A novel *EXT2* frameshift mutation identified in a family with multiple osteochondromas

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Abstract. Multiple osteochondromas (MO) is an autosomal inherited disease that is characterized by benign bone tumors. However, the underlying mechanism of MO at a molecular level requires further investigation. The majority of mutations associated with MO occur in the exostosin glycosyltransferase genes (*EXT)1* or *EXT2*. In the present study, the genetic causes of the disease were investigated. Polymerase chain reaction amplification, followed by DNA sequencing of the complete *EXT1* and *EXT2* coding regions, were conducted in a family with MO (n=5). A novel frameshift mutation in exon 3 of *EXT2* (c.660delG) was detected. The production of a defective EXT2 protein, lacking 450 C-terminal amino acid residues is predicted to be caused by the c.660delG mutation, located within the exostosin domain of *EXT2*. The missing residues contain the exostosin and glycosyltransferase family 64 domains, which are critical for the function of EXT2. The novel c.660delG frameshift mutation in the *EXT2* gene extends the etiological understanding of MO and may provide an effective reference for genetic counseling and prenatal diagnosis in this family.

Introduction

Multiple osteochondromas (MO), also referred to as multiple hereditary exostoses, is an autosomal dominant bone disease with a prevalence of 1/50,000 in the Western population (1). Although MO cases can be sporadic, ~80% of patients with MO have a family history of the disease (2,3). The size and

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number of osteochondroma tumors continually increase prior to adulthood (4). The disease is characterized by ≥1 overgrown exostoses capped by cartilage, usually occurring at the metaphyses of the long bones and bilateral limbs (1). Although the majority of cases of MO have been reported to involve benign bone tumors and a lack of typical symptoms, osteochondromas can cause deformities, functional limitation, compression of the nerves and blood vessels, and pain by placing pressure on adjacent tissues (5). Malignant transformation into chondrosarcoma or osteosarcoma has been reported as the most severe complication, occurring in 0.5-5% of patients (6).

Although the molecular mechanisms of MO require further investigation, previous studies have reported that mutations in the exostosin glycosyltransferase genes, *EXT1* and *EXT2*, serve an important role in the pathogenesis of MO (7-9). *EXT1* and *EXT2* are members of the exostosin family and are located on chromosome 8q24.11-q24.13 and 11p12-p11, respectively. It has been reported that EXT genes encode proteins involved in the biosynthesis of heparan sulfate (HS), a key molecule in adjusting chondrocyte proliferation and bone growth (10). In addition, it has been demonstrated that dysfunction of EXT proteins may trigger the occurrence of MO (11,12). The majority of mutations that have been identified in *EXT1* and *EXT2* are nonsense, frameshift or splice-site (13), which have been reported to cause truncation, premature termination or premature degradation of EXT proteins (9).

In the present study, a genetic analysis of a family with a history of MO was conducted using polymerase chain reaction (PCR) amplification of the entire coding regions of *EXT1* and *EXT2*, followed by DNA sequencing. A novel frameshift mutation, c.660delG (p.L221Cfs*82), was identified in exon 3 of the *EXT2* gene.

Materials and methods

Participants. A total of 3 generations of a family with MO from Zhejiang (China) were investigated in the present study (Fig. 1). A total of 4 members of the family were affected with MO, including a deceased individual (I-1). The proband (III-3) was a 17-year-old female with MO, involving the proximal and distal tibiae, distal femur and proximal fibula (Figs. 1 and 2). The proband underwent an operation for osteochondroma on

the left femoral and tibial condyle at the Zhejiang Provincial People's Hospital (Hangzhou, China), due to pain and functional limitations. Clinical information of the family was collected by a surgical resident, with each participant receiving a physical and/or radiographic examination by two attending physicians. Venous blood samples (5 ml) were collected from five family members (II-2, II-3, II-4, III-2 and III-3) using ethylenediami-netetraacetic acid-anticoagulant vacuum blood collection tubes (Zhejiang Gongdong Medical Technology, Hangzhou, China). The present study was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (Hangzhou, China) and informed written consent was obtained from all patients.

Genetic analysis by PCR. Genomic DNA was purified from peripheral blood leukocytes using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The primers listed in Table I were used to amplify all exons of the *EXT1* and *EXT2* genes using PCR. The DNA sequences of the amplified *EXT1* and *EXT2* coding regions were then analyzed by Sanger sequencing using a BigDye® Terminator V3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an ABI 3730XL sequencer (SoftGenetics LLC, State College, PA, USA), according to the manufacturer's protocols. The PCR (KAPA2G Fast Multiplex mix; Roche Applied Science, Penzberg, Germany) was performed at 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 6 min. Mutation Surveyor Demo software 4.0 (SoftGenetics LLC) was used to analyze the sequence data, with the National Center for Biotechnology Information reference sequences (*EXT1*: NM_000127.2; *EXT2*: NM_000401) serving as control sequences.

In silico analysis. Mutation Taster (http://www.mutationtaster. org) was used to indicate possible changes in amino acids, which may result from mutations identified in patients with MO.

Results

A novel EXT2 mutation in a family with MO. No abnormalities were detected in the *EXT1* gene, following sequencing of each exon of the *EXT1* and *EXT2* genes in the proband. However, a heterozygous G deletion in exon 3 of the *EXT2* gene at c.660 was identified, which was predicted to result in a frameshift (Fig. 3). Subsequently, family members, who were also affected with MO, were identified to exhibit this mutation. None of the unaffected family members exhibited this mutation. Therefore, this indicates that the novel *EXT2* c.660delG frameshift mutation is the genetic determinant for the occurrence of MO in this family.

In silico analyses. *In silico* analyses were performed in order to understand the potential functional impact of the c.660delG mutation in the *EXT2* gene. The EXT2 protein was predicted to contain one transmembrane region (aa 26-46), an exostosin domain (aa 100-380) and a glycosyltransferase family 64 domain (aa 456-701) (Fig. 4) (14). The c.660delG mutation resulted in a frameshift, where leucine was replaced with cysteine at amino acid position 221 and a termination codon was introduced at position 302. Compared with the normal
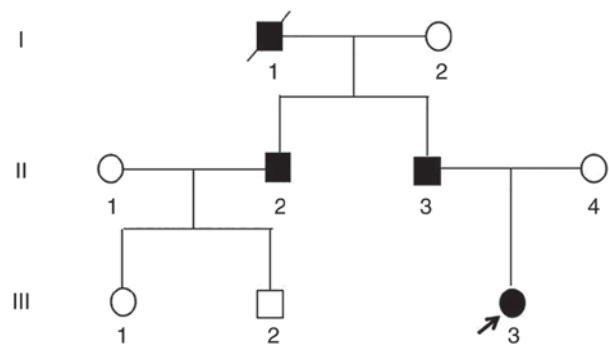

Figure 1. Family pedigree with multiple osteochondromas. The black arrow notes the proband (III-3). Empty symbols indicate healthy individuals and filled symbols indicate affected individuals with multiple osteochondromas. The diagonal line indicates a deceased individual.
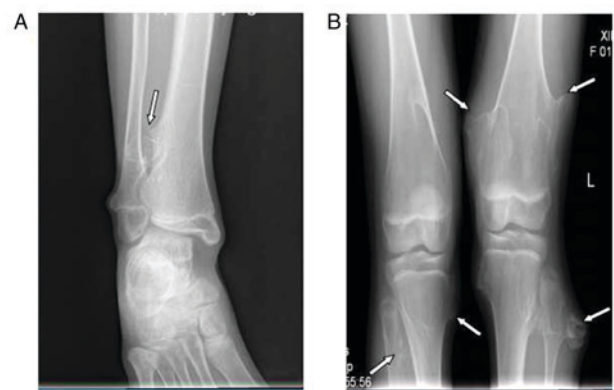

Figure 2. Clinical features of the proband patient with MO. (A) Osteochondroma in the right tibia (white arrow). (B) Osteochondromas in the femurs, tibiae and fibulae of the legs (white arrows). MO, multiple osteochondromas.
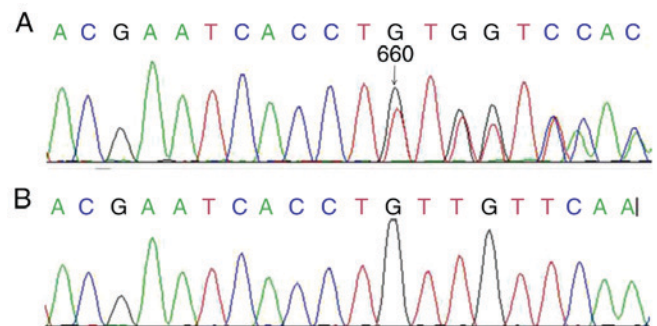

Figure 3. Identification of a novel frameshift mutation (c.660delG) in the *EXT2* gene. (A) A novel deletion mutation was identified in the *EXT2* gene of the proband. (B) No mutations were present at the same location in unaffected family members. EXT, exostosin glycosyltransferase.

EXT2 protein sequence, the mutated protein possessed an aberrant exostosin domain and lacked the glycosyltransferase family 64 domain (Fig. 4).

Discussion

EXT1 and *EXT2* are tumor-suppressor genes that encode endoplasmic reticulum-type II transmembrane glycoproteins.

Table I. Primer sequences used for sequencing the exons of the *EXT1* and *EXT2* genes.

| Primer	Reverse	Forward
EXT1		
Exon1	5'-TCTAGCTGCACACCCGAAC-3'	5'-TGGAGCTGAAAGTGTTGATTGG-3'
Exon2	5'-AGATCCTCAAGGGAAACCACAC-3'	5'-TGCCAGAACGATCAGACTTGT-3'
Exon3	5'-GGGACCTGGGGAGATTTTGTT-3'	5'-ACGTACAACCTTCGTGTGACT-3'
Exon4	5'-TCCTTTGTGGAGTTTGTCAGGA-3'	5'-GCAGCTGACACTTCTTAAGGT-3'
Exon5	5'-GCAATCTTCAATGCAGGGTGT-3'	5'-AGTGGGAGGGAGGGTAGAGT-3'
Exon6	5'-AGGGTGTAACGAGGCAGGAT-3'	5'-AGGCAGTAGCTAACCAGCAG-3'
Exon7	5'-AATCCAGGAACAGGGAGAAGAT-3'	5'-TCTGCCGTTTTGTCTTGCTG-3'
Exon8	5'-GGTGAGGCCCAGTTATGCAA-3'	5'-CTTCGGTGTTGAGGGGTGAC-3'
Exon9	5'-CAGCAAAACTTAAGCGGGGATAC-3'	5'-TTATGGGGCAAAATGTCAAGCAG-3'
Exon10	5'-CCCTGCACCAATCATACACTCT-3'	5'-AGGGATTCAAAGAATGGGTATGTGT-3'
Exon11	5'-GGATAGTTGGCACAATCTGGC-3'	5'-AAGTCGTGGACTGACCAGAG-3'
EXT2		
Exon1	5'-CCAGCAATCTAGTCCGCAGT-3'	5'-CTGCTTCTCCTTCTCCTCCG-3'
Exon2	5'-TGCAAGTCAGTCCCTCCAAC-3'	5'-TTCACCACACCACTTAAGGGAA-3'
Exon3	5'-GTGCACAATCCAGAGTGGGA-3'	5'-TGTCTGATGTGCTGTTGGGA-3'
Exon4	5'-CTGCATGGAGGTGGGAAAGA-3'	5'-GATAGAACGCAGCTGATGGC-3'
Exon5	5'-ACACAAGACACCAGACATCCA-3'	5'-GTGGAGGTGAAGACTGGTAAGG-3'
Exon6	5'-GCCTTGGTTTGTGAACTGCT-3'	5'-TGTGAGCTGTTGTCTTTTGGC-3'
Exon7	5'-TGCATCTGCTATGGTAAGACCC-3'	5'-ATACTTACCGGAAGGGATGTGG-3'
Exon8	5'-CCCTTATCAGGCCCCAAGTA-3'	5'-CACCCCCATCCCTACAACTT-3'
Exon9	5'-CCACCCTAGAACAAGAATGAGATGA-3'	5'-GAGGGGAAGACTTTGAGCAGTTG-3'
Exon10	5'-CTTACGCACACCTTTTGGACTC-3'	5'-TGCATCTGTGAGAATCTCCCCTG-3'
Exon11	5'-TCTACTATGAGCGTGCATTAC-3'	5'-TTTGGCAGAATAACTAACACC-3'
Exon12	5'-AGCTTCAATTTCCCAATGTGACC-3'	5'-AATGCCTCCTTTTACCCTTCCTA-3'
Exon13	5'-CTGGAGCTTAAGGCCCACTAT-3'	5'-CACGCGCATGCAACATCTC-3'
Exon14	5'-ACACGCTTCTCTTCTCCAGC-3'	5'-CCAAGTACCCCCAGTTCACC-3'

EXT, exostosin glycosyltransferase.
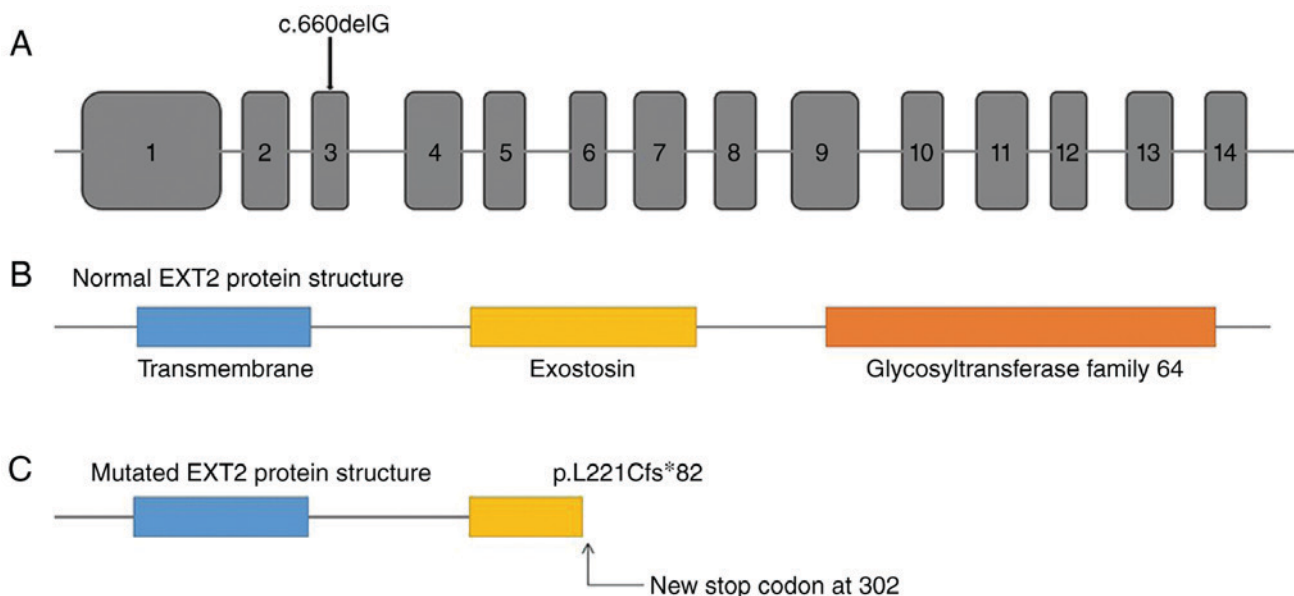
Figure 4. *EXT2* gene and the result of the c.660delG frameshift mutation on protein structure. (A) Exon structure of the *EXT2* gene. (B) Functional domains of the EXT2 protein produced from normal *EXT2* genes. (C) Functional domains of the EXT2 protein produced from mutated *EXT2* genes. EXT, exostosin glycosyltransferase.

Dysregulation or loss of function of these genes results in multiple exostoses (13,15). EXT1 and EXT2 combine to form a stable hetero-oligomeric complex, catalyzing the polymerization of HS chains in the Golgi apparatus and endoplasmic reticulum (16). HS is universally expressed on the surface of cells and is a major component of glycoproteins in the extracellular matrix. It also functions as a regulator of cell adhesion, the receptor-ligand binding process and signal transduction (17-20). Previous studies have reported that HS is a crucial determinant in the growth and differentiation of chondrocytes, and irregularities in its production may be associated with MO (21,22).

Mutations in *EXT1* and *EXT2* are associated with MO pathogenesis, although *EXT1* mutations have been reported to be more common than *EXT2* mutations (13). However, a reverse effect is observed in the Chinese population, in which *EXT2* mutations have been indicated to occur at a higher frequency than *EXT1* mutations (23). In the present study, a three-generation family with a history of MO was investigated and an analysis of mutations in *EXT1* and *EXT2* was performed in five of the family members (three affected by MO and two unaffected individuals). In the proband and the other two affected members of the family, a heterozygous deletion of one nucleotide (G) was identified in exon 3 of *EXT2* at c.660 (p.L221Cfs*82). The mutation was absent in the unaffected family members, whose *EXT* genes were sequenced. In addition, according to the distribution of patients with MO in the family pedigree (Fig. 1), the c.660delG mutation indicates an autosomal dominant pattern of inheritance from the proband's grandfather to the proband's father.

The novel mutation (c.660delG) was identified to be located in the exostosin domain of the *EXT2* gene, resulting in a frameshift and an introduction of a premature termination codon at position 302. At only 301 amino acids in size, the truncated EXT2 was identified to be shorter compared with the normal EXT2 protein. In addition, as the c.660delG mutation was indicated to be located within the exostosin domain, the mutant EXT2 protein indicated a defective exostosin domain and was absent in the glycosyltransferase family 64 domain. The glycosyltransferase domain serves a crucial role in HS biosynthesis, and therefore its absence may abolish the function of the EXT2 protein. The novel mutation introduced a stretch of 81 amino acids and may result in a novel function of the mutant EXT2 protein. Further investigation is required to examine the functional change in EXT2 protein caused by the c.660delG mutation.

In conclusion, a novel frameshift mutation in the *EXT2* gene was identified (c.660delG), and *in silico* analysis revealed that this mutation may produce a dysfunctional EXT2 protein. The present study extends the existing knowledge of mutations that occur in *EXT2* and provides novel insight into the genetic determinants underlying the pathogenesis of MO. Furthermore, the present study offers valuable information for genetic counseling and prenatal diagnosis in this family.

Acknowledgements

Not applicable.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZHC and QB performed most of the experments and data analysis. LC and MXK collected information of this family. WWR helped with biochemical experiments.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital (Hangzhou, China) and informed written consent was obtained from all patients participating in the present study.

Patient consent for publication

All patients provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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