Identification of a novel EXT2 frameshift mutation in a family with hereditary multiple exostoses by whole-exome sequencing

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Abstract

Background: Hereditary multiple exostoses (HME), also referred to as multiple osteochondromas, is an autosomal dominant skeletal disease characterized by the development of multiple overgrown benign bony tumors capped by cartilage and is associated with bone deformity, joint limitation, and short stature. Mutations in exostosin glycosyltransferase (EXT1) and EXT2 genes, which are located on chromosomes 8q24.1 and 11p13, contribute to the pathogenesis of HME.

Methods: In the present study, a genetic analysis of a four-generation Chinese family with HME was conducted using whole-exome sequencing (WES), followed by validation using Sanger sequencing.

Results: A novel heterozygous frameshift mutation in exon 5 of EXT2 (c.944dupT, p.Leu316fs) was identified in all affected individuals but was not detected in any unaffected individuals. This mutation results in a frameshift that introduces a premature termination codon at position 318 (p.Leu316fs) with the ability to produce a truncated EXT2 protein that lacks the last 433 amino acids at its C-terminal to indicate a defective exostosin domain and the absence of the glycosyltransferase family 64 domain, or to lead to the degradation of mRNAs by nonsense-mediated mRNA decay, which is critical for the function of EXT2.

Conclusion: Our results indicate that WES is effective in extending the EXT mutational spectra and is advantageous for genetic counseling and the subsequent prenatal diagnosis.

KEYWORDS
bone tumor, EXT2, frameshift mutation, hereditary multiple exostoses, whole-exome sequencing

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1 | INTRODUCTION

Hereditary multiple exostoses (HME), also referred to as multiple osteochondromas, is an autosomal dominant skeletal disease. The disease is characterized by the development of multiple overgrown benign bony tumors capped by cartilage, generally occurring at the metaphyses of the long bones. Exostoses may be asymptomatic or result in pain, bone deformity, joint limitation, and short stature. The incidence of exostoses is estimated to be 1/50,000 in the western population, which occurs more frequently in males than in females (~1.5:1). The most serious complication of HME is malignant transformation into chondrosarcoma or osteosarcoma, occurring in 1%-2% of the patients.

Although the molecular mechanisms associated with HME are still not fully understood, previous studies have reported that mutations in the exostosis glycosyltransferase 1 (EXT1) (OMIM: 608177) and EXT2 (OMIM: 608210) genes, which are located on chromosome 8q24.1 and 11p13, respectively, contribute to the pathogenesis of HME. To date, more than 862 mutations in EXT1 (568) and EXT2 (294) have been identified, most of which are nonsense, frameshift, or splice site mutations that result in the synthesis of truncated EXT protein with dysfunctional activity.

With the rapid development of next-generation sequencing technologies, whole-exome sequencing (WES) has already been applied in the investigation of rare genetic diseases to reveal novel pathogenic single-nucleotide variants (SNVs). In this study, a genetic analysis of a four-generation Chinese family with HME was conducted using WES, followed by validation using Sanger sequencing. A novel heterozygous frameshift mutation (c.944dupT) was identified in exon 5 of EXT2, which may lead to structural changes in the EXT2 protein (p. Leu316fs).

2 | MATERIALS AND METHODS

2.1 | Patients and clinical data

A four-generation Chinese family with 10 affected members, including a deceased individual, was investigated in this study. The proband was a 28-year-old woman who sought genetic counseling before pregnancy, whose chief complaint was a left forearm deformity, which led to limited physical activity, including pronation and supination, as well as bony prominences around the right knee without movement limitation. Her height was 1.65 m, weight was 48 kg, and body mass index was 17.6 kg/m². The pedigree of this family was drawn, and the complaint was found to be related to a typical autosomal dominant disorder (Figure 1). The diagnostic criteria for HME were at least two exostoses detected by radiography or palpated in the metaphysis of long bones. After the related clinical examinations, including X-ray imaging (Figure 2) and physical examinations, were carried out in three affected individuals (II-2, III-5, III-6), the diagnosis of HME was confirmed. Blood samples (4 ml) were collected from the affected members (II-2, II-6, II-7, II-9, III-5, III-6) and unaffected members (II-3, II-4, II-10). Clinical data of the patients with HME in the family are shown in Table 1.

This study was approved by the Medical Ethics Committee of West China Second University Hospital, Sichuan University, and written informed consent was obtained from all participants.

2.2 | Whole-exome sequencing

Genomic DNA of the family members was extracted from the blood according to the manufacturer's instructions (Qiaamp DNA Blood Mini Kit).
Genomic DNA of the family members in trio (proband and both parents [III-5, II-9, II-10]) was subjected to WES. The Nano WES Human Exome V1 (Berry Genomics) was used to capture the sequences. The Illumina NovaSeq6000 platform with 150-bp paired-end reads was used to sequence the enriched library. Next, the Burrows–Wheeler Aligner software tool was used to align the sequencing reads with hg18/GRCh38. Subsequently, local alignment and recalibration of the base quality of the Burrows–Wheeler aligned reads were performed using the GATK Indel Realigner and the GATK Base Recalibrator, respectively (broadinstitute.org/). Then, the GATK Unified Genotyper (broadinstitute.org/) was used to identify SNVs and small insertions or deletions (InDels). Finally, variants were annotated and interpreted using ANNOVAR and the Enliven Variants Annotation Interpretation System, which was authorized by Berry Genomics. The quality control of WES has been illustrated in supplementary table (Table S1).

During the interpretation and analysis of the data, we selected the variations if their minor allele frequencies were <0.05 in gnomAD (http://gnomad.broadinstitute.org/), the 1000 Genomes Project (1000G) (http://browser.1000genomes.org), and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/).

SNVs with minor allele frequency (MAF) ≥1% for a recessive model or ≥0.1% for a dominant model were excluded. The detected SNVs were systemically evaluated for pathogenicity based on scientific and medical literature and disease databases obtained from the following resources: PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), OMIM (http://www.omim.org), Human Gene Mutation Database (http://www.hgmd.org), and Human Genome Variation Society (http://www.hgvs.org/dblist/dblist.html). SNVs were classified into five categories: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign, according to the guidelines of ACMG based on population data, computational and predictive data, functional data, segregation data, de novo data, and allelic data. The detailed process for identifying candidate variants is shown in Figure 3.

2.3 | Sanger sequencing

To validate the identified variants by WES, Sanger sequencing was performed to confirm candidate variants in each independent gDNA

TABLE 1 | Clinical data on patients with HME in the family

| Patient | Gender | Age, years | Age of onset, years | Height, m | Involved sites |
|---------|--------|------------|---------------------|-----------|---------------|
| I-1     | Male   | decreased  | unknown             | unknown   | forearm, humerus, femur |
| II-2    | Female | 51         | <10                 | 1.58      | humerus, ulna, femur |
| II-6    | Female | 46         | <10                 | 1.54      | humerus, ulna, femur |
| II-7    | Male   | 45         | <10                 | 1.67      | humerus, femur, tibiae, fibulae |
| II-9    | Male   | 48         | <10                 | 1.65      | humerus, ulna, radius, femur, tibiae, fibulae |
| III-1   | Male   | 21         | 2                   | 1.72      | humerus, femur |
| III-3   | Male   | 29         | 2                   | 1.71      | humerus, femur, ulna |
| III-5   | Female | 26         | 3                   | 1.65      | ulna, fibulae |
| III-6   | Male   | 22         | 2                   | 1.74      | humerus, femur |
| IV-1    | Male   | 0.5        | At birth            | 0.62      | rib |

FIGURE 2 | Radiograph images of patients, III-5 (the proband) (A, B), III-6 (C), and II-9 (D–F). Exostoses were indicated by white arrows. (A) A relatively curved left ulna and radius, with exostoses on the ulna; (B) exostoses on the right proximal fibulae; (C) exostosis on the right proximal humerus; (D) exostosis on the left humerus; (E) exostosis on the left proximal humerus radius; (F) exostoses on the left distal femur, proximal tibiae, and fibulae.
Genomic DNA was extracted from the blood samples of proband and both parents (III-5, II-9, II-10)

Whole-exome sequencing was performed in trio

Burrows-Wheeler Aligner software tool was used for aligning the sequencing reads with hg18/GRCh38

GATK Unified Genotyper (broadinstitute.org/) was used for identifying SNVs and InDels

Variants were annotated and interpreted using ANNOVAR and the Enliven Variants Annotation Interpretation System

Identification and analysis all variants with minor allele frequencies are less than 0.05 in gnomAD, 1000 Genomes Project, and ExAC

Variants were classified according to the guidelines of ACMG

Identification of de novo / dominant variants and segregation analysis

Evaluation with databases and literature review (PubMed / ClinVar / Human Gene Mutation Database)

Characterization of the gene / variants structure and function

c.944dupT in EXT2 gene in proband (III-5) and affected father (II-9)

**FIGURE 3** The detailed process for identifying candidate variants

Sample (II-9, II-10, III-5). The segregation analysis of candidate variants was traced through other affected individuals (II-2, II-6, II-7, III-6) and unaffected individuals (II-3, II-4), also by using Sanger sequencing.

The primers were designed for standard polymerase chain reaction assays using Primer 3 software (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/). For the EXT2 mutation (GenBank NM_000401.3, c.944dupT, p. Leu316fs), primers were designed as follows: forward 5’-TGG AGG TGA AGA CTG GTA A-3’, reverse 5’-TTG CGA GAG GTA ATG AAT GA-3’. PCR was performed in a total volume of 25 μl containing 5 μl of DNA, 2.5 μl of 10x buffer (MgCl₂ plus), 0.5 μl of dNTPs, 1.5 μl of each primer, 0.5 μl of Taq, and 13.5 μl of H₂O. The PCR program was performed on a Veriti® 96-Well Thermal Cycler (Thermo Fisher) as follows: 95°C for 10 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 45 s, and 72°C for 5 min. PCR products were sequenced using an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific) for c.944dupT.

2.4 | In Silico analysis

Possible changes in the amino acids, which may result from the mutation c.944dupT identified in patients with HME, were predicted using PROVEAN (Protein Variation Effect Analyzer, version 1.1.3).
3 | RESULTS

3.1 Novel EXT2 mutation in a Chinese family with HME

To reveal the genetic determinants of HME in this family, WES was performed in triplicate. A heterozygous frameshift mutation in exon 5 of EXT2 was detected (NM_000401.3, c.944dupT) in the proband, which was inherited from the patient’s affected father; no mutation was detected in her unaffected mother. No secondary findings associated with the genes recommended by ACMG were detected in our study.17

The presence of the mutation was further validated by Sanger sequencing and was confirmed in all affected members (II-2, II-6, II-7, II-9, III-5, III-6) but was not detected in any unaffected members (II-3, II-4, II-10) (Figure 4).

3.2 In silico analysis

To understand the potential impact of the c.944dupT mutation on EXT2 function, an in silico analysis was performed. The mutation (c.944dupT) resulted in a frameshift that led to a change in leucine to threonine at amino acid position 316 and a premature stop codon at amino acid position 318 (p. Leu316fs). This mutation produces a truncated EXT2 protein that lacks the last 433 amino acids at its C-terminus.

The EXT2 protein (NP_000392.3), composed of 751 amino acids, contains two vital domains [the protein families (Pfam) database (http://pfam.xfam.org)]: an exostosin domain (134–413 amino acids) and a glycosyltransferase family 64 domain (490–723 amino acids). Amino acid 316 is located within the exostosin domain and prior to the glycosyltransferase domain.

The mutated protein contained an aberrant exostosin domain and lacked the glycosyltransferase family 64 domain (Figure 5). PROVEAN (Protein Variation Effect Analyzer, version 1.1.3) was used to predict the pathogenicity of the mutation (p. Leu316fs) in EXT2, which revealed the mutation to be pathogenic (PROVEAN: deleterious, with a score of −1267.928).

4 | DISCUSSION

HME is an autosomal dominant disorder with variable clinical characteristics, including symmetrical bony protrusions in long bones, disproportionate limbs, and short stature,18 even with a wild-type clinical heterogeneity in the same family.1,4 Although the exact etiology of HME is still under investigation, approximately 70%–95% of patients with HME have mutations in the EXT1 or EXT2 gene.19,20 Mutations in EXT1 account for 56%–78% of the patients with HME, whereas mutations in EXT2 are detected in 21%–44% of the patients.5 However, EXT2 mutations are more common than EXT1 mutations in the Chinese population.21 Several studies have indicated that patients with EXT1 mutations have a more severe phenotype than those with EXT2 mutations.22-24 The most commonly affected bones with exostoses were the long bones of the upper and lower limbs, while the second most commonly affected bones were those in the chest.24

Whether the severity of HME is associated with sex is controversial. In our study, it seemed that a larger number of lesions and more severe phenotypes were observed in men than in women, which is consistent with previous literature.25,26 However, Porter et al. and Fusco et al. demonstrated that the severity of HME did not differ significantly with sex.23,24 Thus, a larger cohort study should be conducted in the future.

The EXT gene encodes a type II transmembrane protein that forms a heterooligomeric complex that catalyzes the polymerization of heparan sulfate (HS), a key component in the adjustment of chondrocyte proliferation and endochondral ossification.27 The complex encoded by the EXT gene is an essential factor in a signal transduction cascade that regulates chondrocyte differentiation, ossification, and apoptosis.28,29 Thus, loss-of-function mutations in EXT1 and EXT2 cause HS deficiency to trigger the initial development of exostoses.30,31 The majority of mutations in EXT1 and EXT2 are frameshift (42%–44%), nonsense (22%–24%), and splice site mutations (11%–13%), while in the EXT2 gene, a majority of the mutations are frameshift (42%) and nonsense mutations (22%).6

In this study, a four-generation family with HME was investigated, and trio-WES was performed in the proband and her parents. All nine affected individuals (except one deceased individual) were confirmed to carry a novel heterozygous frameshift mutation (c.944dupT) in EXT2 genes (p. Leu316fs). This mutation was, however, not detected in any of the unaffected members by Sanger sequencing. In addition, according to the distribution of the individuals with HME in this family pedigree (Figure 1), the mutation indicates an autosomal dominant pattern of inheritance from the proband’s deceased grandfather to her father.

The novel mutation (c.944dupT) was identified to be located in the exostosin domain of the EXT2 gene, resulting in a frameshift and the introduction of a premature termination codon (PTC) at position 318 (p. Leu316fs). We proposed the possible mechanisms inducing HME in the family to be as follows: (1) the mutation (c.944dupT) produces a truncated EXT2 protein that lacks the last 433 amino acids at its C-terminus, indicating a defective exostosin domain and the absence of the glycosyltransferase family 64 domain. The glycosyltransferase family 64 domain plays a crucial role in HS biosynthesis; therefore, its absence leads to the loss of the function of the EXT2 protein. PROVEAN (deleterious, with a score of −1267.928) predicted that this mutation was a disease-causing mutation. (2) The PTC at position 318 (p. Leu316fs) may lead to the degradation of mRNAs by nonsense-mediated mRNA decay (NMD). It is well known that NMD is a quality control pathway that degrades mRNAs containing PTCs to exert protective effects by preventing the production of a toxic
Whole-exome sequencing identified a novel EXT2 frameshift mutation (c.944dupT, p. Leu316fs) in a family with hereditary multiple exostoses (HME). The mutation was detected in all affected individuals (II-2, II-6, II-7, II-9, III-5, III-6) but not in any unaffected members (II-3, II-4, II-10) by Sanger sequencing. The black arrows indicate the point of mutation (dupT).
truncated protein to ameliorate disease.\textsuperscript{32,33} Conversely, in some conditions, NMD tends to aggravate the clinical phenotype in genetic diseases and increase the deleterious effects of the truncating variants.\textsuperscript{34,35} NMD may have a negative effect on the HME patients of the family. Thus, further functional tests, such as the RNA synthesis rate, are needed to determine the exact mechanism of the mutation.

In conclusion, a novel heterozygous frameshift mutation in \textit{EXT2} (c.944dupT, p. Leu316fs) was identified in a four-generation family with HME, which was revealed to produce a dysfunctional EXT2 protein following in silico analysis. The present study indicates that WES is effective in the diagnosis of monogenic disorders, such as HME, and extends the EXT mutational spectra. Furthermore, our results are advantageous for genetic counseling and the subsequent prenatal diagnoses.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
MY, TH and SLL designed the study. HBX, BCX, and QQX performed the experiments. MY, HW, and TH conducted data analysis. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
The present study was approved by the Medical Ethics Committee of West China Second University Hospital of Sichuan University. Written informed consent to participate was obtained from the family members of the patient.

PATIENT CONSENT FOR PUBLICATION
Written informed consent for publication was obtained from the family members of the patient.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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