrelated Spanish patients, most of them with moderate phenotype, and looked for genotype-phenotype correlations. We found the mutant allele in 37 patients, 29 in *EXT1* and 8 in *EXT2*. Five of the *EXT1* mutations were deletions identified by MLPA. Two cases of mosaicism were documented. We detected a lower number of exostoses in patients with missense mutation versus other kinds of mutations. In conclusion, we found a mutation in *EXT1* or in *EXT2* in 95% of the Spanish patients. Eighteen of the mutations were novel.

Multiple osteochondromas (MO, MIM#133700, #133701), also known as multiple hereditary exostoses, is an autosomal dominant skeletal disorder characterized by the formation of multiple cartilage-capped tumours (exostoses or osteochondromas). The prevalence of MO is estimated at 1/50,000 in the Western population¹. Osteochondromas are the result of excessive chondrocyte proliferation and bone growth at the juxtaepiphyseal regions of long bones and are the most common benign bone tumour. MO is characterized by an important inter- and intra-familial phenotypic variability, including variations in the number and size of osteochondromas and in the number and location of bones involved. Secondary complications are heterogeneous too, and may consist of deformities, functional limitations, compression of nerves and blood vessels, pain caused by pressure on neighbouring tissues and short stature². The most serious secondary complication is the malignant transformation toward a secondary peripheral chondrosarcoma, occurring in 0.5–5% of patients³.

MO is a genetically heterogeneous disease. In almost 90% of MO patients, germline mutations in *EXT1* (MIM #608177)⁴ or *EXT2* (MIM #608210)^{5,6} are found. *EXT1* maps to chromosome 8q24.11–q24.13, comprises 11 exons and spans approximately 350 kb⁷, while *EXT2*, located at 11p11–p11.2, consists of 16 exons and spans almost 108 kb⁸. Both genes act as tumour suppressors. Involvement of other genes has been suggested, since there are families with no mutation identified in either *EXT1* or *EXT2*.

The *EXT* genes encode ubiquitous glycosyltransferases, catalyzing heparan sulfate (HS) polymerization at HS proteoglycans (HSPGs). These HSPGs play a major role in the diffusion of signalling molecules such as Indian Hedgehog (Ihh), which is an important regulator of chondrocyte proliferation and differentiation in the growth plate⁹.

Approximately 10% of patients have a *de novo* mutation¹⁰. Loss of the wild-type allele in hereditary cases indicates that inactivation of both *EXT* alleles is required for osteochondroma formation¹¹, confirming their tumour suppressor action that results in a loss of chondrocyte polarization¹². However, the inactivation of both alleles probably occurs only in some of the cells in the cartilaginous cap of osteochondromas^{12,13}.

Several studies have reported on MO causing mutations in different populations. These are being gathered in the Multiple Osteochondromas Mutation Database (MOdb) (<http://medgen.ua.ac.be/LOVDv.2.0/>), currently listing over 400 and 200 different mutations in *EXT1* and *EXT2*, respectively. Most of the mutations (80%) are nonsense, frame-shift and splice-site mutations, resulting in a premature termination of translation, or involve partial or total deletion of the gene. Although it has been suggested that mutations in *EXT1* are associated with a



more severe phenotype than mutations in *EXT2*^{14–16}, many aspects of the phenotypic variability observed in patients have yet to be understood at the genetic level. As suggested by the Human Variome Project initiative, characterization of causative mutations in familial and sporadic cases in diverse populations is needed for full understanding of Mendelian diseases¹⁷.

Here we present the mutational analysis of 39 unrelated Spanish MO patients and the clinical features of most of them. This is the first report of MO mutations in the Spanish population.

Results

Clinical features. The main clinical features of the patients are detailed in Table 1. Most of the patients (77%) had deformities (classes II and III). More than half the cases (51%) had more than 20 exostoses. Mean age of onset was 2 years and clinical evaluation was performed, on average, 12 years later. Only one of the 39 cases developed a malignant transformation.

***EXT1* and *EXT2* point mutations.** On sequencing all exons and flanking regions of the *EXT1* and *EXT2* genes in the samples of 39 unrelated patients, 31 pathogenic point mutations were identified (Table 2). Twenty-four different mutations were found in the *EXT1* gene, while 7 were found in *EXT2*. One of them (*EXT2*, c.544C > T) was found in two unrelated patients. Eighteen out of the 31 mutations were novel, two of them missense in *EXT1*: p.Asp231Val and p.Pro337Arg. Bioinformatic predictions suggested a pathogenic role for both of them [PolyPhen, probably_damaging (1); SIFT, deleterious (0)]. The mutation screening also detected two changes that were interpreted as non-pathogenic (*EXT1*, c.962+8_962+11delTCTG and *EXT2*, c.1178G > A), indicated in italics in Table 2. These two changes were found in patients BCN33 and BCN29, respectively, together with additional mutations, as indicated in Figure 1 and Table 2. In the case of BCN33, the patient inherited the *EXT1*, c.962+8_962+11delTCTG change

from his unaffected mother, while the other mutation, *EXT2*, c.544C > T (p.Arg182*), was putatively inherited from his affected father (from whom a sample was not available). Patient BCN29 inherited the *EXT2*, c.1178G > A (p.Arg393Gln) change from her unaffected father, while she presented with a *de novo*, previously described mutation in the *EXT1* gene: c.1019G > C (p.Arg340Pro). After sequencing and thorough manual checking of chromatograms, seven patients remained undiagnosed at the molecular level.

***EXT1* deletions.** Exon dosage for *EXT1* and *EXT2* was assessed by MLPA analysis of the seven samples without identified point mutations. Five unrelated patients (BCN06, BCN07, BCN19, BCN20/25 and BCN22) were found to bear different *EXT1* deletions (Figure 2 and Table 2). No large deletion was found in the *EXT2* gene. The proband from family 20/25 was hemizygous for all *EXT1* exons (i.e., he presented with half a dose), while probands BCN06 and BCN07 (the latter not shown in Figure 2) were hemizygous for exons 2 to 11, and patient BCN19 was hemizygous only for exon 8. In contrast, patient BCN22 showed a partial loss of dose for exons 2 to 11, consistent with a mosaic constitution (see Figure 2). Relatives of patient BCN06, including the affected father, were available for MLPA examination. As seen in Figure 2, the father had a partial loss of dose of exons 2 to 11, compared with the unaffected relatives (proband's mother and brother), consistent with a case of mosaicism for the deletion present in his son. For two cases (BCN21 and BCN28) no mutation was found after sequencing and MLPA analyses.

Phenotype-genotype correlations. We examined the *EXT1* and *EXT2* mutations in relation to the various phenotypic aspects shown in Table 1. Gene distribution within the three clinical classes showed a higher proportion of *EXT2* mutations in class III than in classes I and II (Figure 3A). No significant differences were found when comparing patients with *EXT1* and *EXT2* mutations for

Table 1 | Clinical and genetic data of the 39 patients with multiple osteochondromas

	All patients n = 39	With <i>EXT1</i> mutations n = 29 (74.4%)	With <i>EXT2</i> mutations n = 8 (20.5%)	No mutation n = 2 (5.1%)
Clinical class				
I	7 (18.9%)	6 (21.4%)	1 (14.3%)	0
II	17 (45.9%)	14 (50.0%)	2 (28.6%)	1 (50.0%)
III	13 (35.1%)	8 (28.6%)	4 (57.1%)	1 (50.0%)
NA	2	1	1	0
Number of exostoses				
≤5	3 (8.1%)	3 (10.7%)	0	0
6–20	14 (37.8%)	9 (32.1%)	4 (57.1%)	1 (50.0%)
>20	20 (54.1%)	16 (57.1%)	3 (42.9%)	1 (50.0%)
NA	2	1	1	0
Malignant transformation	1	1	0	0
Gender				
Male	24 (61.5%)	17 (58.6%)	5 (62.5%)	2 (100%)
Female	15 (38.5%)	12 (41.4%)	3 (37.5%)	0
Disease onset				
Range	0.5–5	0.5–5	0.5–3	2
Mean (SD)	2 (1.53)	2 (1.60)	1 (1.21)	2 (0)
Lumbar densitometry				
Impaired	6 (33.3%)	4 (28.6%)	2 (50%)	-
Normal	12 (66.6%)	10 (71.4%)	2 (50%)	-
NA	21	15	4	2
Type of mutation				
Missense	6 (15.4%)	6 (20.7%)	0	-
Other	31 (79.5%)	23 (79.3%)	8 (100%)	-
Not found	2 (5.1%)	0	0	2

NA: not available.

Table 2 | Mutations in the *EXT1* and *EXT2* genes

Gene	Exon or intron	cDNA change	Protein change	Reference	Family
<i>EXT1</i>	Exon 1	c.208C > T	p.Gln70*	Present study	BCN01
	Exon 1	c.228_229delCA	p.Ser76Serfs*111	Present study	BCN36
	Exon 1	c.294C > A	p.Cys98*	Present study	MAD03
	Exon 1	c.369_370delA	p.Glu125Argfs*11	Present study	BCN40
	Exon 1	c.551G > A	p.Trp184*	Present study	MAD04
	Exon 1	c.552G > A	p.Trp184*	Present study	BCN31
	Exon 1	c.692A > T	p.Asp231Val	Present study	BCN11
	Exon 1	c.793delG	p.Val265Tyrfs*8	Jennes et al. ²¹	BCN09 [†]
	<i>Intron 1</i>	c.962+8_962+11delTCTG	Unknown	<i>Signori et al.</i> ²³	BCN33
	Exon 2	c.967_972del	p.Asp323_Tyr324del	Present study	BCN39
	Exon 2	c.1010C > G	p.Pro337Arg	Present study	BCN35
	Exon 2	c.1016G > A	p.Gly339Asp	Philippe et al. ²⁸	BCN15
	Exon 2	c.1019G > C	p.Arg340Pro	LOVD ^{&}	BCN29
	Exon 2	c.1019G > A	p.Arg340His	Raskind et al. ²⁹	MAD01
	Exon 2	c.1021A > G	p.Arg341Gly	LOVD ^{&}	BCN26
	Intron 2	c.1057-3C > G	Unknown	LOVD ^{&}	MAD02
	Intron 2	c.1057-2A > C	Unknown	Present study	BCN32
	Intron 3	c.1164+1G > A	Unknown	Present study	BCN38
	Exon 4	c.1261A > T	p.Lys421*	Present study	BCN14
	Exon 6	c.1468delC	p.Leu490Argfs*9	<i>Signori et al.</i> ²³	BCN42
	Exon 6	c.1469delT	p.Leu490Argfs*9	Ahn et al. ⁴	BCN41
	Exon 8	Deletion exon 8	p.Val545_Glu574del	Jennes et al. ²¹	BCN19
	Intron 9	c.1883+2T > G	Unknown	Seki et al. ³⁰	BCN05
	Exon 10	c.1896C > G	p.Tyr632*	Heinritz et al. ³¹	BCN27
	Exon 10	c.1896C > A	p.Tyr632*	Lonie et al. ³²	BCN37
	Exon 10	c.2051_2053del3insA	p.Gly684Glufs*10	Present study	BCN03
Exon 1-11	Exon 1-11 deletion	Unknown	Jennes et al. ²¹	BCN20/25	
Exon 2-11	Exon 2-11 deletion	Unknown	Jennes et al. ²¹	BCN06; BCN07	
<i>EXT2</i>	Exon 2-11	Mosaic deletion	Unknown	Szuhai et al. ²⁴	BCN22 [#]
	Exon 2	c.415_416delGA	p.Asp139Glnfs*2	Present study	BCN17
	Exon 2	c.424_425insT	p.Tyr142Leufs*4	Present study	BCN34
	Exon 3	c.540G > A	p.Trp180*	Present study	BCN04
	Exon 3	c.544C > T	p.Arg182*	Dobson-Stone et al. ²⁵	BCN18; BCN33
	Exon 5	c.783_789del7	p.His262Serfs*6	Present study	BCN10
	Exon 6	c.1073G > A	p.Trp358*	LOVD ^{&}	BCN30
	<i>Exon 8</i>	c.1178G > A	<i>p.Arg393Gln</i>	<i>Present study</i>	BCN29
	Exon 8	c.1278T > A	p.Tyr426*	Present study	BCN13

In italics: non-pathogenic variants (see text and Figure 1 for details).

[†]Patient with malignant transformation.

[&]<http://medgen.ua.ac.be/LOVDv.2.0/>.

[#]A similar mosaic deletion was found in the father of patient BCN06.

age of disease onset or number of osteochondromas. In *EXT1* patients, we compared the group of 6 individuals bearing missense mutations with 18 patients carrying other type of mutations (nonsense and small or large insertions and deletions) in relation to the number of exostoses. As shown in Figure 3B, a significant difference was found (mean number of exostoses for missense =

14.33; SD = 8.24; mean for other = 26.50; SD = 12.66; $p = 0.04$). However, no significant difference in the age of onset of the disease was found between these two groups (not shown). Lumbar densitometry was performed in 18 patients: results showed impairment in 6 cases, which harboured non-missense mutations, 4 in *EXT1* gene and 2 in *EXT2* gene.



Figure 1 | Segregation analysis in the BCN33 and BCN29 families, in which the probands were found to bear two mutations each, one in *EXT1* and one in *EXT2*. Italics indicate mutations considered non-pathogenic. NA: DNA not available.



BCN06 family

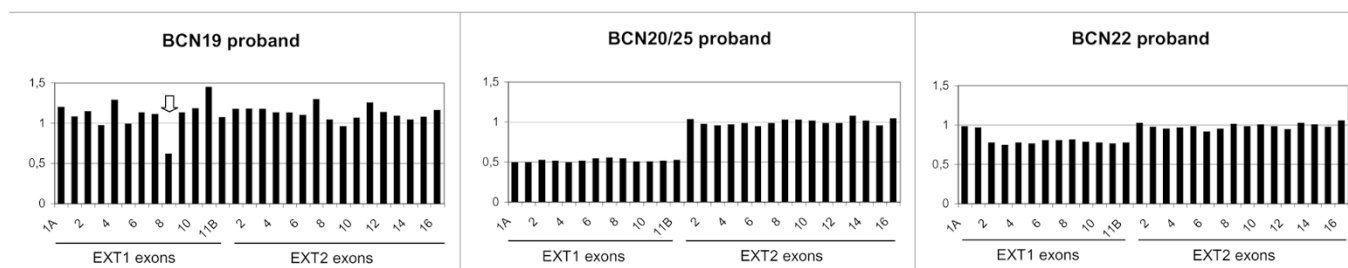
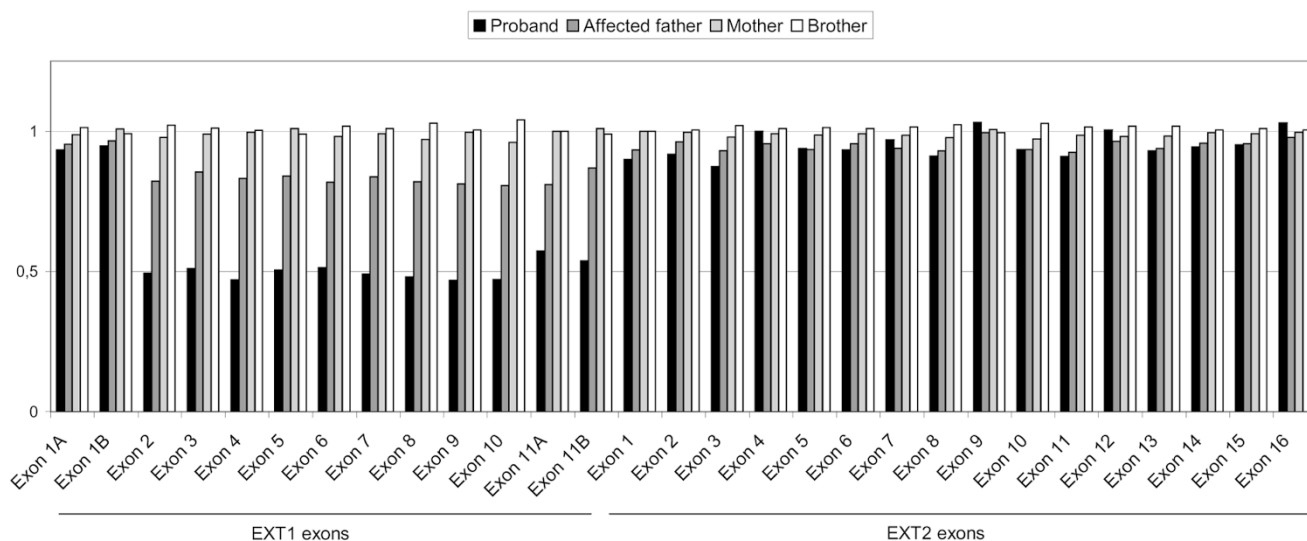


Figure 2 | MLPA results for some of the patients bearing deletions of different size in the *EXT1* gene. In the Y axis, 1 and below 0.6 correspond to full dose (two copies) and half dose (one copy) of the corresponding exons, respectively. For the BCN06 family, results for different members of the family are shown. Arrow in BCN19 proband indicates the deletion of exon 8.

Patients without identified mutations in *EXT1* or *EXT2*. Details of the two unrelated patients (BCN21 and BCN28) with no identified *EXT1* or *EXT2* mutations are included in Table 1. Patient BCN21 was a son of an unaffected couple, while BCN28 inherited the disease from his father. Both presented with a number of exostoses and an onset age that are within the range of the rest of the patients.

Discussion

Thirty-nine Spanish unrelated patients were analyzed and the mutant allele was identified in 37 of them. Twenty-nine patients

(74%) had mutations in *EXT1*, while 8 (21%) had mutations in *EXT2* (Figure 4A). Only two patients remained undiagnosed at the molecular level. Genotype-phenotype correlations were analysed. Patients bearing *EXT1* missense mutations correlated with a lower number of exostoses.

The greater number of *EXT1* mutations is in agreement with most other studies of different populations (Figure 4A)^{10,14,18,21–23}. The proportion of missense mutation (15.4%) agrees with the large study by Pedrini et al.¹⁸. All the missense mutations were in *EXT1* and 5 out of the 6 clustered in residues 337–341, while the novel missense

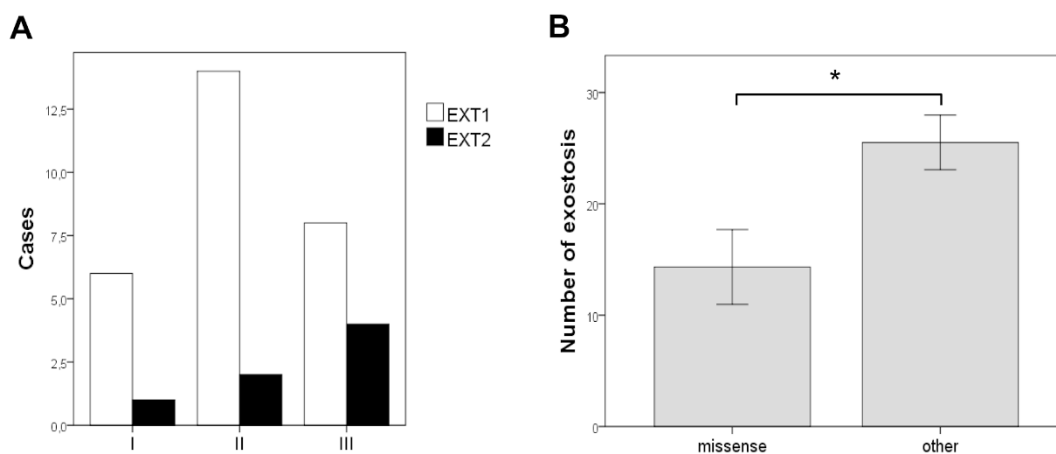


Figure 3 | Genotype-phenotype correlations. (A) Distribution of cases with mutations in *EXT1* or *EXT2* among the three clinical classes. (B) Average number of exostoses among patients bearing missense mutations in *EXT1* ($n = 6$) or other type of mutations (nonsense and small or large insertions and deletions) in the same gene ($n = 18$).

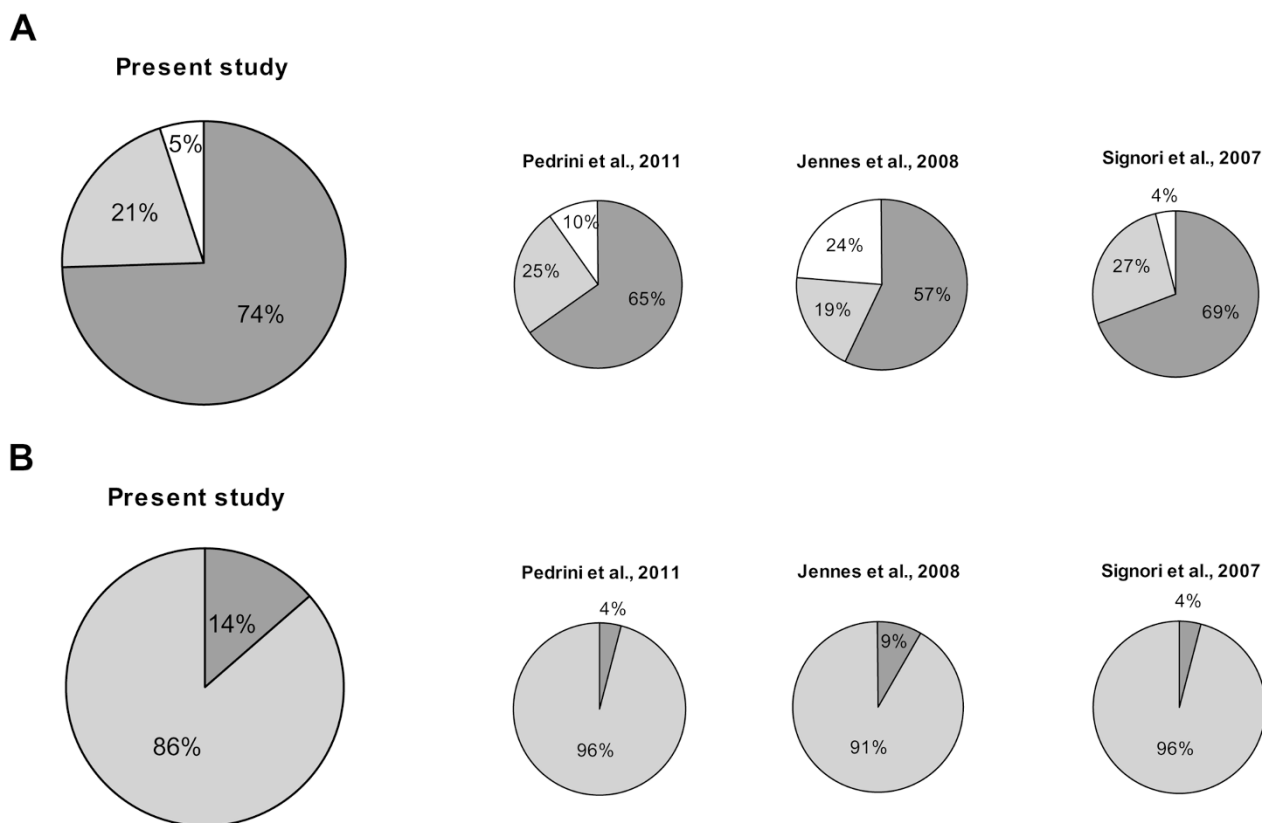


Figure 4 | Comparison of mutation frequencies with previous studies. (A) Proportion of *EXT1* mutation cases (dark grey), *EXT2* mutation cases (light grey) and cases with no mutation identified (white). (B) Among cases with identified mutations, proportion of large rearrangements (at least one exon) are shown in dark grey, while point mutations and small insertions and deletions are shown in light grey.

mutation (p.Asp231Val), lay outside this region. Five probands (14% of the cases) were found to carry partial or whole *EXT1* deletions. This proportion is higher than that found by Jennes et al.²¹ and Signori et al.²³ (Figure 4B). All these deletions had been previously described²¹. However, whether the breakpoints in all cases are the same or not remains to be studied. Only one publication has addressed this issue and found that two cases with deletion of exons 2 to 11 of *EXT1* (and two cases with an exon 8 deletion in *EXT2*) bore different breakpoints²¹.

We found two cases of mosaicism for the exon 2–11 deletion, which deserve further discussion. In one case, the patient (BCN06) bore the deletion, while the father, from whom he had inherited the disease, was discovered to be a mosaic thanks to the previous finding of the son's mutation. The other case (BCN22) showed results that were consistent with a deletion, although the MLPA values for exons 2 to 11 were above the threshold for hemizyosity (see Figure 2). These cases are similar to those described by Szuhai et al.²⁴. Both mosaic cases (BCN06f and BCN22) had an early disease onset (2 years) and neither was affected less than the average (BCN22 belongs to class IIB). This suggests that mosaic mutations play a role in the pathology: it seems that the deletion of one allele (or of a large part of it) in a relatively small number of cells is enough to trigger the phenotype. However, mosaicism was observed in blood cells, while the actual status in bone remains to be studied.

Two patients bore two changes each, one in *EXT1* and one in *EXT2*. In patient BCN33, the *EXT2* nonsense mutation is undoubtedly pathogenic, has been previously described²⁵ and was also found in another patient in our series (BCN18). On the other hand, the pathogenic effect of the donor splice site deletion in intron 1 of *EXT1* is dubious. It was described as a pathogenic mutation by Signori et al.²³, in an Italian MO patient, and was absent in 100 Spanish control chromosomes. However, the splice score was not affected

by the change (data not shown). A novel putative donor site generated by the 4-bp deletion has a very low score, making its use unlikely. Moreover, the patient inherited the disease from his father (Figure 1B) and there are several affected relatives on the paternal side. However, the patient inherited the intron 1 4-bp deletion from his healthy mother (and there are no affected relatives on the mother's side). The best explanation with the available data is that the 4-bp deletion in intron 1 is a rare non-pathogenic variant. Patient BCN29 inherited the *EXT2* c.1178G > A (p.Arg393Gln) from her unaffected father (Figure 1B). Additionally, she presented with a *de novo* *EXT1* mutation: c.1019G > C (p.Arg340Pro). This mutation has been previously described by several authors and reviewed by Jennes et al.¹⁰. That the father had the *EXT2* p.Arg393Gln change and is unaffected and that the other mutation was shown to be pathogenic strongly suggest that the former is a non-pathogenic variant. Protein sequence alignments show that *Drosophila melanogaster* bears a Gln at this position (data not shown). Additionally, the p.Arg393Gln change was found in the Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: <http://evs.gs.washington.edu/EVS/>) [June, 2012] with a frequency of 16/13,004 (0.12%).

At the genotype-phenotype level, our data showed that the class with more severe clinical presentations (class III) had a higher proportion of *EXT2* mutations than the other classes. However, it must be noted that this may be due to a relatively small number of patients with *EXT2* mutations in our study, all of whom bore truncating mutations. When the average number of exostoses in patients with different types of mutations was compared, a significant difference ($p < 0.05$) was detected between missense mutations and other types of mutation (nonsense and small or large insertions and deletions). Patients with missense mutations had fewer osteochondromas than the rest. A hypothesis to explain this is that some residual activity of the mutant protein may remain only in the case of mutations of



amino acid change, which may be enough to produce a small amount of heparan sulfate. This is in agreement with the continuum model suggested by Berger et al.²⁶ which states that protein function can be a continuum related to the level of expression or activity of the tumour suppressor genes rather than to discrete step-by-step changes in gene copy number. One patient out of 39 developed a malignant tumour (2.6%). This figure is within the range of 2–5% described by several authors (see, for example, Bovee³; Pedrini et al.¹⁸). There were two other patients in whom no disease-causing mutation could be identified (5.1%). This proportion is lower than that found in most studies, including the large one by Pedrini et al.¹⁸. There are different explanations for the undetected mutations, including a possible third locus, the presence of mosaic point mutations gone undetected or the presence of mutations in unexplored regions such as the promoter, recently characterized by Jennes et al.²⁷, or deep intronic.

Overall, our phenotype-genotype correlation results do not agree with those of Pedrini et al.¹⁸, which consisted of an association of a mild phenotype with female sex, *EXT2* mutations and absence of *EXT1/2* mutations. As stated above, the main limitation of our study was the small sample size. Comparisons between groups had not enough statistical power and we could not detect differences, for example, in the association between sex and the severity of the disease. The main challenge, therefore, will be to collect more cases to further investigate the genotype-phenotype correlation in Spanish patients.

To conclude, this is the first genetic study of MO performed in a Spanish cohort. We described a collection of mutations in *EXT1* and *EXT2* and it was possible to offer diagnosis and genetic counselling to the MO patients and their families. This is an essential first step, in order to gain insight into the bases of the disease and then to develop novel strategies in the search for possible therapies.

Methods

Patients. In this study we investigated patients from 39 Spanish families with MO. Diagnosis, performed at 2 centres (Hospital Sant Joan de Déu, Barcelona, $n = 35$ families, and Hospital Universitario "La Paz", Madrid, $n = 4$ families), was based on the presence of MO confirmed by physical and/or radiographic examinations. Blood samples were obtained from patients and available relatives for genomic DNA extraction, after informed consent. Samples from patients were obtained in accordance with the Helsinki Declaration, as revised in 2000. The experiments were approved by the Ethics Committee of Hospital Sant Joan de Déu.

Clinical studies. A clinical diagnosis of MO was achieved after obtaining an accurate medical history and physically examining the patient, including the evaluation of all palpable lesions, height, long bone and trunk deformities, and functional limitations.

Patients were classified in three clinical classes based on the presence of deformities and functional limitations (I: no deformities and no functional limitations; II: deformities and no functional limitations; and III: deformities and functional limitations), as reported elsewhere¹⁸. The subcategories were defined by the number of osteochondromas (A: less than 20; B: more than 20).

Genotyping and mutation analysis. Genomic DNA mutation screening of the *EXT1* and *EXT2* genes was performed for each patient. New primers were designed to amplify all exons and flanking intronic regions of both the *EXT1* and the *EXT2* genes, so that all fragments, except those corresponding to exon 1 of *EXT1*, could be amplified by polymerase chain reaction (PCR) simultaneously. Primers are listed in on-line Supplementary Table S1. Exon 1 of *EXT1* and exon 4 of *EXT2* were split into several overlapping fragments, to obtain amplification products that did not exceed 650 bp. PCR was performed in a 50 μ l reaction volume, containing ~100 ng of genomic DNA, 1 \times PCR buffer, 1–2 mM MgCl₂, 0.2 mM of each dNTP, 0.4 μ M of each forward and reverse primer and 0.7 U of GoTag[®] Flexi polymerase (Promega, Madison, WI). All PCR programs included an initial denaturation of 4 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at annealing temperature (Ta) and 10 sec at 72°C. Finally, an extension at 72°C was performed for 5 min. Annealing temperature was 60°C for all primer combinations, with the exception of primers for the amplification of overlapping regions of exon 1 of *EXT1*. For these primer combinations, Ta was set at 55°C for ex1.1 and 57°C for ex1.2 and ex1.3. After amplification, the PCR products were purified using a PCR purification kit (GE Healthcare) and then sequenced with BigDye 3.1 (Applied Biosystems; life technologies) in the following PCR programme: denaturation 1 min at 96°C, 25 cycles of 10 sec at 96°C, 5 sec at 55°C and 4 min at 60°C. The sequences were analyzed with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems life technologies). The presence of all the mutations detected was confirmed by digestion of each PCR product with the corresponding enzyme. None of the novel mutations

were found in 50 control samples. All chromatograms of *EXT1* and *EXT2* exons of negative cases were manually re-analyzed.

The mutations were given the official HGVS nomenclature (www.hgvs.org). As reference sequences, NM_000127.2 for *EXT1* and NM_000401.3 for *EXT2* were used.

MLPA. The number of copies of the *EXT1* and *EXT2* genes present in genomic DNA samples was analyzed by the multiplex ligation-dependent probe amplification (MLPA) technique designed by MRC-Holland. We used the commercial kit #P215-B1 and followed the manufacturer's instructions. PCR products were run on an ABI 3730 DNA Analyzer capillary sequencer (Applied Biosystems, Forster City, CA, USA). Peaks were analyzed by means of the Coffalyser v9.4 software. The proportion of each peak relative to the height of all peaks was calculated for each sample and then compared to proportions for the corresponding peak averaged for a set of at least ten normal DNA samples. Ratios of 1.0 were treated as normal copy number. Ratios below 0.6 were considered as deletions. Each positive result was confirmed in a second independent MLPA reaction.

Assessment of functionality of missense mutations. Disease causing potential of missense mutations was evaluated using two different bioinformatic tools: *SIFT* and *PolyPhen*^{19,20}.

Statistical analysis. Normal distribution of the data and variance differences were assessed by the Kolmogorov-Smirnoff and Lavenne tests, respectively. Assessment of mean differences was performed using ANOVA. A p value <0.05 was considered significant. The statistical analysis used the PASW 18.0 program.

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Additional information

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