A broad spectrum of genomic changes in Latin American patients with EXT1/EXT2-CDG

M. A. Delgado1, G. Martínez-Domenech1, P. Sarrión2, R. Urreizti2, L. Zechini3, H. H. Robledo4, F. Segura5, R. Dodelson de Kremer1, S. Balcells2, D. Grinberg2 & C. G. Asteggiano1,6,7

1Centro de Estudio de las Metabolopatías Congénitas (CEMECO), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Hospital de Niños de la Santísima Trinidad, Córdoba, Argentina, 2Universitat de Barcelona, IBUB, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Departament de Genètica, Facultat de Biologia, Barcelona, España, 3Servicio de Traumatología, Hospital de Niños de la Santísima Trinidad, Córdoba, Argentina, 4Servicio de Bioimágenes, Hospital de Niños de la Santísima Trinidad, Córdoba, Argentina, 54th Cátedra de Ortopedia y Traumatología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina, 64th Cátedra de Farmacología, Facultad de Medicina, Universidad Católica de Córdoba, Argentina, 7Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Multiple osteochondromatosis (MO), or EXT1/EXT2-CDG, is an autosomal dominant O-linked glycosylation disorder characterized by the formation of multiple cartilage-capped tumors (osteochondromas). In contrast, solitary osteochondroma (SO) is a non-hereditary condition. EXT1 and EXT2 are tumor suppressor genes that encode glycosyltransferases involved in heparan sulfate elongation. We present the clinical and molecular analysis of 33 unrelated Latin American patients (27 MO and 6 SO). Sixty-three percent of all MO cases presented severe phenotype and two malignant transformations to chondrosarcoma (7%). We found the mutant allele in 78% of MO patients. Ten mutations were novel. The disease-causing mutations remained unknown in 22% of the MO patients and in all SO patients. No second mutational hit was detected in the DNA of the secondary chondrosarcoma from a patient who carried a nonsense EXT1 mutation. Neither EXT1 nor EXT2 protein could be detected in this sample. This is the first Latin American research program on EXT1/EXT2-CDG.
was shown to have a significant effect on \textit{EXT1} promoter activity (the C-allele resulting in a 56% rise in promoter activity) compared to the G-wild-type allele\textsuperscript{16}. The presence of an additional MO-causing gene has been proposed to explain the absence of an \textit{EXT1} or \textit{EXT2} mutation in a small percentage of MO patients (15–30\%)\textsuperscript{17,18,24}. To date, more than 600 different \textit{EXT1} and 345 \textit{EXT2} mutations have been found worldwide and an update on all reported mutations is deposited at http://medgen.ua.ac.be/LOVD\textsuperscript{20}.

This study represents the first Latin American research program in MO, with a broad spectrum of genomic changes detected, including 10 novel pathogenic mutations identified in \textit{EXT1}/\textit{EXT2}-CDG patients. Twenty-one different mutant alleles in the \textit{EXT1} or \textit{EXT2} genes were found in a cohort of 27 MO patients, most of them with a severe phenotype, including two patients with malignant transformation to chondrosarcoma. No mutation was found in six MO patients after performing sequencing and MLPA analyses.

**Results**

**Phenotypic characterization.** We observed multiple osteochondromas in 27 out of 33 patients, who ranged from 3 to 55 years at diagnosis. Orthopedic deformities of the forearm, shortening of limbs, ankle, varus or valgus of the knee, short metacarpal bones, scoliosis, synostosis, arthritis, and vessel or nerve compression were some common manifestations. The lesions were located in the femur (71\%), tibia (67\%), humerus (67\%), fibula (62\%), radius (52\%) and pelvis (29\%), a frequent site of malignant transformation to chondrosarcoma. Phenotypic data were available for 78\% of the MO patients (n = 21), of whom four presented with a moderate phenotype (15\% of all MO patients) and 17 with a severe presentation of the disease (63\% of all MO patients) (Table 1, Figure 1A). A severe phenotype ranging from grade IS to IVS was observed in most of the MO patients (Figure 1B). Seventy-six percent of them presented an age of onset below 5 years and 59\% manifested familial inheritance (Table 1). Two patients developed malignant transformation as a large chondrosarcoma on the pelvis that led to severe vascular and organ compression: P06, a 32 year-old female with a type IV severe phenotype, reported by Delgado et al. and P38, a 42 year-old male with a type IIS severe phenotype (Table 1)\textsuperscript{22}.

**Gene sequence and dose analyses of \textit{EXT1} and \textit{EXT2} exons.** Exons and flanking regions of the \textit{EXT1} and \textit{EXT2} genes were sequenced from the genomic DNA of the 33 patients and MLPA analysis was performed in DNA samples of those with negative results for sequencing analysis. The mutant allele was found in 78\% of the MO patients including one patient with solitary presentation (P36) (Table 1). We identified 21 pathogenic mutations, 15 in \textit{EXT1} and 6 in \textit{EXT2} (five nonsense, six frame-shift, four missense, three splice-site mutations, and three large deletions identified by MLPA) listed in Table 2. Six of the \textit{EXT1} mutations were novel (p.Val78Glyfs*111, p.Leu264Pro, p.Lys306*, p.Arg346Thr, c.1164 + 1C > A, and p.Gln583Arg) as were four of the \textit{EXT2} mutant alleles (p.Asp307Valfs*45, p.Trp394*, p.Asp539Glnfs*5 and a deletion of exon 4 to 14).

Bioinformatic predictions for the \textit{EXT1} missense mutations suggested a pathogenic role for these genomic changes. In particular, the p.Leu264Pro change was considered “\textit{disease causing}” by Mutation Taster (score: 0.999, amino acid sequence changed, protein features might be affected with potential luminal loss and splice site changes) and “\textit{probably damaging}” by PolyPhen2 (score: 0.997 sensitivity: 0.27; specificity: 0.98), while ESE Finder predicted an increased level of the enhancer splicing proteins SF2/ASF (score changed from −0.21685 to 1.24048), and SF2/ASF (score changed from 0.497 to 1.75265). The novel missense mutation, p.Arg346Thr, change from a basic amino acid (Arg) to a non-polar one (Thr) and it was predicted to be “\textit{disease causing}” by Mutation Taster (score 0.999, amino acid sequence changed, protein features might be affected with potential luminal loss and splice site changes) and “\textit{probably damaging}” by PolyPhen2 (score: 0.993, sensitivity: 0.47; specificity: 0.96). ESE Finder predicted diminished levels of enhancer splicing protein SRSF2 (SC35). The other novel \textit{EXT1} missense mutation, p.Gln583Arg, is a change from an uncharged polar amionic acid (Gln) to a basic one (Arg) and it was predicted to be “\textit{disease causing}” by Mutation Taster (score: 0.999, amino acid sequence changed, protein features might be affected with potential luminal loss and splice site changes) although PolyPhen2 predicted it to be “\textit{benign}” (score: 0.002 sensitivity: 0.99; specificity: 0.30). The Protein Homology Fold Recognition Engine Phyre2, (http://www.sbg.bio.ic.ac.uk/ phyre2/html, last accessed March 2014) was used to predict the effect of missense mutations on 3D structure and the missense mutation p.Arg346Thr removes two fragments of alpha helix between aa 345 and 347, and from aa 635 to 639, and a beta sheet from aa 361 to 368 in \textit{EXT1} protein. The p.Leu264Pro mutation adds an alpha helix structure from aa 161 to 166 and removes an alpha helix from aa 344 to 346, while removing a beta sheet structure from aa 360 to 365 and introducing a segment of beta sheet from aa 724 to 726. The other novel missense mutation, p.Gln583Arg removes two fragments of alpha helix between aa 39 and 41, and from aa 635 to 639 in the \textit{EXT1} protein.

**In silico analyses for one novel intronic mutation (c.1164 + 1 G > A) predicted the use of cryptic donor splice sites: Human Splice Finder (http://www.umd.be/HSF/), last accessed March 2014), considered the use of a cryptic donor splice site (score: 91.85%) located 74 nucleotides downstream from the wild-type sequence, while NetGene2 (http://www.cbs.dtu.dk/cgi-bin/webface/jobid=netgene2/ last accessed March 2014) predicted the use of a cryptic donor splicing site (score: 0.76) 201 nucleotides downstream from the wild-type (score:0.83).

In three patients large deletions were detected by MLPA (Tables 1 and 2). In \textit{EXT1} (exon 1, P36) and a deletion of 11 exons (6–16) in (P12), and the third one was a deletion in \textit{EXT2} (exon 6, P04). Normal MLPA profiles were obtained for 19 patients.

No mutation was found in 12 cases (6 MO and 6 SO) after performing sequencing and MLPA analyses. Most of these patients did not have a positive family history of osteochondromatosis (Table 1).

**Analysis of the \textit{EXT1} promoter.** We sequenced 435 bp upstream of the \textit{EXT1} gene including the 123-bp region described to contain the basic promoter elements\textsuperscript{26} in samples from patients and 9 controls, but no mutation was detected. We found that four patients (P18, P21, P34 and P41), and one control individual, were heterozygous carriers of the C-allele of SNP rs34016643, which has been previously shown to have a significant effect on \textit{EXT1} promoter activity, with the C-allele resulting in a 56\% rise in promoter activity compared to the G (wild-type) allele\textsuperscript{36}. No pathogenic mutation was identified in \textit{EXT1} or \textit{EXT2} in three of these four patients, while patient P41 bore a nonsense mutation (c.1219C > T, p.Gln407*) in exon 4 of the \textit{EXT1} gene (Table 1).

**Loss of heterozygosity analysis in a chondrosarcoma.** We had access to a chondrosarcoma sample from P06. We have detected the heterozygous p.L283* mutation in \textit{EXT1} in the tumor sample. We further analysed both genes by MLPA and we did not detect any dose alteration in the chondrosarcoma from this patient. The patient was heterozygous for the single nucleotide polymorphism rs11546829 in exon 3 of the \textit{EXT1} gene. Loss of heterozygosity for this marker was not observed in the analysis of DNA in the tumor tissue.

**Discussion**

This work represents the first clinical, biochemical and molecular research on multiple hereditary osteochondromatosis (\textit{EXT1}/\textit{EXT2}-CDG) in Latin American patients. Thirty-three unrelated patients
### Table 1 | Overview of EXT1 and EXT2 mutations and the phenotype found in this cohort

| Patient | Sex | Gene | DNA | Deduced protein change | EXT1 promoter SNP r.s340,166,43 | MLPA | Family History | Phenotype | Age of onset | Other clinical features |
|---------|-----|------|-----|-------------------------|--------------------------------|------|----------------|------------|--------------|------------------------|
| P01     | male | EXT2 | c.1182G > A   | p.Thr394*                  | Wt                              | NA   | No             | MO/IIIS    | 1.5 y-o      | Vertebral location     |
| P02     | fem  | EXT1 | c.1469delT    | p.Leu490Argfs*9            | Wt                              | NA   | No             | MO/IIIS    | 5 y-o        | Surgery/Sinostoses     |
| P03     | fem  | ND   | .............. | ..........................   | Wt                              | Normal | No            | SO         | 3 y-o        | Exostoses in humerus   |
| P04     | male | EXT2 | ex 6 del     | Unknown                    | Wt                              | Abnormal | No            | MO/IIIS    | 5 m          | Shortening of limbs    |
| P05     | male | EXT1 | c.152delT     | p.Leu52*                   | Wt                              | NA   | Yes            | MO/IVS     | 4 y-o        | Surgery/Axial deviations (cubito and radius) |
| P06     | fem  | EXT1 | c.848T > A    | p.Leu283*                  | Wt                              | Normal | Yes            | MO/IVS     | 12 y-o       | Chondrosarcoma /Surgery |
| P07     | fem  | ND   | .............. | ..........................   | Wt                              | Normal | No            | SO         | 6 y-o        | Surgery            |
| P08     | fem  | EXT1 | c.1037G > C   | p.Arg346Thr                | Wt                              | NA   | No             | MO/IIIS    | 3 y-o        | Scholiosis           |
| P09     | male | EXT2 | ex 4-14del    | Unknown                    | Wt                              | Abnormal | Yes            | MO/IVS     | 2 m          | Surgery/Scapular and ribs location. Abnormal karyotype (18q deletion) |
| P10     | fem  | EXT1 | c.920_929del10insTGG | p.Asp307Valfs*45          | Wt                              | NA   | Yes            | MO/IIIS    | 2 m          | Scapular osteochondromas |
| P11     | fem  | EXT1 | c.369_370delAG | p.Lys126Asnfs*62           | Wt                              | Normal | Yes            | MO/IS      | 1 y-o        | Deformity of the heel  |
| P12     | male | EXT1 | c.916A > T    | p.Val78Glyfs*111           | Wt                              | NA   | No             | MO/M       | 8 y-o        | Decreased bone density |
| P13     | male | EXT2 | c.626 + 1G > A | --                          | Wt                              | Normal | Yes            | MO/NA      | 4 y-o        | Ribs location         |
| P14     | fem  | EXT1 | c.791T > C    | p.Leu264Pro                | Wt                              | NA   | No             | MO/NA      | 1 y-o        | Ribs location         |
| P15     | ND   | EXT2 | .............. | ..........................   | G/C                             | Normal | No            | SO         | 14 y-o       | NA                   |
| P16     | ND   | EXT2 | c.626 + 1G > A | --                          | Wt                              | Normal | Yes            | MO/NA      | 8 y-o        | NA                   |
| P17     | ND   | EXT2 | c.1164 + 1G > A | --                          | Wt                              | Normal | No             | MO/NA      | 2 y-o        | Surgery/Shortening and deformities of limbs |
| P18     | ND   | EXT2 | c.1164 + 1G > A | --                          | Wt                              | Normal | Yes            | MO/NA      | 10 y-o       | Surgery/Shortening and deformities of limbs |
| P19     | ND   | EXT2 | c.1722 + 1G > A | --                          | Wt                              | Normal | No             | MO/IVS     | 1 m          | Scoliosis            |
| P20     | ND   | EXT1 | c.248_249insA | p.Gln84Alafs*105           | Wt                              | NA   | No             | MO/NA      | 3 y-o        | Bilateral valgus, vertebral |
| P21     | ND   | EXT1 | .............. | ..........................   | Wt                              | Normal | Yes            | MO/NA      | 10 y-o       | Bilateral valgus, vertebral |
| P22     | ND   | EXT1 | c.1164 + 1G > A | --                          | Wt                              | Normal | Yes            | MO/NA      | 5 m          | Distrophy in ribs     |
| P23     | ND   | EXT1 | .............. | ..........................   | Wt                              | Normal | Yes            | MO/NA      | 10 y-o       | Chondrosarcoma/Severe vascular compression, phlebitis |
| P24     | ND   | EXT1 | c.1164 + 1G > A | --                          | Wt                              | Normal | Yes            | MO/NA      | 2 m          | Scapular osteochondromas |
| P25     | ND   | EXT1 | c.1722 + 1G > A | --                          | Wt                              | Normal | No             | MO/NA      | 12 y-o       | Deformity of ankles    |
| P26     | ND   | EXT1 | c.1164 + 1G > A | --                          | Wt                              | Normal | No             | MO/NA      | 2 m          | Pelvic and Scapular Osteochondromas |
| P27     | ND   | EXT1 | c.1722 + 1G > A | --                          | Wt                              | Normal | No             | MO/NA      | 1 y-o        | Pelvic and Scapular Osteochondromas |

Novel mutations are indicated in bold.

1 Patients with malignant transformations to chondrosarcoma.

2 (Wt) wild type = G/G. (ND) No mutation detected by sequencing and MLPA analysis; (NA) Not Available; (S) Severe phenotype; (M) Mild phenotype; (MO) Multiple osteochondroma; (SO) Solitary osteochondroma.
were studied, 27 of which presented with MO. The mutant allele was identified in 21 of these patients (78%). 

EXT1 mutations (71%) were more common than EXT2 mutations (29%) and most of the EXT1 mutations were located in the first six exons. These results are consistent with recent studies reporting that EXT1 is responsible for 65–75% of MO cases20,27.

The 67% of EXT1 mutations (10/15), were located in exon 1 or 2, which encode the exostosin domain of the EXT1 protein (from amino acid 111 to 396). Most of these patients presented a severe phenotype (67%). Twenty per cent of EXT1 mutations (n = 3) were located in the glycosyltransferase domain (from amino acid 480 to 729) (Table 2). In contrast, EXT2 mutations (n = 6) were more frequent in the last exons. Thirty three per cent of the EXT2 mutations (n = 2) were found in the exons that encode the glycosyltransferase domain (from exon 10 to 14) (Table 2). The structure of the different EXT1 and EXT2 protein domains was analyzed using Phyre2 to predict a decrease or loss of protein function according to the detected mutations and the altered structure of protein Figure 1| Genotype–phenotype association in MO patients (n = 27). (A) Graph showing the proportion of severe phenotype (blue), mild phenotype (red) and patients with phenotype not available (green) and the distribution of EXT1 and EXT2 mutations or no mutations identified (NM) within each category. (B) Grade of phenotype severity among severely affected patients and distribution of EXT1 and EXT2 mutations or no mutations identified (NM) within each category.

| Gene | Patient | Exon-Intron | DNA | Deduced protein change | Mutation Type | Publication |
|------|---------|-------------|-----|------------------------|---------------|-------------|
| EXT1 | P37     | Ex 1        | c.248_249insA, | p.Gln84Alafs*105 | Frameshift    | Francannet, et al 2001 |
| EXT1 | P15     | Ex 1        | c.232dupG       | p.Val78Glufs*111 | Frameshift    | Ciavarella, et al, 2013 |
| EXT1 | P14     | Ex 1        | c.369_370delAG  | p.Lys126Asufs*62 | Frameshift    | Ciavarella, et al, 2013 |
| EXT1 | P05     | Ex 1        | c.752delT       | p.Leu251*       | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P17     | Ex 1        | c.791T>C        | p.Leu251*       | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P06'    | Ex 1        | c.848T>A        | p.Leu283*       | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P16     | Ex 1        | c.916A>T        | p.Lys306*       | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P36     | Ex 1        | ex1del          | Unknown         | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P40     | Ex 2        | c.1018C>T       | p.Arg340Cys     | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P08     | Ex 2        | c.1037G>C       | p.Arg346Thr     | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P28     | In 3-4      | c.1164 + 1G > A | --              | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P41     | Ex 4        | c.1219C>T       | p.Gln407*       | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P02     | Ex 6        | c.1469delT      | p.Leu490Argfs*9 | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P29     | In 8-9      | c.1722 + 1G > A | --              | Frameshift    | Delgado, et al, 2012 |
| EXT1 | P25     | Ex 9        | c.1748A>G       | p.Gln583Arg     | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P19     | In 3-4      | c.626 + 1G > A  | --              | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P13     | Ex 5        | c.920_929del10insTG | p.Asp307Valfs*45 | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P04     | Ex 6        | exdel           | Unknown         | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P01     | Ex 8        | c.1182G>A       | p.Trp394*       | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P27     | Ex 10       | c.1616_1623del8ins10 | p.Asp539Glnfs*5 | Frameshift    | Delgado, et al, 2012 |
| EXT2 | P12     | Ex 4-14     | ex4-14del       | Unknown         | Frameshift    | Delgado, et al, 2012 |

Table 2 | List of mutations in EXT1 or EXT2 gene in MO patients

Novel mutations are indicated in bold. We considered as new mutations those not published and/or not mentioned in the LOVD databases. LOVD: http://medgen.ua.ac.be/LOVDv.2.0/

*Patient with malignant transformation to chondrosarcoma.
domains32 (Protein Homology Fold Recognition Engine, http:// www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Regarding the 3-D prediction for two novel EXT1 missense mutations, p.Leu264Pro and p.Arg346Thr, the Phyre2 bioinformatic tool showed that both mutations produce impairment in protein folding or alterations in the exostosin or glycosyltransferase domains. For the third one, p.Gln583Arg, Phyre2 showed the removal of alpha helix structures with possible alterations in EXT1 glycosyltransferase domain. A type II severe phenotype (IIIS) was found in patients carrying the two first missense mutations (P17 and P08), and a severe phenotype (IIIS) was observed in P25 carrying the p.Gln583Arg mutation.

Six out of the 14 mutations in the EXT1 gene (p.Val78Glyfs*111, p.Leu264Pro, p.Lys306*, p.Arg346Thr, c.1164 + 1C > A and p.Gln583Arg) and four of the six EXT2 mutant alleles (p.Asp307Valfs*45, p.Trp394*, p.Asp359Glnfs*5, and exon4-14del) were novel. Although some mutation hotspots have been reported20 (http://medgen.ua.ac.be/LOVDv2.0/), we did not observe recurrent EXT1 or EXT2 mutations in patients in this study. The missense mutation c.1018C > T (p.Arg340Cys) observed in P40 and the exon 6 deletion c.1469delT (p.Leu490Argfs*9) found in P02 were previously described to cause the impairment of heparan sulfate synthesis20,37.

The MLPA analysis in gDNA of patient P36, showed the complete deletion of EXT1 exon 1. This patient had previously been reported as an SO case because he only had one lesion, but this detection in germline DNA allow us to change the diagnosis into MO patient with very mild symptoms (only a single osteochondroma lesion)30 (Table 1 and 2). Two molecular defects in EXT2 were detected by MLPA: the deletion of exon 6 (P04) and of 10 exons (from exon 4 to exon 14) in patient P12. This patient also carried an abnormal karyotype (an 18 q deletion).

Splice-site mutations were detected in EXT1 in two patients. One of them, c.1164 + 1G > A in intron 3, was a novel mutation observed in patient P28. This novel mutation was analyzed in silico and the use of alternative cryptic donor sites was predicted. The phenotype in these patients was severe, presenting deformity of the limbs, valgus, restricted joint movement and scoliosis. Furthermore, one splice site mutation previously described was detected in P19 in EXT2. Clinical data were not available for this patient39. There are several possible explanations for the lack of identification of mutations in some of the MO patients (22%). The mutation may have been in the EXT1 or EXT2 genes but in regions that were not analyzed. We did not look for mutations in deep intronic regions or in the 5’ and 3’ UTR sequences. Instead, the promoter region was genotyped and no mutation was detected. A recent study described a regulatory role for a G/C SNP (rs3401643) located at position ~1158 bp, within a USF1 transcription factor binding site30. These authors observed that the presence of the C-allele resulted in a ~56% increase in EXT1 promoter activity. The effect of this allele in the four patients of the present study who are heterozygous for it will require further studies. It is well established that methylation of cytosine residues in the promoter region leads to transcription repression in tumor suppressor genes; nevertheless this does not seem to be the case for EXT1 and EXT2 promoters in osteochondromas or in chondrosarcomas44,45. Finally, the possible existence of other genes responsible for MO should also be considered. A putative EXT3 gene, located on the short arm of chromosome 19, has been proposed to explain the absence of an EXT1 or EXT2 mutation in a small percentage of MO patients (15–30%). Nevertheless, the existence of this third locus is generally accepted to be a false linkage result.

Inactivating mutations in the EXT1 and EXT2 genes were previously reported as the most common event in MO patients resulting in the formation of non-functional EXT1 or EXT2 proteins with a variable degree of expression in tissues34,35. We observed 11 truncating mutations that create premature stop codons presenting a high grade of severity in patient’s phenotype (Table 1 and 2). One of these patients (P06) presented malignant transformation to chondrosarcoma and we detected the p.Leu283* mutation in the EXT1 gene. Very low or null levels of EXT1 and EXT2 proteins were detected by Western blot in this patient. However, in these experiments, the bands corresponding to GAPDH (control protein) were very weak and the lack of additional sample precluded repetition. We think that in spite of the technical problems, this observation should be reported to allow comparisons with other studies. Obviously, further cases should be analyzed to confirm these findings. The loss of EXT2 protein suggests that EXT1 mutations probably interfere with the function of exostosin’s complexes in the Golgi, inactivating the holoenzyme, degrading the whole protein, or interfering in some other function in the Golgi34.

Several studies have suggested that MO patients present a more severe phenotype due to EXT1 mutations than EXT2 mutations16,18,21 while other studies could not confirm this observation23,36. Pedrini et al 2011 recently performed a genotype–phenotype association study in a large cohort of MO patients and identified some specific correlation according to a new clinical classification system34. Our patients presented some of the most common manifestations, including orthopedic deformities of the forearm, ankle, varus or valgus of the knee, arthritis, vessels and nerve compression and very short stature (below the third percentile). The bones most often affected were tibia, femur, radius, humerus and fibula. Nevertheless, we observed a severe phenotype (12% type IS, 53% type IIS, 12% type IIIS and 23% type IVS) in 63% of MO patients (Figure 1), the remaining 15% presented with a moderate phenotype without a family history of the disease. We observed that the grade of severity differed between the proband and other affected members in the family, according to previously reported intra-familial variabilities18,39. Nevertheless, no family history for MO was reported in 56% of MO patients. Patients with a mutation in the EXT2 gene showed a smaller number of affected bones (data not shown) consistent with a recent study40. The most frequently observed skeletal deformations in our patients were shortening of limbs, varus or valgus knee, short metacarpal bones, scoliosis, shortened stature and synostosis, with no evidence of differences between the grade of severity in the phenotype observed in patients with EXT1 or EXT2 (Figure 1). Genotype–phenotype correlations are difficult to establish in MO patients because most of the EXT1 and EXT2 variants are private mutations20.

Malignant transformation to a chondrosarcoma is the most important complication in MO, and has been estimated to occur in 0.5–5% of patients32. Patients P06 and P38 developed malignant tumors, which gives a frequency of malignant transformation of 7% in our cohort of patients. Patient P06 bore the pathogenic mutation c.848T > A (p.Leu283*) in the first exon of the EXT1 gene, while no mutation was detected in P38, neither in EXT1 nor EXT2 (Table 1). It has been shown that hereditary osteochondromas and secondary chondrosarcomas are associated with a second mutational hit in the EXT genes46,47. We thus investigated this possibility in DNA extracted from the osteochondroma tissue resected from P06 by Sanger sequence and MLPA but we found no evidence of a somatic mutation as a second hit in any of these genes. The presence of genetic rearrangements at the EXT1 and EXT2 loci (as the second mutational hit) in P06 osteochondromas and secondary chondrosarcomas was ruled out.

In conclusion, we have identified the disease-causing mutation in 21 out of 27 MO patients, including 10 mutations described for the first time. No mutation was identified in SO cases. Structural analyses predicted a disruption of important domains of EXT1 proteins bearing missense mutations. A potentially functional promoter polymorphism was found in three patients with no other mutation, in one patient with a disease-causing mutation and in one control. No second hit was identified in a sample from a chondrosarcoma. Further studies are needed to identify the molecular bases of the
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Author contributions
Study design: S.B., D.G., C.G.A. Collection and data samples: M.A.D., G.M.-D., P.S., L.Z., H.H.R., F.S. Performance of experiments: M.A.D., G.M.-D., C.G.A. Data interpretation and analysis: M.A.D., G.M.-D., P.S., R.U., R.D.-K., S.B., D.G., C.G.A. Draft composition: M.A.D., G.M.-D., S.B., D.G. C.G.A. conceived of the study, and participated in its design, coordination and helped to draft the manuscript. All authors reviewed the manuscript.

Additional information
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