Identification of mutations in *EXT1* and *EXT2* genes in six Chinese families with multiple osteochondromas

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Abstract. The aim of the present study was to identify mutations of major causative genes in six unrelated Chinese families with multiple osteochondromas (MO). Radiographic examinations and genetic analyses were performed in 8 patients exhibiting typical features of MO. Analysis was also performed on unaffected members of the six families and 250 healthy volunteers. Radiographies of the patients revealed multiple exostoses in the cartilage of long bones. A total of five different mutations were identified, one in exostosin-1 (*EXT1)* and four in exostosin-2 (*EXT2)*. Two novel mutations were detected in *EXT2*: A missense mutation, c.1385G>A, in exon 8, resulting in p.Trp462X; and a splice site mutation, c.725+1G>C, which consisted of a heterozygous guanine-to-cytosine transition at nucleotide 725+1 in intron 3. Three common *EXT* mutations were also detected: c.1036C>T in exon 5 of *EXT2* resulting in p.Gln346X; c.1299C>A in exon 8 of *EXT2* resulting in p.Phe433Leu; and c.1038A>T in exon 2 of *EXT1* resulting in p.Arg346Ser. In conclusion, the present study identified a novel missense mutation (c.1385G>A) in exon 8 and a splicing mutation (c.725+1G>C) in intron 3 of the *EXT2* gene, which are responsible for MO in certain Chinese patients. The findings are useful for expanding the database of known *EXT2* mutations and understanding the genetic basis of MO in Chinese patients, which may improve genetic counseling and the prenatal diagnosis of MO.

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Abbreviations: MO, multiple osteochondromas; *EXT1*, exostosin-1; *EXT2*, exostosin-2; SNP, single nucleotide polymorphism; HGMD, Human Gene Mutation Database; HS, heparan sulfate

Key words: multiple osteochondromas, *EXT1, EXT2*, mutations

Introduction

Multiple osteochondromas (MO) is an autosomal dominant disease with a prevalence of 1 in 50,000 and the significant characteristic of the disease is the formation of multiple benign cartilaginous exostoses in the metaphyses of long bones (1,2), and the ratio of male-to-female is ~3:2 (3). MO is accurately diagnosed by radiological observation of exostoses and clinical investigation. In 90% of patients with MO, sessile or pedunculated exostoses are located around the distal femur (1,4). The prevalence of exostoses in other anatomical regions includes 85% in the proximal tibia, 76% in the fibula and 72% in the humerus (2,5,6). The exostoses are formed prior to adolescence, and the size and number increase over time until the closure of the growth plate (7). The major clinical symptoms are localized pain, short stature, restricted joint motion and bone deformities, including discrepancy in leg length, valgus deformities in the knees or ankles, asymmetric pelvis or pectoral region, and bending of the radius bone resulting in carpal subluxation (8). The complications caused by exostoses include pressure on neighboring tissues, nerves or vessels, and the most severe complication is the transformation of a benign osteochondroma into a malignant chondrosarcoma, however the risk of this is 1-2% (2,6). Surgical excision is the critical treatment to prevent progressive deformities and improve functional impairment. Furthermore, certain researchers propose that bisphosphonate therapy may relieve pain in children with MO (9).

MO is a genetically heterogeneous disorder, and linkage analysis has identified at least two loci involved, including exostosin-1 (*EXT1*) in 8q24.11-q13 and exostosin-2 (*EXT2*) in 11p11-p13 (10-12). The *EXT1* and *EXT2* genes have been cloned and validated as disease-causing genes (12). Previous studies have identified *EXT1* and *EXT2* as tumor suppressor genes that encode glycosyltransferases responsible for heparan sulfate synthesis (13,14). *EXT1* and *EXT2* contain 11 and 16 exons, respectively, and their protein products are EXT1, with 746 amino acids and EXT2, with 718 amino acids, respectively; there is a 30.9% homology between *EXT1* and *EXT2* (15). The Multiple Osteochondroma Mutation Database

(medgen.ua.ac.be/LOVDv.2.0/home.php?%20action5switch_db) has described 432 mutations in *EXT1* (updated December 2013) and 223 in *EXT2* (updated April 2012). *EXT2* mutations have been reported to occur more frequently in Chinese patients, however Caucasian and Japanese patients harbor *EXT1* mutations more often (16,17). The mutations of *EXT1* and *EXT2* include frameshift, nonsense, missense and splicing mutations. We have previously identified five mutations in *EXT1* and four in *EXT2* in Chinese patients, and in that study, we first reported a proband carrying mutations in both *EXT1* and *EXT2* simultaneously (18). Novel mutations in *EXT1* and *EXT2* have been identified in previous studies of different populations, including Chinese, Polish and Taiwanese populations (19-21). In addition to point mutations, which account for 70-75% of MO cases, deletions involving in single or multiple exons of *EXT1* or *EXT2* result in the pathogenesis of 10% of MO cases (22-25). The remaining 10-15% cases are caused by intronic changes, partial exon deletions, somatic mosaicism, positional changes, including insertion, inversion or translocation not leading to copy number alteration, and alterations affecting *EXT1* or *EXT2* promoter function (26).

The present study aimed to identify the gene mutations in six unrelated Chinese families with MO to extend the known mutations of *EXT1* and *EXT2*. Identifying more mutations is useful for revealing the genetic basis of MO in Chinese patients and contributes to prenatal counseling and diagnosis.

Patients and methods

Patients. This study was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China). Six Chinese families with MO diagnosed using the typical radiological observations of exostoses in the juxta-metaphyseal region of long bones were analyzed in the present study. The pedigrees and radiographies of these families are presented in Figs. 1 and 2, respectively.

In family 1 (Fig. 1A), the proband (II-1) was a 13-year-old boy from Shanghai who complained of the asymmetry of his bilateral lower extremities. The X-rays revealed pelvis tilting, asymmetry of the bilateral lower extremities, and multiple exostoses on the right proximal femur, bilateral distal femurs, right proximal tibia and fibula, and the lower sternum. Multiple exostoses were also identified in the father of the proband. In family 2 (Fig. 1B), the proband (II-1) was a 19-year-old female from Gansu (China). She was admitted to hospital due to deformation of the tibia and fibula, where a pseudarthrosis had formed. The X-rays revealed multiple exostoses on bilateral proximal tibias and fibulas, and the right distal femur. Prior to the present study, the patient had received surgical treatment for the multiple exostoses near both knees. In family 3 (Fig. 1C), the proband (II-2) was a 47-year-old female from Shanghai (China). The proband and her 74-year-old mother (I-2) exhibited exostoses on their skulls. In family 4 (Fig. 1D), the proband (II-1) was a 17-year-old boy from Zhejiang (China). The proband exhibited exostoses on the right femur and right proximal tibia and fibula. A 10x8 cm hard lump was observable on the lateral right thigh, with no tenderness. Prior to admission to the clinic, the proband had undergone five operations to remove multiple exostoses at the right axillary fossa, the left knee, ankle, scapula and neck. In family 5

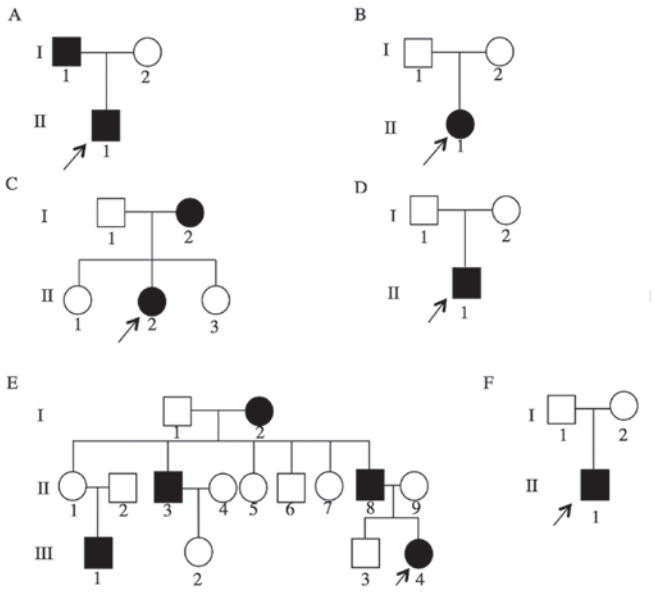

Figure 1. Pedigrees of six Chinese families with multiple osteochondromas. (A) Family 1, (B) family 2, (C) family 3, (D) family 4, (E) family 5 and (F) family 6. Black symbols represent the affected individuals and open symbols represent the unaffected individuals. Circles and squares indicate females and males, respectively. Arrows identify the probands in the families.

(Fig. 1E), the proband (III-4), a 5-year-old girl from Anhui (China), was referred to the clinic for a deformity of her right *digitus annularis* and left elbow. X-rays revealed the epiphysis of the right *digitus annularis* was broken and an exostosis at the left proximal ulna, which resembled Madelung deformity. The proband's father (II-8) and grandmother (I-2) had similar deformities of the elbows or hands. In family 6 (Fig. 1F), the proband (II-1) was a 19-year-old boy from Zhejiang. The proband complained of asymmetry of the bilateral lower extremities and genu valgum. X-rays revealed multiple exostoses at knees, distal femurs, tibias and fibulas. Prior to the study, the proband had received two operations to remove the exostoses at interior left leg, right crus and both knees.

Methods. Informed consent was obtained from the 6 families and 250 healthy ethnically-matched volunteers prior to blood sampling and DNA analysis. The 250 volunteers (125 males and 125 females; age, 24.7-65.3 years) were recruited from the Department of Osteoporosis of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. A QuickGene DNA Whole Blood kit (Kurabo Industries Ltd., Osaka, Japan) and a Nucleic Acid Isolation system (QuickGene-610L; AutoGen, Inc., Holliston, MA, USA) were used to extract genomic DNA from 2 ml peripheral blood. The sequences of *EXT1* and *EXT2* genes were attained from the online database (GenBank accession no. NM 000127 and NC 000011.9). All exons and the exon-intron boundaries of *EXT1* and *EXT2* genes were amplified via polymerase chain reaction (PCR) using the primers designed by Primer 3 software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences are presented in Table I. The reaction mixture (20 μl) contained 1X GC buffer I (Takara Bio, Inc., Otsu, Japan), 2.5 mM Mg^{2+}, 0.2 mM dNTP, 0.2 μM of each primer, 1 unit HotStarTaq polymerase (Takara Bio, Inc.) and 1 μl template DNA. The thermocycling conditions

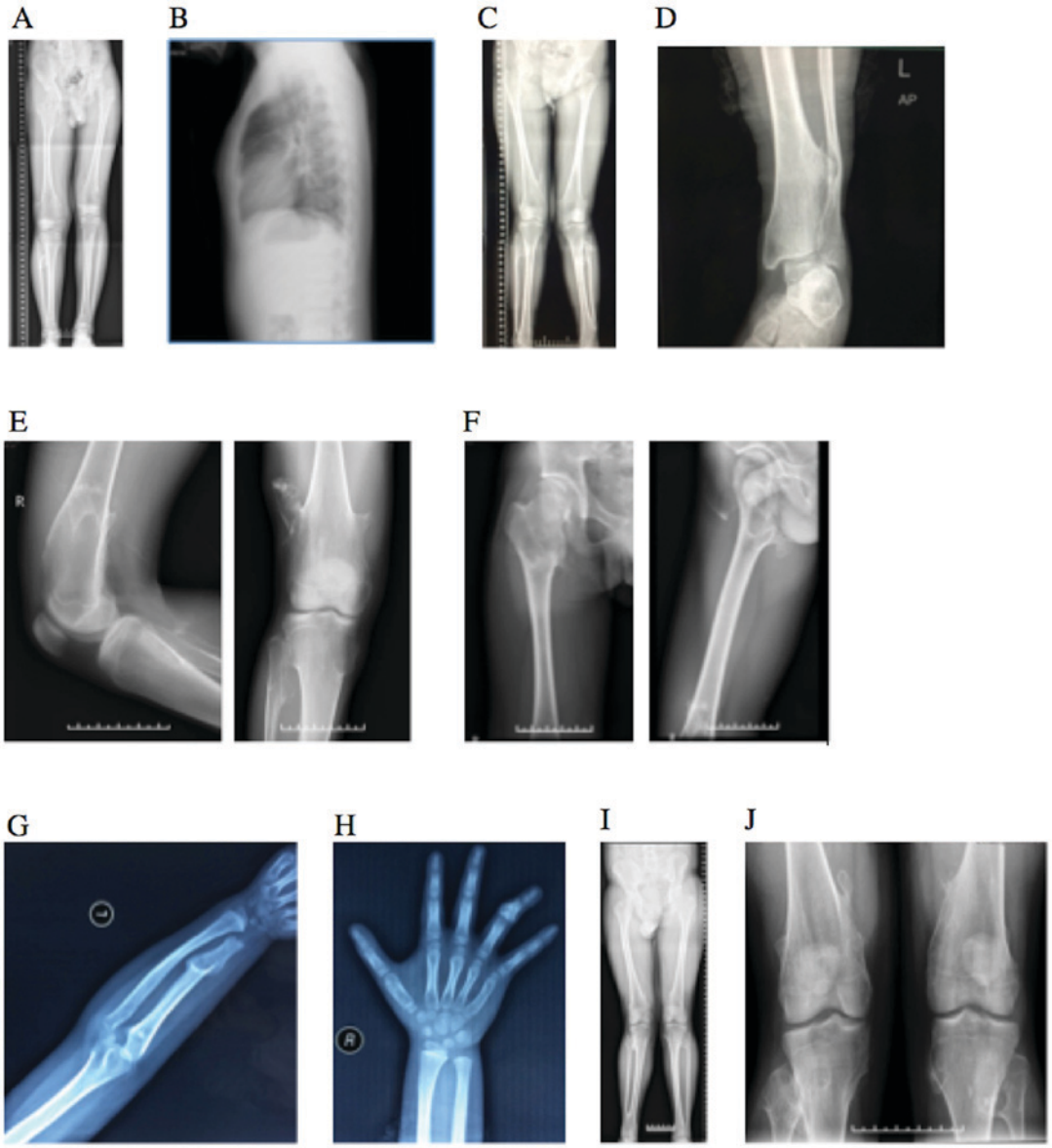

Figure 2. Radiographies of the Chinese patients with multiple osteochondromas. (A) X-ray images of the lower limbs (anteroposterior view) of the proband of family 1, which reveals a tilt of the pelvis, bilateral lower extremities asymmetry, multiple exostoses on the right proximal femur, bilateral distal femurs, right proximal tibia and fibula. (B) Chest radiography of the proband of family 1 demonstrating exostosis on the lower sternum. (C) X-ray images of the lower limbs (anteroposterior view) of the proband of family 2, revealing multiple exostoses on the bilateral proximal tibias and fibulas and the right distal femur. (D) Radiography of left calf of the family 2 proband demonstrating a pseudoarticulation on the left tibia and fibula. (E) The right knee radiographies from the proband of family 4 demonstrating exostoses on the right knee. (F) The right femur radiographies of the proband in family 4 that presenting exostoses on the right femur. (G) Radiographies of the left forearm of the proband of family 5 demonstrating an exostosis at the left proximal ulna and Madelung deformity. (H) The right hand radiography of the proband in family 5 revealing the broken epiphysis of the *digitus annularis*. (I) X-ray images of the lower limbs (anteroposterior view) of the proband of family 6 that reveals the asymmetry of bilateral lower extremities and genu valgum. (J) Radiograph of knees from the proband of family 6 demonstrating multiple exostoses at both knees and distal femurs.

were as follows: Initial denaturation at 95˚C for 2 min followed by 11 cycles at 94˚C for 20 sec, at 64.5˚C for 40 sec, at 72˚C for 1 min, and 24 cycles at 94˚C for 20 sec, at 58˚C for 30 sec, at 72˚C for 1 min and at 72˚C for 2 min. Subsequently, direct sequencing was performed on DNA from 8 patients using the BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.1; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and analyzed the sequences by an ABI Prism 3130 automated sequencer (Thermo Fisher Scientific, Inc.) (18). Subsequently, the identified mutation sites were screened among the unaffected members of six families and 250 healthy volunteers. Single nucleotide mutations were checked using the Polyphred program (droog.gs.washington.edu/polyphred/). Novel mutations were recognized with the Human Gene Mutation Database program (http://www.hgmd.cf.ac.uk/). Disease-causing mutations were predicted using MutaionTaster (www.mutationtaster.org).

Results

In total, five mutations were identified in the *EXT1* and *EXT2* genes in six Chinese families. In F1, a nonsense mutation, p.Gln346X (c.1036C>T), was identified in exon 5 of *EXT2* in the proband (II-1) and his father (I-1). In F2 and F3, the

Table I. Primer sequences used for polymerase chain reaction amplification of the *EXT1* and *EXT2* genes.

Gene	Forward primer	Reverse primer
EXT1-1	5'-GGAAAGGCATCCAGAGAAGGT-3'	5'-GCACATACTGAGGTGACAACTGG-3'
EXT1-2	5'-ACTGGGCAAACCAAATTGTTG-3'	5'-TAGGCCAAGCTGGCAATTAGA-3'
EXT1-3	5'-CAAGGCCAGTCGTCTCTATGG-3'	5'-GCTCCCATTCTTTACCTGCAA-3'
EXT1-4	5'-GGTTGTTCATGTGCAAGGTCA-3'	5'-CCATGGCAAAGCAGGTAAAAG-3'
EXT1-5	5'-TTTTGGAATGAGCATGGACTC-3'	5'-TGCAATGCTCTGCTCTGTTTT-3'
EXT1-6	5'-GGCAAAGGATGTCAAAGCAAG-3'	5'-AACGAGGCAGGATGAATGAAA-3'
EXT1-7	5'-CCGGACACAGTTGGTTTTGTT-3'	5'-TCAAGACCCAGATTTCCCTGA-3'
EXT1-8	5'-GTTGCTCCATCCTGTGGTCTC-3'	5'-GCAAGGTGCTAACAGGAATCG-3'
EXT1-9	5'-TTATGGGGCAAAATGTCAAGC-3'	5'-TGCCAAGAGGTTTCACTGGTT-3'
EXT1-10	5'-CCTGCCTTGTAGGCTCCTTATG-3'	5'-TGGGTGGAACAGCTAGAGGAA-3'
EXT1-11	5'-CTTGGTCCCAAGTGCAAAGAG-3'	5'-CACAATCTGGCTCTGCTGATG-3'
EXT2-1	5'-TTCAAGTGTCATTTGCCATCC-3'	5'-CCCTTCCCTTTAGTTCCCTGA -3'
EXT2-2	5'-GCAGGTCTGTATGGGACAAGC-3'	5'-GCACAATCCAGAGTGGGAAAA-3'
EXT2-3	5'-GGGAGGTAGCAGAGAGGCTGT-3'	5'-CTCAGTGCCTCAAGGACCCTA-3'
EXT2-4	5'-CATGCGCTCTCAGCTTAGCAT-3'	5'-TTCGCTGGGCTCAATTTTAAC-3'
EXT2-5	5'-TTTCAGAAGGCCAACAGTGGT-3'	5'-GCCTTGGTTTGTGAACTGCTC-3'
EXT2-6	5'-TGGAGGCAGGGTGAAAGATTA-3'	5'-CATTCAGCTCCTGTCCCTCTG-3'
EXT2-7	5'-CACCCCCATCCCTACAACTTT-3'	5'-AAGTCACCGGGATGTCTTTGA-3'
EXT2-8	5'-GCAAATTTTGAGGAGGGGAAG-3'	5'-GAGAAAAATGGAGGCATGCTG-3'
EXT2-9	5'-AGAGCCGTGGATACAAGCTGA-3'	5'-GCACAGTTGCCATTTTGGAAT-3'
EXT2-10	5'-GGAACATCTCCAGAATCCCATT-3'	5'-GCAAGCTGGAAATAGCACCTG-3'
EXT2-11	5'-GGTCACTTGACCAAAAGCATTC-3'	5'-CAATGTGACCGCATCAATCAT-3'
EXT2-12	5'-TCGCCCTTATGGCTACAAGAA-3'	5'-TGCACATGGAGGTGACTATGG-3'
EXT2-13	5'-AGAACCTGGGAGCAGACTGTG-3'	5'-CTTCCACTTGGCATTTTCGAG-3'

EXT, exostosin.

Table II. Clinical data and mutations identified in *EXT1* and *EXT2* from patients with multiple osteochondromas.

Family	Patient	Gender	Gene	DNA change	Site	Protein change	Type of mutation	Novel
F1	II-1	M	*EXT2*	c.1036C>T	Exon 5	p.Gln346X	Nonsense	No
F1	I-1	M	*EXT2*	c.1036C>T	Exon 5	p.Gln346X	Nonsense	No
F2	II-1	F	*EXT2*	c.1299C>A	Exon 8	p.Phe433Leu	Missense	No
F3	II-2	F	*EXT2*	c.1299C>A	Exon 8	p.Phe433Leu	Missense	No
F4	II-1	M	*EXT2*	c.1385G>A	Exon 8	p.Trp462X	Nonsense	Yes
F5	III-4	F	*EXT2*	c.725+1G>C	Intron 3	Unknown	Putative aberrant splicing	Yes
F6	II-1	M	*EXT1*	c.1038A>T	Exon 2	p.Arg346Ser	Missense	No

EXT, exostosin; M, male; F, female.

same missense mutation in exon 8 of *EXT2*, p.Phe433Leu (c.1299C>A), was present in the two probands (II-1 of F2, II-2 of F3). However the proband's mother in F3, who had similar features to her daughter, harbored no mutations. In F4, a nonsense mutation in exon 8 of *EXT2*, p.Trp462X (c.1385G>A), was identified in the proband (II-1). In F5, the proband (III-4) had a splice site mutation, c.725+1G>C, which consisted of a heterozygous guanine-to-cytosine transition at nucleotide 725+1 in intron 3 of the *EXT2* gene. The proband's father and grandmother who had similar deformity refused to provide the blood samples. In F6, the missense mutation in exon 2 of *EXT1*, p.Arg346Ser (c.1038A>T), was present in the proband (II-1). The mutation results are presented in Fig. 3, and the clinical data and mutations identified in *EXT1* and *EXT2* from patients with MO are summarized in Table II.

The above mutations identified in *EXT1* and *EXT2* genes were not detected in the unaffected family members or in the 250 healthy volunteers.

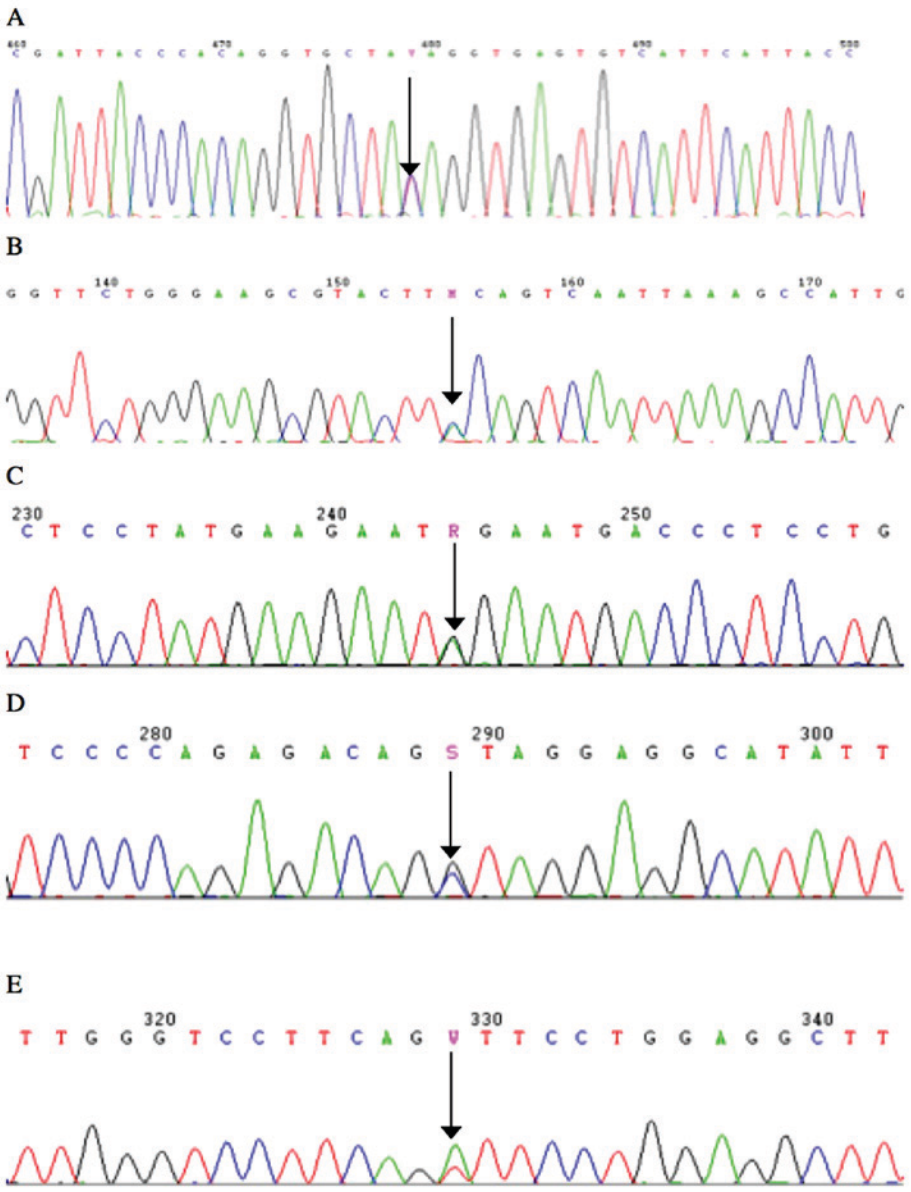

Figure 3. Mutation analysis. (A) A nonsense mutation, p.Gln346X, in exon 5 of *EXT2* was identified in the proband (II-1) of family 1. (B) A missense mutation, p.Phe433Leu, in exon 8 of *EXT2* was identified in the proband (II-1) of family 2 and the proband (II-2) of family 3. (C) A nonsense mutation, p.Trp462X, in exon 8 of *EXT2* was identified in the proband (II-1) of family 4. (D) A splice site mutation, c.725+1G>C, in intron 3 of the *EXT2* was identified in the proband III-4 of family 5. (E) A missense mutation, p.Arg346Ser, in exon 2 of *EXT1* was identified in the proband (II-1) of family 6. EXT, exostosin.

Discussion

Previous studies have revealed that *EXT1* or *EXT2* mutations are detected in majority of patients with MO (14). In general, *EXT1* mutations are more frequently reported than *EXT2* mutations; however, in Chinese patients, *EXT2* mutations have been reported to occur more frequently. The products of *EXT* genes are heparan sulfate (HS)-synthesizing enzymes, EXT1 and EXT2, which are glycosyltransferases responsible for HS synthesis in the Golgi apparatus. HS is an essential component of cell surface- and matrix-associated proteoglycans (12). HS can regulate the distribution and availability of growth factors and signaling proteins, and influence various critical processes in skeletal growth and morphogenesis. The glycosyltransferase encoded by *EXT* genes is a heterodimer complex of EXT1 and EXT2, and it only has enzymatic activity as the EXT1/EXT2 complex. The disease-causing mutations of the majority of MO cases are loss-of-function mutations in the *EXT1* or *EXT2* gene. The mutations result in premature termination of EXT protein translation and premature degradation. EXT2 is a structural component and acts as a chaperone for EXT1 (27). However, EXT1 is more important in controlling cartilage growth, which is a critical regulator of the perichondrium phenotype (28). This may explain why *EXT1* mutations are associated with more severe phenotypes of MO compared with *EXT2* mutations, including shorter stature, increased skeletal deformities and more severe impairments in forearm rotation, elbow flexion and knee flexion (29,30). In the present study, all the probands were at or before adolescence except the proband of F3. Their clinical symptoms occurred prior to puberty, and the number and size of exostoses increased with age, even when surgical excision had been performed. This is

consistent with previous reports (7,31). The proband of F6 with an *EXT1* mutation had asymmetric lower extremities, genu valgum deformity, and at least six exostoses at knees, distal femurs, tibias and fibulas. The other five probands from the five different families with *EXT2* mutations also had multiple exostoses in different anatomical regions and had deformities of varying degrees. There was no evident difference of severity in the patients, which may be caused by the limited number of cases. An increased case number is required to observe the different clinical manifestations of patients with MO.

Nonsense, frame shift and splice site mutations constitute a large proportion of the inactivating mutations in *EXT* genes (14,32). In the present study, one mutation in *EXT1* and four mutations in *EXT2* were identified, including two nonsense, two missense and one splicing mutation. This corresponds with previous research demonstrating that in Chinese patients with MO, *EXT2* mutations may be more frequent than *EXT1* mutations (16,17). The detected nonsense mutations led to premature termination at amino acid 346 (p.Gln346X) and amino acid 462 (p.Trp462X) in *EXT2*, which cosegregated with the disease phenotype in F1 and F4, and the latter had not been previously reported. In the probands of F2 and F3, a missense mutation (p.Phe433Leu) was identified in exon 8 of *EXT2*, and in the proband of F6, a missense mutation (p.Arg346Ser) was identified in exon 2 of *EXT1*. The missense mutations may pinpoint the key domains in *EXT1* and *EXT* that contribute to the MO disease mechanism. In proband of F5, a novel splicing mutation (c.725+1G>C) was detected, which consisted of a guanine-to-cytosine transition at nucleotide 725+1 in intron 3 of *EXT2*. A splicing mutation can cause the alteration of splice sites or formation of new splice sites, and result in alternative transcription and translation. A previous study identified a splice site mutation (c.743+1G>A) in *EXT2*, which leads to an aberrantly spliced transcript with a premature termination codon and nonsense-mediated decay of mRNA (19). In addition, in F3, no mutations in *EXT1* and *EXT2* were identified in the mother of the proband, although both of the proband and the mother had exostoses on the skull. As for this mutation negative case, large deletions in single or multiple exons should be taken into account. Large exon deletions, which cannot be detected using direct sequencing, may be present and can be identified by multiple ligation-dependent probe amplification (MLPA). Signori *et al* (23) demonstrated that MLPA combined with PCR-based methods can improve the detection rate of mutations in patients with MO. Intronic deletions or duplications, partial exon deletions not reaching the detection threshold of MLPA, somatic mosaicism, and alterations influencing *EXT1* or *EXT2* promoter function may also be the causative mechanism in the mutation negative case of the F3 mother proband. For the aforementioned alterations, which are not detected by direct sequencing and MLPA, array-comparative genomic hybridization (CGH) is a useful method for their detection. Waaijer *et al* (33) used array-CGH to detect an insertion/deletion within the first intron of *EXT1* in mutation negative patients with MO, which indicates the importance of array-CGH. Furthermore, positional changes, not involving copy number alterations, such as translocations, insertions and inversions, may also be the cause of pathogenesis in this mutation negative case. Such translocations, insertions and inversions will not be detected by array-CGH, thus, next-generation sequencing could be used for the detection of these types of mutation. According to the various possibilities mentioned, the DNA of mutation negative mother of F3 in the present study should be analyzed using MLPA, array-CGH or next-generation sequencing to identify a genetic cause of pathogenesis. However, MLPA and array-CGH were not performed, which is a limitation of the current study. Furthermore, in F2, F4 and F6, mutations were observed in probands, but not in the parents. They may be *de novo* cases, but for the parents, the possibility of a somatic mosaic mutation condition cannot be excluded. Similarly, *de novo* somatic mutation depending on the tissue distribution may be present even in the probands. Szuhai *et al* (34) were the first to identify somatic mosaic large genomic deletions as an underlying mechanism of MO in mutation negative patients using a resolution array-CGH. They reported the presence of a mosaic deletion in ~10-15% of the patient blood cells. In view of this, mutation analysis of the probands and parents to detect the presence of mutations in other tissues and detecting low mutation proportion is important. However, because obtaining other tissues from probands is invasive, the probands and their family members refused to provide their tissues. Due to the limitations in techniques and available samples, the presence of mutations in other tissues and low mutation proportions were not detected.

To conclude, two novel mutations in *EXT2* and three known mutations in *EXT1* and *EXT2* were identified in six MO probands of unrelated Chinese families. Although the majority of MO cases can be easily diagnosed by radiological observations and clinical investigations, the reduced penetrance and intrafamilial variability may cause atypical symptoms. Mutation screening of the *EXT* genes can be used to confirm the diagnosis in atypical patients. The current results add to the known mutations of *EXT2*, and contribute to the understanding of the genetic basis of MO in Chinese patients. Furthermore, the mutation screening can be used in genetic counseling and prenatal diagnosis to reduce the burden caused by MO.

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References

1. Wuyts W, Van Hul W, De Boulle K, Hendrickx J, Bakker E, Vanhoenacker F, Mollica F, Lüdecke HJ, Sayli BS, Pazzaglia UE, *et al*: Mutations in the EXT1 and EXT2 genes in hereditary multiple exostoses. Am J Hum Genet 62: 346-354, 1998.
2. Schmale GA, Conrad EU III and Raskind WH: The natural history of hereditary multiple exostoses. J Bone Joint Surg Am 76: 986-992, 1994.
3. Ahn J, Lüdecke HJ, Lindow S, Horton WA, Lee B, Wagner MJ, Horsthemke B and Wells DE: Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). Nat Genet 11: 137-143, 1995.

4. Shapiro F, Simon S and Glimcher MJ: Hereditary multiple exostoses. Anthropometric, roentgenographic, and clinical aspects. J Bone Joint Surg Am 61: 815-824, 1979.
5. Hennekam RC: Hereditary multiple exostoses. J Med Genet 28: 262-266, 1991.
6. Wicklund CL, Pauli RM, Johnston D and Hecht JT: Natural history study of hereditary multiple exostoses. Am J Med Genet 55: 43-46, 1995.
7. Greenspan A: Tumors of cartilage origin. Orthop Clin North Am 20: 347-366, 1989.
8. Pierz KA, Stieber JR, Kusumi K and Dormans JP: Hereditary multiple exostoses: One center's experience and review of etiology. Clin Orthop Relat Res: 49-59, 2002.
9. Winston MJ, Srivastava T, Jarka D and Alon US: Bisphosphonates for pain management in children with benign cartilage tumors. Clin J Pain 28: 268-272, 2012.
10. Cook A, Raskind W, Blanton SH, Pauli RM, Gregg RG, Francomano CA, Puffenberger E, Conrad EU, Schmale G, Schellenberg G, *et al*: Genetic heterogeneity in families with hereditary multiple exostoses. Am J Hum Genet 53: 71-79, 1993.
11. Wuyts W, Ramlakhan S, Van Hul W, Hecht JT, van den Ouweland AM, Raskind WH, Hofstede FC, Reyniers E, Wells DE, de Vries B, *et al*: Refinement of the multiple exostoses locus (EXT2) to a 3-cM interval on chromosome 11. Am J Hum Genet 57: 382-387, 1995.
12. Legeai-Mallet L, Margaritte-Jeannin P, Lemdani M, Le Merrer M, Plauchu H, Maroteaux P, Munnich A and Clerget-Darpoux F: An extension of the admixture test for the study of genetic heterogeneity in hereditary multiple exostoses. Hum Genet 99: 298-302, 1997.
13. Esko JD and Selleck SB: Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Annu Rev Biochem 71: 435-471, 2002.
14. Jennes I, Pedrini E, Zuntini M, Mordenti M, Balkassmi S, Asteggiano CG, Casey B, Bakker B, Sangiorgi L and Wuyts W: Multiple osteochondromas: Mutation update and description of the multiple osteochondromas mutation database (MOdb). Hum Mutat 30: 1620-1627, 2009.
15. Wuyts W, Van Hul W, Wauters J, Nemtsova M, Reyniers E, Van Hul EV, De Boulle K, de Vries BB, Hendrickx J, Herrygers I, *et al*: Positional cloning of a gene involved in hereditary multiple exostoses. Hum Mol Genet 5: 1547-1557, 1996.
16. Seki H, Kubota T, Ikegawa S, Haga N, Fujioka F, Ohzeki S, Wakui K, Yoshikawa H, Takaoka K and Fukushima Y: Mutation frequencies of EXT1 and EXT2 in 43 Japanese families with hereditary multiple exostoses. Am J Med Genet 99: 59-62, 2001.
17. Xu L, Xia J, Jiang H, Zhou J, Li H, Wang D, Pan Q, Long Z, Fan C and Deng HX: Mutation analysis of hereditary multiple exostoses in the Chinese. Hum Genet 105: 45-50, 1999.
18. Kang QL, Xu J, Zhang Z, He JW, Fu WZ and Zhang ZL: Mutation screening for the EXT1 and EXT2 genes in Chinese patients with multiple osteochondromas. Arch Med Res 44: 542-548, 2013.
19. Tian C, Yan R, Wen S, Li X, Li T, Cai Z, Li X, Du H and Chen H: A splice mutation and mRNA decay of EXT2 provoke hereditary multiple exostoses. PLoS One 9: e94848, 2014.
20. Jamsheer A, Socha M, Sowinska-Seidler A, Telega K, Trzeciak T and Latos-Bielenska A: Mutational screening of EXT1 and EXT2 genes in Polish patients with hereditary multiple exostoses. J Appl Genet 55: 183-188, 2014.
21. Lin WD, Hwu WL, Wang CH and Tsai FJ: Mutant EXT1 in taiwanese patients with multiple hereditary exostoses. Biomedicine (Taipei) 4: 11, 2014.
22. Wuyts W and Van Hul W: Molecular basis of multiple exostoses: Mutations in the EXT1 and EXT2 genes. Hum Mutat 15: 220-227, 2000.
23. Signori E, Massi E, Matera MG, Poscente M, Gravina C, Falcone G, Rosa MA, Rinaldi M, Wuyts W, Seripa D, *et al*: A combined analytical approach reveals novel EXT1/2 gene mutations in a large cohort of Italian multiple osteochondromas patients. Genes Chromosomes Cancer 46: 470-477, 2007.
24. Lonie L, Porter DE, Fraser M, Cole T, Wise C, Yates L, Wakeling E, Blair E, Morava E, Monaco AP and Ragoussis J: Determination of the mutation spectrum of the EXT1/EXT2 genes in British Caucasian patients with multiple osteochondromas, and exclusion of six candidate genes in EXT negative cases. Hum Mutat 27: 1160, 2006.
25. Jennes I, Entius MM, Van Hul E, Parra A, Sangiorgi L and Wuyts W: 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. J Mol Diagn 10: 85-92, 2008.
26. Vink GR, White SJ, Gabelic S, Hogendoorn PC, Breuning MH and Bakker E: Mutation screening of EXT1 and EXT2 by direct sequence analysis and MLPA in patients with multiple osteochondromas: splice site mutations and exonic deletions account for more than half of the mutations. Eur J Hum Genet 13: 470-474, 2005.
27. Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J and Zako M: Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68: 729-777, 1999.
28. Huegel J, Sgariglia F, Enomoto-Iwamoto M, Koyama E, Dormans JP and Pacifici M: Heparan sulfate in skeletal development, growth and pathology: The case of hereditary multiple exostoses. Dev Dyn 242: 1021-1032, 2013.
29. Francannet C, Cohen-Tanugi A, Le Merrer M, Munnich A, Bonaventure J and Legeai-Mallet L: Genotype-phenotype correlation in hereditary multiple exostoses. J Med Genet 38: 430-434, 2001.
30. Porter DE, Lonie L, Fraser M, Dobson-Stone C, Porter JR, Monaco AP and Simpson AH: Severity of disease and risk of malignant change in hereditary multiple exostoses. A genotype-phenotype study. J Bone Joint Surg Br 86: 1041-1046, 2004.
31. Philippe C, Porter DE, Emerton ME, Wells DE, Simpson AH and Monaco AP: Mutation screening of the EXT1 and EXT2 genes in patients with hereditary multiple exostoses. Am J Hum Genet 61: 520-528, 1997.
32. Ciavarella M, Coco M, Baorda F, Stanziale P, Chetta M, Bisceglia L, Palumbo P, Bengala M, Raiteri P, Silengo M, *et al*: 20 novel point mutations and one large deletion in EXT1 and EXT2 genes: Report of diagnostic screening in a large Italian cohort of patients affected by hereditary multiple exostosis. Gene 515: 339-348, 2013.
33. Waaijer CJ, Winter MG, Reijnders CM, de Jong D, John Ham S, Bovée JV and Szuhai K: Intronic deletion and duplication proximal of the EXT1 gene: A novel causative mechanism for multiple osteochondromas. Genes Chromosomes Cancer 52: 431-436, 2013.
34. Szuhai K, Jennes I, de Jong D, Bovée JV, Wiweger M, Wuyts W and Hogendoorn PC: Tiling resolution array-CGH shows that somatic mosaic deletion of the EXT gene is causative in EXT gene mutation negative multiple osteochondromas patients. Hum Mutat 32: E2036-E2049, 2011.