Identification of a novel frameshift mutation of the *EXT2* gene in a family with multiple osteochondroma

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Abstract. Multiple osteochondroma (MO), also known as multiple hereditary exostoses, is an autosomal dominant skeletal disorder with characteristic multiple cartilage-capped tumours (osteochondromas or exostoses) growing outward from the metaphyseal region of the long tubular bones. Mutations in exostosin glycosyltransferase 1 (*EXT1)* or *EXT2* are the most commonly associated mutations with MO and are responsible for 70-95% of cases. In the present study, a genetic analysis was performed on a large family with MO using polymerase chain reaction and direct DNA sequencing of the entire coding regions of *EXT1* and *EXT2*. Sanger sequencing identified a novel heterozygous frameshift mutation, c.119_120delCT (p.Thr40ArgfsX15), in exon 2 of the *EXT2* gene in the proband and all other affected individuals, while this deleterious mutation was not detected in the healthy family members and normal controls. The c.119_120delCT mutation is located in the transmembrane region of the EXT2 protein and results in a truncated EXT2 protein lacking 665 amino acids at the C-terminus, which includes the critical exostosin and glycosyltransferase family 64 domains. Thus, the present study identified a novel causative frameshift mutation in *EXT2* from a large MO family. This study is useful for extending the known mutational spectrum of *EXT2*, for understanding the genetic basis of MO in the patients studied, and for further application of mutation screening in the genetic counseling and subsequent prenatal diagnosis of this family.

Introduction

Multiple osteochondroma (MO; MIM #133700 and #133701), also known as multiple hereditary exostoses, is an autosomal dominant skeletal disorder with characteristic multiple cartilage-capped tumours (exostoses or osteochondromas) growing outward from the metaphyseal region of the long tubular bones. The estimated prevalence of MO has been recorded to range from 1/100 in a small Guam population (1) to 1.3/100,000 in a European population (2) and at least 1/50,000 in a Western population (3). Osteochondromas are a consequence of the superfluous proliferation of chondrocytes and bone growth at the juxta-epiphyseal regions of the long bones, and are the most frequently identified benign bone tumour. Osteochondromas are slow-growing during the first decades of life, growing in number and size until the closure of the growth plates upon skeletal maturation at the end of puberty. Characteristic features of MO consist of significant inter- and intra-familial phenotypic variability, including variability in the size and number of the osteochondromas, the location and number of the associated bones, and the degree of the deformity. Although benign, osteochondromas can result in a number of secondary complications, including compression of the blood vessels, tendons and nerves. Individuals with osteochondroma are often of short stature, with deformities leading to problems in functionality. The most serious secondary complication is the malignant transformation toward a secondary peripheral chondrosarcoma, which occurs in 0.5-5% of cases (4).

MO is a genetically heterogeneous disease. At least 2 loci have been isolated by linkage analysis and positional cloning: *EXT1* (MIM #608177) maps to chromosome 8q24.11-q24.13, spans ~350 kb and is comprised of 11 exons (5), while EXT2 (MIM #608210) maps to chromosome 11p11-p11.2, spans almost 108 kb and is comprised of 16 exons (6). *EXT1* and *EXT2* belong to the putative tumour-suppressor *EXT* gene family, which also contains three homologous EXT-like (EXTL) genes: *EXTL1, EXTL2* and *EXTL3* (7-9). All *EXT* gene family members share a homologous carboxyl terminus and encode glycosyltransferases that are involved in heparin sulfate (HS) biosynthesis (10). A number of studies have investigated MO-causing mutations in a variety of populations (11-13). These data are being gathered in the Multiple Osteochondromas Mutation Database (http://medgen.ua.ac.be/LOVDv.2.0/). To date, >400 and 200 mutations have been identified in *EXT1* and *EXT2*, respectively. The majority of the mutations (80%) are nonsense, frameshift and splice site mutations, which lead to the premature termination of translation, or are involved in the partial or total deletion of the gene (14).

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The present study screened for mutations in the *EXT1/EXT2* genes using polymerase chain reaction (PCR) and direct sequencing in three generations of a family with MO. A novel frameshift mutation, c.119_120delCT (p.Thr40ArgfsX15), was consequently identified in exon 2 of the *EXT2* gene.

Patients and methods

Patients. Three generations of a MO family from Jilin, China, were investigated in the present study (Fig. 1). From the family, 8 members were affected with MO, including 2 deceased individuals. The proband (III-16) was a 28-year-old man suffering from multiple exostoses involving the bilateral proximal humeri, the left ulnar, the bilateral proximal and distal tibiae, the bilateral distal femurs and the right proximal femur (Fig. 2). On January 13, 2014, the proband underwent osteochondroma surgery for the right femur at Country Hospital of Fusong (Fusong, China) due to pain and functional limitations. At 15 days post-surgery (January 28, 2014), the proband suffered a subtrochanteric fracture of the right femur and was referred to the Second Hospital of Jilin University (Changchun, China), where internal fixation of the fracture site was performed.

Clinical data of each subject was recorded by nurse-administered questionnaires. Each participant underwent careful physical and/or radiographic examinations by two or more experienced experts at the Department of Orthopedics, The Second Hospital of Jilin University (Changchun, Jilin, China). MO was classified as at least 2 osteochondromas, arising from the lateral ends of the humeri, ulnae, femurs, tibiae, fibulae or knee joints. A group of 50 unrelated healthy subjects, matched for geographical ancestry, were included as controls. EDTA-anticoagulated peripheral blood (5 ml) was drawn from 10 family members (Table I) and from the 50 healthy controls. Written informed consent was obtained from all subjects or their respective guardians prior to their participation in the experimental protocol. This study was approved by the Ethics Review Committee of the Second Hospital of Jilin University.

Genetic analysis. Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. All exons and flanking intronic regions of the *EXT1* and *EXT2* genes were amplified by PCR using the primers listed in Table II. The amplifications were performed to a final volume of 50 µl, containing 1 µl genomic DNA, 25 µl 2X Premix Taq™ (Takara Biotechnology Co., Ltd., Dalian, China), 1 µl of each primer and 22 µl sterilized distilled water. All PCR programs included an initial denaturation of 5 min at 95˚C, followed by 35 cycles of 30 sec at 95˚C, 45 sec at annealing temperature (Ta) and 30 sec at 72˚C. Finally, an extension at 72˚C was performed for 10 min. Ta was 60˚C for all primer combinations, with the exception of primers for the amplification of overlapping regions of exon 1 of *EXT1*. For these primer combinations, Ta was set at 57˚C for ex1-2 and ex1-3. Direct sequencing of the PCR products was performed using BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) with the ABI 3730 automated sequencer. Sequences generated from patients were compared with the published *EXT1* and *EXT2* reference sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/; GenBank accession

Table I. Characteristics of study subjects in the MO family.

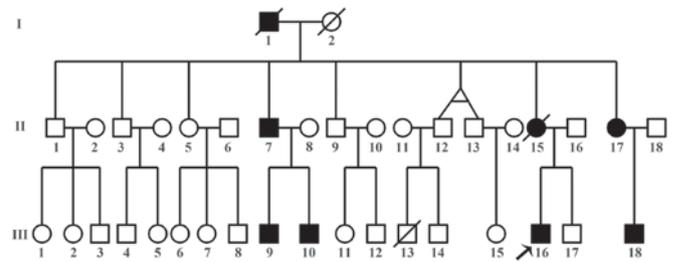| Subject	Age, years	Gender	MO status
II-5	62	Female	Unaffected
II-7	61	Male	Affected
II-17	48	Female	Affected
III-9	37	Male	Affected
III-10	33	Male	Affected
III-11	30	Female	Unaffected
III-14	29	Male	Unaffected
III-16	28	Male	Affected
III-17	24	Male	Unaffected
III-18	25	Male	Affected

MO, multiple osteochondroma.

Figure 1. Pedigree of the family with multiple osteochondroma. The proband (III-16) is noted with an arrow. ■, affected male; ●, affected female; □, healthy male; ○, healthy female. Boxes or circles with an oblique line mean that the individual is deceased.

nos. NM_000127.2 and NM_207122.1, respectively). When an insertion/deletion mutation was found, the amplified products with mutations were first ligated to a pMD-18T vector (Takara Biotechnology Co., Ltd.) and transformed into *Escherichia coli*. Next, sequencing was performed with plasmids extracted from *E. coli* using the AxyPrep Plasmid Miniprep kit (Axygen Biosciences, Union City, CA, USA). In addition, detected mutations were confirmed in another 50 unrelated healthy controls.

In silico analysis and homology study. When a mutation that had never been reported was found, the functional consequences of amino acid changes were predicted using the Mutation Taster software (http://www.mutationtaster.org). Regarding the homology study, ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to compare the human EXT2 protein sequence (NP_997005.1) with the homologs from *Pan troglodytes*, *Macaca mulatta*, *Mus musculus*, *Rattus norvegicus*, *Canis lupus familiaris*, *Bos taurus*, *Gallus gallus*, *Danio rerio* and *Xenopus* (sequences acquired from http://www.ensembl.org/).

Results

Genetic analysis identifies a novel frameshift mutation (c.119_120delCT) in EXT2 as the causative mutation in the MO family. Following sequencing of all the exons and

Table II. Primers used to amplify the exons of the *EXT1* and *EXT2* genes.

Gene	Exon	Upstream primer (5'-3')	Downstream primer (5'-3')	Amplicon length, bp	Ta, °C
EXT1	Ex1-1[a]	GGAGAGTTTGAAGTCTTTACAGGC	TGGGGTCCGAGGTGTAGAAC	561	60
	Ex1-2[a]	GGCTTGCAGTTTAGGGCATCGAG	TTGTTCCACAAGTGGAGACTCTG	485	57
	Ex1-3[a]	CCAGTTGTCACCTCAGTATGTGC	AACTTCACACCTGGACCAAG	543	57
	Ex2	TGCGTAAATTCATGCACATGG	GGAGAGGTGATAATGTTAAACC	268	60
	Ex3	TGCCAGTCATTGAGTTTGTAC	GGAGATTTTGTTGGAAAGTGAA	386	60
	Ex4	CTATATGCTAGAAGCCAAATGC	CAATATATCCAAGTACAGGAATC	372	60
	Ex5	TCCATTACTCTCTCTGTCTTG	ATGCAGGGTGTTAGATGGAC	410	60
	Ex6	CCTGTCAGGACATAAGAAGC	TGAAAGGGTGTAACGAGGC	426	60
	Ex7	TCTGCCGTTTTGTCTTGCTG	ATACACACAAGGTCACAAAGC	450	60
	Ex8	GTGAGGATGGGAGAATTGTC	AGGAATCGGGCTGATTAAAAC	334	60
	Ex9	GAATTAATGTTTCGCCACAGTC	AAACACACATTTGACACATCAG	435	60
	Ex10	CATCATCATTATCATTACCATTC	GGAGAGCTTTACATCCTTGG	525	60
	Ex11	TTGCTGCTTGCTCATTTGCC	TTTGTCATTCTGCTCATCTAAG	420	60
EXT2	Ex2-1[a]	TGAGTGACAGAGTGAAACCC	CAGAGCATAGATATACACCTTG	488	60
	Ex2-2[a]	AGCCGACAGTCCCATCCC	AAACCAACTCAAGAGCAGAAG	425	60
	Ex3	TGGGATTTCCAGGAGTTTGC	CACTTCTAAATCTTCAGGAGG	390	60
	Ex4	AGCAGAGAGGCTGTCCGTAA	ATAGGAAGCCGTTTCAGCAA	805	60
	Ex5	AGGGACTCAGATGTAACTAAGA	ACGAACACAAGACACCAGAC	792	60
	Ex6	AGTATTGCTTGGCGTCAACC	TAGACCAGTGTACTAACTCTC	401	60
	Ex7	AATGGAGCTGTAAGAGAACTC	ATCTAGTGGAGGAAGTAAACC	408	60
	Ex8	TGGCTTGAACAGCAGGGAG	AATTATGCTGCCCTTATCAGG	446	60
	Ex9	CACCAAGCCTGCCATGTTTG	GGCATGCTGTCTCAGAAATG	408	60
	Ex10	ACCTTTGGATTTGATGAGAGC	TAACCCACACTCTTACGCAC	450	60
	Ex11	TCTCCAGAATCCCATTATGAC	CATATTTTCTACTATGAGCGTG	469	60
	Ex12	GTCACTTGACCAAAAGCATTC	GAGCTTAAAGTTTATCTAGTCC	417	60
	Ex13	CTTGTGAGTTCTGCCGTTGG	ACAAATTGAGTGAGTAGCATTC	471	60
	Ex14	ACCTGTCAACCTTTTTAAGAAC	CCAAGATCCAAGTAGGTCAAC	445	60

[a]Exon 1 of *EXT1* and exon 2 of *EXT2* were amplified in 3 and 2 fragments, respectively. Ta, annealing temperature.

Figure 2. Posterior-anterior radiograph and 3D reconstruction images of osteochondromas (indicated by white arrows) in the proband. (A and B) Single osteochondroma in the bilateral humeri. (C) MOs in the femurs and fibula of the lower limbs. (D) Osteochondroma in the juxta-epiphyseal region of the left ulnar. (E and F) Osteochondromas in the bilateral tibiae. (G) 3D reconstruction images of knees of the MO proband. MO, multiple osteochondroma; 3D, three-dimensional.

flanking intronic regions of the *EXT1* and *EXT2* genes, no mutations were detected in the *EXT1* gene, but a heterozygous deletion of two nucleotides (CT) at c.119_120 in exon 2 of the *EXT2* gene was found in the proband (Fig. 3). Subsequently, this variation was detected in all the 5 affected individuals (II-7, II-17, III-9, III-10 and III-18) in this pedigree by PCR and
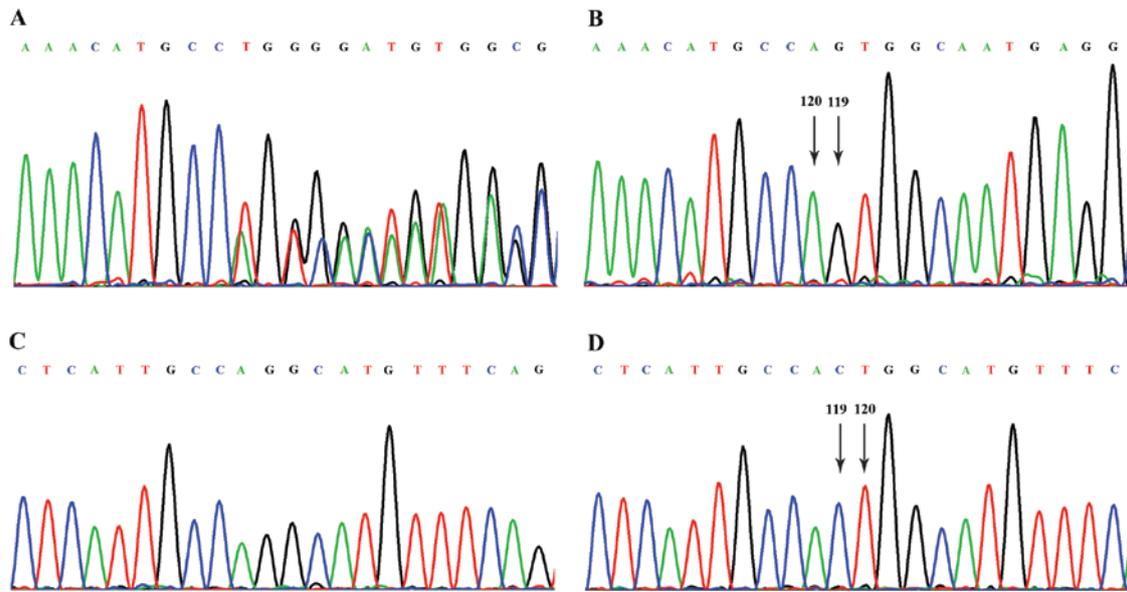
Figure 3. Sanger sequencing results from DNA of the proband's blood and from plasmids extracted from *E. coli*. (A) Reverse-sequencing result of the proband from DNA in blood. (B) Reverse-sequencing result of the unaffected family members and normal controls from DNA in blood. (C) Forward-sequencing result of the plasmid showing the deletion of two nucleotides (CT) at c.119_120 of the *EXT2* gene. (D) Forward-sequencing result of normal *EXT2* sequence from plasmid.
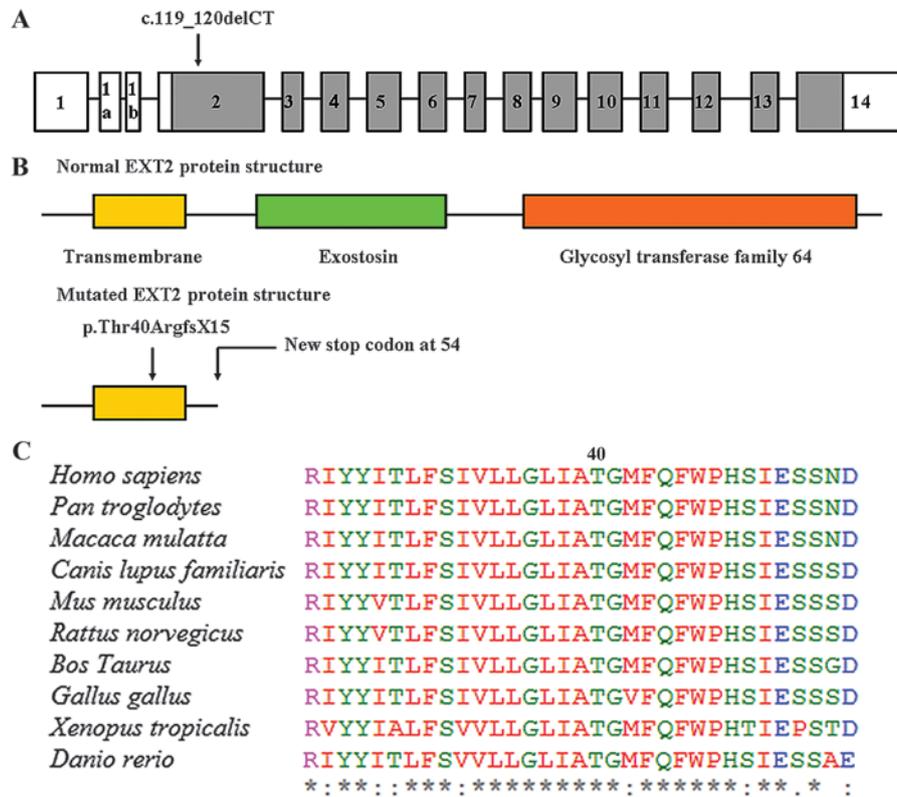

Figure 4. *EXT2* gene and protein structural and homology study result of the identified c.119_120delCT (p.Thr40ArgfsX15) frameshift mutation. (A) Intron-exon structure of the *EXT2* gene. (B) Comparison of the functional domains of EXT2 proteins encoded by mutated and normal *EXT2* genes. (C) Multiple sequence alignment of EXT2 orthologs around codon 40. Codon 40 is highly conserved across various vertebrate species.

direct sequencing, and was not detected in any of the 4 unaffected family members (II-5, III-11, III-14 and III-17) or in the 50 healthy controls. Thus, this finding suggested that the novel c.119_120delCT frameshift mutation in the *EXT2* gene was the causative mutation for MO in this family.

In silico analyses indicate the critical impact of the c.119_120delCT mutation on EXT2 function. The *EXT2* gene consists of 16 exons, and the c.119_120delCT mutation was located in exon 2 (Fig. 4A). To understand the potential impact of the c.119_120delCT frameshift mutation on EXT2

function, *in silico* analyses were performed and the predicted result was disease-causing. The EXT2 protein is composed of 718 amino acids, with one transmembrane region (amino acids 26-46) and two critical domains: The exostosin domain (amino acids 100-380) and the glycosyltransferase family 64 domain (amino acids 456-701) (Fig. 4B). The c.119_120delCT frameshift mutation results in the substitute of amino acid Threonine by Arginine at position 40 and creates a premature translational termination signal at amino acid position 54 of the EXT2 protein (Fig. 4B). Compared with normal EXT2 protein, the mutated EXT2 protein lacks 665 amino acids at the C-terminus, including the two critical exostosin and glycosyltransferase family 64 domains. Thr40 is located in the transmembrane region, which is highly conserved across various vertebrate species (Fig. 4C), indicating its functional importance.

Discussion

EXT1 and *EXT2* are tumour suppressor genes encoding for endoplasmic reticulum-localized type II transmembrane glycoproteins (15). EXT1 and EXT2 form a hetero-oligomeric complex in the Golgi apparatus that catalyses the alternative transfer of *N*-acetyl-glucosamine and D-glucuronic acid residues to the elongating HS glycosaminoglycan chain (16). This observation is consistent with the notion that inherited mutations in either of the two *EXT* genes could result in MO. HS and HS proteoglycans are known as regulators of the distribution and receptor binding of signalling molecule family members such as transforming growth factor-β, fibroblast growth factor, and Indian hedgehog (IHH) (17). Previously, studies showed that mutations in the *EXT1/EXT2* genes impaired the biosynthesis of HS and that a decreased content of HS disturbs the IHH/parathyroid hormone-related peptide signalling pathway in MO; this disturbance leads to abnormal endochondral ossification resulting in exostoses formation (18,19).

EXT1 and *EXT2* gene mutations have been reported to be involved in the pathogenesis of MO and are responsible for 70-95% of MO cases. *EXT1* accounts for 56-78% of the MO families, whereas an *EXT2* mutation is detected in 21-44% of cases (20). *EXT1/EXT2* mutation analysis demonstrated that mutations in *EXT1* are more common in the Caucasian population in North America and Europe. However, these associations were different in the Chinese population, where mutations in *EXT2* were more common than *EXT1* mutations (21). In the past 10 years, 67 Chinese families affected with MO and 10 spontaneous cases affected with MO have been screened for mutations in *EXT1* or *EXT2*. Mutations in the *EXT1* gene account for 37.7% (29/77) of the patients, whereas *EXT2* mutations account for 44.2% (34/77), which is higher than that of *EXT1*. Based on all the aforementioned data, *EXT2* mutations may be responsible for more cases of MO in the Chinese population. *EXT2* mutations tend to be concentrated in the first two-thirds of the coding region, and mutations may occur more commonly in exon 2 compared with other exons of *EXT2*, whereas mutations in *EXT1* are reported to be scattered throughout the complete gene.

In the present study, a large family with MO was investigated and a sequence analysis was performed of the entire coding regions of *EXT1* and *EXT2* for the proband (III-16).

A heterozygous deletion of two nucleotides (CT) was found at c.119_120 in exon 2 of *EXT2* in the proband, and this variation was also present in all the other 5 affected patients (II-7, II-17, III-9, III-10 and III-18) from this pedigree, but was not detected in any of the 4 unaffected family members (II-5, III-11, III-14 and III-17) or in the 50 healthy controls. This mutation (c.119_120delCT; p.Thr40ArgfsX15) results in a frameshift mutation starting from amino acid position 40 and introduces a premature termination signal at amino acid 54, which leads to the production of a truncated EXT2 protein with only 53 amino acids, 665 amino acids shorter than the normal EXT2 protein. Furthermore, the c.119_120delCT mutation was located in the transmembrane region of the EXT2 protein, the mutated EXT2 protein lacking 665 amino acids at the C-terminus, which included the two critical exostosin and glycosyltransferase family 64 domains.

In conclusion, the present study identified a novel deleterious frameshift mutation, c.119_120delCT (p.Thr40ArgfsX15), in the transmembrane region of the *EXT2* gene from a large MO family. This study is useful for extending the known mutational spectrum of *EXT2*, for understanding the genetic basis of MO in the patients studied, and for further application of mutation screening in the genetic counseling and subsequent prenatal diagnosis of this family.

Acknowledgements

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