SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
DISEASE GENETICS
CANCER GENOMICS

Received
7 January 2014

Accepted
22 July 2014

Published
18 September 2014

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

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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.

Multiple osteochondromatosis (MO; MIM# 133700, 133701), also known as *EXT1/EXT2*- CDG in the Congenital Disorder of Glycosylation (CDG) nomenclature[1,2] is an autosomal dominant disease. MO is genetically heterogeneous and 70–90% of patients present mutations in one of two genes: *EXT1* (MIM 608177) (8q24.11-q24.13)[3] or *EXT2* (MIM 608210) (11p12-p11)[4,5]. Both are ubiquitously expressed tumor-suppressor genes of the EXT gene family. All members of this gene family have been cloned encode glycosyltransferases involved in the adhesion and/or polymerization of heparan sulfate chains (HS)[6–10].

Heparan sulfate proteoglycans (HSPG) are ubiquitously expressed at cell surfaces and in extracellular matrices. They are composed of a core protein and one or more heparan sulfate glycosaminoglycan chains (linear polysaccharides formed by alternating N- acetylated or N-sulfated glucosamine units and uronic acid) that interact with numerous proteins, including growth factors, morphogens and extracellular matrix proteins[11]. Each HS binds to a serine unit of a proteoglycan core protein via *O*-linked-xylosylation binding[11,12]. The truncated HSPG disturb specific growth-factor–binding in chondrocytes, resulting in abnormal signaling and altered endochondral ossification, thus leading to MO[13].

MO is characterized by the formation of multiple cartilaginous tumors (osteochondromas), that mainly affect the metaphyses of long bones or the surface of flat bones[14–18]. Complications may involve bone and surrounding tissue deformities, fractures or mechanical joint problems, vascular compression, arterial thrombosis, aneurysm, pseudoaneurysm formation, and venous thrombosis. Pain, acute ischemia, signs of phlebitis or nerve compression occur alongside the most severe complication, the malignant transformation of osteochondroma to secondary peripheral chondrosarcoma (0.5–5% of patients)[16–23]. *EXT1* and *EXT2* have been analysed using different techniques to search for point mutations and structural alterations. Intragenic deletions involving single or multiple exons of *EXT1* or *EXT2* genes have been found in about 10% of cases[24–29]. Additionally, the promoter of *EXT1* was analysed in some cases. The *EXT1* core promoter region was reported to map to approximately −917 bp upstream of the *EXT1* start codon, within a 123-bp region[30]. One SNP within this region, rs34016643,

was shown to have a significant effect on *EXT1* promoter activity (the C-allele resulting in a 56% rise in promoter activity) compared to the G-wild-type allele[30]. The presence of an additional MO-causing gene has been proposed to explain the absence of an *EXT1* or *EXT2* mutation in a small percentage of MO patients (15–30%)[17,31,32]. To date, more than 600 different *EXT1* and 345 *EXT2* mutations have been found worldwide and an update on all reported mutations is deposited at http://medgen.ua.ac.be/LOVD)[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 *EXT1*/*EXT2*-CDG patients. Twenty-one different mutant alleles in the *EXT1* or *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)[22].

Gene sequence and dose analyses of *EXT1* and *EXT2* exons. Exons and flanking regions of the *EXT1* and *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 *EXT1* and 6 in *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 *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 *EXT2* mutant alleles (p.Asp307-Valfs*45, p.Trp394*, p.Asp539Glnfs*5 and a deletion of exon 4 to 14).

Bioinformatic predictions for the *EXT1* missense mutations suggested a pathogenic role for these genomic changes. In particular, the p.Leu264Pro change was considered "*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 "*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 0.4979 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 "*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 "*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 *EXT1* missense mutation, p.Gln-583Arg, is a change from an uncharged polar amionacid (Gln) to a basic one (Arg) and it was predicted to be "*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 "*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 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 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 *EXT1* (exon 1, P36) and a deletion of 11 exons (6–16) in (P12), and the third one was a deletion in *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 *EXT1* promoter. We sequenced 435 bp upstream of the *EXT1* gene including the 123-bp region described to contain the basic promoter elements[30] 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 *EXT1* promoter activity, with the C-allele resulting in a 56% rise in promoter activity compared to the G (wild–type) allele[30]. No pathogenic mutation was identified in *EXT1* or *EXT2* in three of these four patients, while patient P41 bore a nonsense mutation (c.1219C > T, p.Gln407*) in exon 4 of the *EXT1* gene (Table 1).

Loss of hetereozygosity analysis in a chondrosarcoma. We had access to a chondrosarcoma sample from P06. We have detected the heterozygous p.L283* mutation in *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 *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 (EXT1/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 rs34016643	MLPA	Family History	Phenotype	Age of onset	Other clinical features
P01	male	*EXT2*	**c.1182G > A**	p.Trp394*	Wt	NA	No	MO/IIIS	1,5 y-o	Vertebral location
P02	fem	*EXT1*	c.1469delT	p.Leu490Argfs*9	Wt	NA	No	MO/IIS	5 y-o	Surgery/Sinostoses
P03	fem	ND	....	....	Wt	Normal	No	SO	5 y-o	Exostoses in humerous
P04	male	*EXT2*	ex 6 del	Unknown	Wt	*Abnormal*	No	MO/IIS	5 m	Shortenining of limbs
P05	male	*EXT1*	c.752delT	p.Leu251*	Wt	NA	Yes	MO/IVS	4 y-o	Surgery/Axis deviations (cubito and radious)
P06[1]	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/IIS	3 y-o	Scholiosis
P12	male	*EXT2*	**ex 4-14del**	Unknown	Wt	*Abnormal*	Yes	MO/IVS	2 m	Surgery/Scapular and ribs location. Abnormal karyotype (18 q deletion)
P13	male	*EXT2*	**c.920_929del10insTG**	p.Asp307Valfs*45	Wt	NA	Yes	MO/IIS	2 m	Scapular osteochondromas
P14	fem	*EXT1*	c.369_370delAG	p.Lys126Asnfs*62	Wt	Normal	Yes	MO/IS	1 y-o	Deformity of the heel
P15	male	*EXT1*	**c.232insG**	p.Val78Glyfs*111	Wt	NA	No	MO/M	8 y-o	Decreased bone density
P16	male	*EXT1*	**c.916A > T**	p.Lys306*	Wt	NA	No	MO/M	4 y-o	Restricted joint motion
P17	male	*EXT1*	**c.791T > C**	p.Leu264Pro	Wt	NA	Yes	MO/IIS	1 m	Ribs location
P18	male	ND	....	....	G/C	Normal	No	SO	14 y-o	NA
P19	male	*EXT2*	c.626 + 1G > A	–	Wt	Normal	Yes	MO/NA	8 y-o	Ribs location
P21	male	ND	....	....	G/C	Normal	No	MO/NA	9 y-o	Surgery
P24	male	ND	....	....	Wt	Normal	No	MO/IIS	2 y-o	Surgery/Shortening and deformities of limbs
P25	fem	*EXT1*	**c.1748A > G**	p.Gln583Arg	Wt	Normal	Yes	MO/IIIS	5 y-o	Vertebral location
P26	fem	ND	....	....	Wt	Normal	No	MO/M	11 y-o	Surgery/Scoliosis
P27	fem	*EXT2*	**c.1616_1623del8ins10**	p.Asp539Glnfs*5	Wt	Normal	No	MO/NA	2 m	Deformity of the hip
P28	male	*EXT1*	**c.1164 + 1G > A**	–	Wt	Normal	Yes	MO/NA	10 m	Surgery/Shortening and deformities of limbs
P29	fem	*EXT1*	c. 1722 + 1G > A	–	Wt	Normal	No	MO/IVS	1 m	Scoliosis
P30	fem	ND	....	....	Wt	Normal	No	SO	4 y-o	Restricted Joint motion
P31	male	ND	....	....	Wt	Normal	No	SO	8 y-o	Surgery/Bilateral valgus
P32	fem	ND	....	....	Wt	Normal	No	SO	9 y-o	NA
P34	fem	ND	....	....	G/C	Normal	No	MO/IS	3 y-o	Bilateral valgus, vertebral
P36	fem	*EXT1*	ex 1del	Unknown	Wt	*Abnormal*	No	MO/M	10 y-o	NA
P37	male	*EXT1*	c.248_249insA	p.Gln84Alafs*105	Wt	NA	No	MO/NA	5 m	Distrophy in ribs
P38[1]	male	ND	....	....	Wt	Normal	Yes	MO/IIS	10 y-o	**Chondrosarcoma**/Severe vascular compression, phlebitis
P39	fem	ND	....	....	Wt	Normal	NA	MO/NA	12 y-o	Deformity of ankles
P40	male	*EXT1*	c.1018C > T	p. Arg340Cys	Wt	NA	Yes	MO/IIS	2 m	Scapular osteochondromas
P41	male	*EXT1*	c.1219C > T	p.Gln407*	G/C	NA	Yes	MO/IIS	1 y-o	Pelvic and Scapular Osteochondromas

Novel mutations are indicated in bold.
[1]Patients with malignant transformations to chondrosarcoma.
(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.

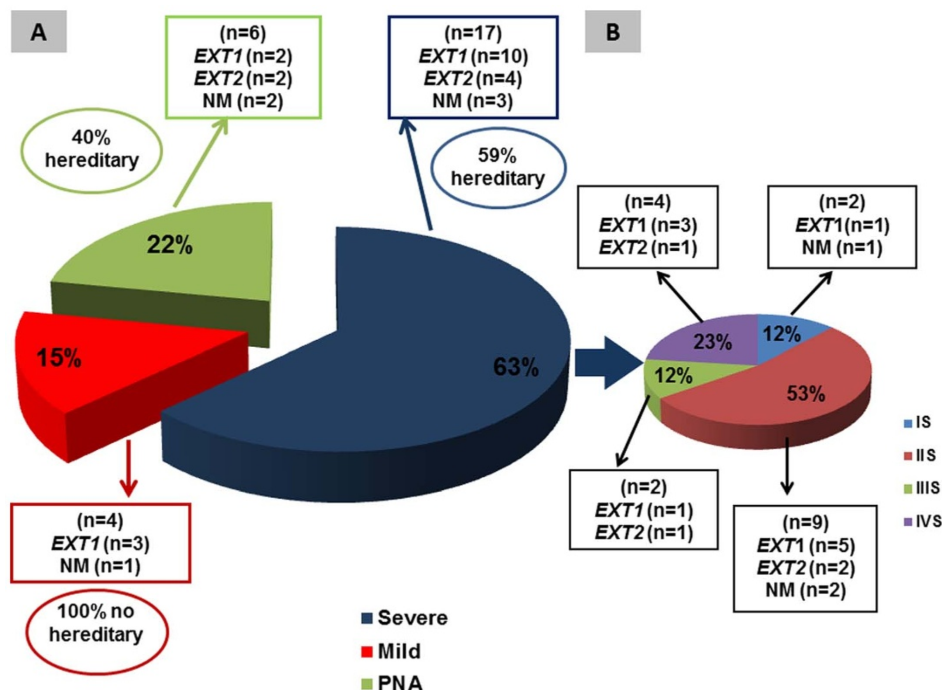

Figure 1 | Genotype–phenotype association in MO patients (n = 27). (A) Graph showing the proportion of severe phenotype (blue), mild phenotype (red) and patients with phenotype not available (green) and the distribution of *EXT1* and *EXT2* mutations or no mutations identified (NM) within each category. (B) Grade of phenotype severity among severely affected patients and distribution of *EXT1* and *EXT2* mutations or no mutations identified (NM) within each category.

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 cases[20,27].

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

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

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.Val78Glyfs*111	Frameshift	**This study**
EXT1	P14	Ex 1	c.369_370delAG	p.Lys126Asnfs*62	Frameshift	Ciavarella, et al, 2013
EXT1	P05	Ex 1	c.752delT	p.Leu251*	Nonsense	Ciavarella, et al, 2013
EXT1	P17	Ex 1	**c.791T > C**	p.Leu264Pro	Missense	**This study**
EXT1	P06[1]	Ex 1	c.848T > A	p.Leu283*	Nonsense	Delgado, et al, 2012
EXT1	P16	Ex 1	**c.916A > T**	p.Lys306*	Nonsense	**This study**
EXT1	P36	Ex1	ex1del	Unknown	Deletion	LOVD
EXT1	P40	Ex 2	c.1018C > T	p.Arg340Cys	Missense	Philippe, et al, 1997
EXT1	P08	Ex 2	**c.1037G > C**	p.Arg346Thr	Missense	**This study**
EXT1	P28	In 3-4	**c.1164 + 1G > A**	--	Splice site	**This study**
EXT1	P41	Ex 4	c.1219C > T	p.Gln407*	Nonsense	LOVD
EXT1	P02	Ex 6	c.1469delT	p.Leu490Argfs*9	Frameshift	Anh, 1995
EXT1	P29	In 8-9	c. 1722 + 1G > A	--	Splice site	Jennes I et al, 2008
EXT1	P25	Ex 9	**c.1748A > G**	p.Gln583Arg		**This study**
EXT2	P19	In 3-4	c.626 + 1G > A	--	Splice site	Vink et al. 2004
EXT2	P13	Ex 5	**c.920_929del10insTG**	p.Asp307Valfs*45	Frameshift	**This study**
EXT2	P04	Ex 6	ex6del	Unknown	Deletion	Leube, et al, 2008
EXT2	P01	Ex 8	**c.1182G > A**	p.Trp394*	Nonsense	**This study**
EXT2	P27	Ex 10	**c.1616_1623del8ins10**	p.Asp539Glnfs*5	Frameshift	**This study**
EXT2	P12	Ex 4-14	**ex4-14del**	Unknown	Deletion	**This study**

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/
[1]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 (IIS) 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.Asp539Glnfs*5, and exon4-14del) were novel. Although some mutation hotspots have been reported20 (http://medgen.ua.ac.be/LOVDv.2.0/), 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 synthesis[20,33].

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)[33] (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 patient[25], 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 *EXT*2 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 site[30]. 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 chondrosarcomas[34,35]. 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 tissues[27,36]. 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 Golgi[37].

Several studies have suggested that MO patients present a more severe phenotype due to *EXT1* mutations than *EXT2* mutations[16,18,21] while other studies could not confirm this observation[23,36]. 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 system[31]. 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 variability[18,38]. 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 study[39]. 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 mutations[20].

Malignant transformation to a chondrosarcoma is the most important complication in MO, and has been estimated to occur in 0.5–5% of patients[17]. 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[22], 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 genes[40,41]. 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

disease in 22% of the patients of this cohort and to understand the mechanisms underlying the malignant transformation process.

Methods

Patients and control individuals. We investigated 33 patients (18 males and 15 females), from unrelated pedigrees with osteochondromatosis from Chile and Argentina (27 MO and 6 SO). Nine control samples from healthy subjects were included in the promoter studies. Diagnosis was made on the basis of clinical manifestations and confirmed by physical and/or radiographic examinations at the Orthopedic and Imaging Departments, Children's Hospital of Córdoba, National University of Córdoba, Argentina. DNA and tissues samples from patients and their relatives were obtained together with their informed consent in accordance with the Helsinki Declaration as revised in 2000. The study was approved by the Ethics Committee (CIEIS) Act N° 95/2007. Genomic DNA was obtained from peripheral blood leukocytes and tissue samples from discarded tissues obtained by surgery, using the Wizard Genomic DNA purification Kit (Promega, Madison, WI). DNA was extracted according to the manufacturer's instructions.

Clinical studies and phenotypic data. Clinical variables were analyzed according to a scale established by the Musculoskeletal Tumoral Society with some modifications[18]. This scale includes the evaluation of all palpable lesions, patient's height, deformities, and functional limitations. Lesion quality and the severity of the disease were assessed according to age of onset (before/after 3 years), number of exostoses (more/less than 10 osteochondromas), vertebral location of the exostoses (absence or presence), stature (above/below 10th percentile), and functional rating (good or fair). The degree of severity was classified as mild (M) or severe (S). Four subcategories were defined in patients with a severe phenotype (from types IS to IVS)[16,18].

Genotyping and mutation analysis. The 11 *EXT1* and 13 *EXT2* coding exons and their intronic flanking regions were amplified by PCR from genomic DNA. Primer sequences and PCR conditions were as described by Sarrión et al 2013[42]. All fragments, except those corresponding to exon 1 of *EXT1*, could be amplified by PCR simultaneously. Exon 1 of *EXT1* and exon 2 of *EXT2* were split into several overlapping fragments, to obtain amplification products that did not exceed 650 bp. PCR was performed in a 50-μl reaction volume, containing ~100 ng of genomic DNA, 1–2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each forward and reverse primer and 0.7 U of GoTaqR 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 1 min at 72°C. An extension at 72°C was then performed for 5 min. The annealing temperature was 60°C for all primer combinations, except during 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. The *EXT1* promoter region, between positions −1285 and −851, was also analyzed by sequencing. The PCR reaction was performed as described above with a Ta of 55°C. All PCR products were purified using a PCR purification kit (GE Healthcare) and sequenced with BigDye 3.1 (Applied Biosystems Life Technologies). The sequences were analyzed in an ABI PRISM 3730 DNA Analyzer (Applied Biosystems Life Technologies).

The presence of all detected mutations was confirmed by digestion with the appropriate restriction enzyme. Novel mutations were confirmed by analyzing 100 control alleles. The mutations were given the official HGVS nomenclature (www.hgvs.org). The reference sequences were NM_000127.2 for *EXT1* and NM_000401.3 for *EXT2*.

MLPA. The number of copies of the *EXT1* and *EXT2* exons present in the patient's genomic DNA was analyzed using the multiplex ligation-dependent probe amplification (MLPA) technique designed by MRC-Holland (code #P215-B1 EXT, MRC-Holland, Amsterdam, The Netherlands) following 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 using Coffalyser v9.4 software (MRC-Holland Vs 05; 30-08-2007). 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. Samples with ratios between 0.7 and 1.3 were considered as bearing a normal copy number. Ratios below 0.7 were considered to correspond to deletions, and above 1.3 to duplications. Each positive result was confirmed in a second independent MLPA reaction.

Assessment of functionality of missense mutations. In order to assess the possible pathogenic effect of the new missense mutations, the changes were analyzed using three in-silico online tools: PolyPhen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/, last accessed March 2014), Mutation Taster (http://www.mutationtaster.org/, last accessed March 2014, and ESE Finder 3.0 (ESE: Exonic Splicing Enhancer; http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home, last accessed March 2014). Protein Homology Fold Recognition Engine (http://www.sbg.bio.ic.ac.uk/phyre2/html, last accessed March 2014) was used to predict the implications of missense mutations for EXT1 3D structure Human Splice Finder (http://www.umd.be/HSF/, last accessed March 2014) and NetGene2 (http://www.cbs.dtu.dk/cgi-bin/webface?jobid=netgene2, last accessed March 2014) online tools were used to assess the possible effect of novel intronic mutations on splicing.

Ethics statement. The methods were carried out in accordance with the approved guidelines and in accordance with the Helsinki Declaration as revised in 2000. The study was approved by the Ethics Committee (CIEIS) Act N° 95/2007.

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Acknowledgments

The authors thank the patients and their families for their cooperation. They wish to acknowledge Joanne Ferrier for revising the English. The authors are also grateful for the support from the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), which is an initiative of the ISCIII. Grant support: Scientific and Technical National Research Council (CONICET); FONCyT PICT 2006–2350 and BID N° 2437/OC-AR PICT2010-2824, Catholic University of Cordoba Grant 2010/2011/2012/2013, Spanish Ministry of Education and Science (SAF2011-25431, PIB2010AR00473) and the Catalan Government (2009SGR971).

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

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Delgado, M.A. *et al*. A broad spectrum of genomic changes in latinamerican patients with EXT1/EXT2-CDG. *Sci. Rep.* **4**, 6407; DOI:10.1038/srep06407 (2014).

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