Identification of a Novel EXT Mutation in A Kindred With Hereditary Multiple Exostoses Using Whole-Exome Sequencing and Overview of Mutation Spectrum

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Research Article

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Abstract

Background: Hereditary Multiple Osteochondromas(HMO) is a rare genetic musculoskeletal disorder characterized by multiple osteochondromas that form near to the growth plates of many bones. Loss-of-function mutations in *EXT1* or *EXT2* that encode glycosyltransferases are the causal mutations for most HMO patients.

Methods: After collecting the family history and clinical information, we used Whole-Exome Sequencing to find the pathogenic mutations in one Chinese Hereditary Multiple Exostoses pedigree. Sanger sequencing and relevant online databases were used to validate the screened variants. Lollipop plots were drew to map the reported mutations from online databases (Multiple Osteochondroma Mutation Database and clinvar)on a linear protein domains by MutationMapper.

Results: A novel heterozygous splicing-site mutation in gene *EXT1* (NM_000127:exon5:c.1417+1G>C [chr8:118834703]) was found in this pedigree and mutation spectrum of genes *EXT1* and *EXT2* were demonstrated.

Conclusions: Our results help this pedigree to identify the pathogenic variant and guide the prenatal diagnosis, also expand the mutation spectrum in Hereditary Multiple Osteochondromas.

Introduction

Hereditary multiple osteochondromas (HMO), also called hereditary multiple exostoses (HME), is a rare genetic disease, inherited in an autosomal dominant manner. Genes *EXT1* (8q24.11) and *EXT2*(11p12) are the causal genes[1, 2], with a penetrance nearly to 100%. Another gene designated *EXT3* on chromosome 1 between D19S413 and D19S221 still needs further supported data.[3]

The prevalence of HMO in western countries is estimated around 0.4 to 1 in 50000, peak in Guam 50 in 50000[4]. The major symptoms of the HMO are multiple osteochondromas and related skeletal deformities. These bony exostoses could affect all metaphyses and diaphyses of bones, commonly found at knees, arms, forearm, thigh, pelvis and shoulder. Patients with HME show symptoms at a young age with a palpable mass near long bones, often are diagnosed before 12 years old. The number of the exostoses and functional deformity grow with the time. The exostoses burden per individual is about 31.5 with the mean age of 28.2. Patients with an *EXT1* genotype were reported to have significantly greater number of exostoses compared to *EXT2* genotype.[5]

Osteochondroma is usually a benign bone mass with a malignant degeneration rates about 0.38-7.0% toward chondrosarcoma.[6, 7] About 3/4 occurred between ages 20–40 and more than half at the pelvis and proximal femur. [7] Surgical excision of the tumor with free margin is suggested and local recurrence is lower than 2% if it was completely resected. Somatic mutation of *EXT1* was the molecular basis of chondrosarcoma. Complications of HMO include local compression of adjacent tissues, joints deformity

and restricted joint motion, discrepancies of limb-length, shortened stature and osteoarthritis. Pain and joints dysfunction require surgical treatment to relieve symptoms.

Both *EXT1* and *EXT2* gene products are glycosyltransferases, which play an important role in the chain elongation step of heparan sulfate synthesis. HS binds with core proteins to produce heparin sulfate proteoglycans (HSPGs), which are abundant in both cell membrane and extracellular matrix and also function with vital signaling proteins, including bone morphogenetic protein (BMP), to regulate cell function.[8, 9] Animal studies showed that both *Ext1*^{+/-} and *Ext2*^{+/-} heterozygous knockout mice developed osteochondromas at a low penetrance.[10, 11] In contrast, mice with stochastic inactivation of *ext1* in some chondrocytes demonstrate multiple exostoses and bone deformity mimicking human HMO.[12, 13] And also compound heterozygous *Ext1*^{+/-} *Ext2*^{+/-} mice and *Ext1*-deficient mice displayed long-bone-associated exostoses.[14, 15] In line with the observation of three-dimensional chondrogenic pellet model, osteochondroma formation arises via a “second hit” that would further lower heparan sulfate level.[16]

Considering the genetic causes and the disease burden of HME, genetic counseling and PGD/PGS (Preimplantation Genetic Diagnosis/Screening) is highly suggested. In this study, we collect family history, clinical data, radiological images of a four-generation Chinese HME kindred. Whole-Exome Sequencing was used to identify the pathogenic mutation in this family. Moreover, we review the published literature and two online Multiple Osteochondroma Mutation databases to figure out the hot-spot and mutation spectrum of *EXT1* and *EXT2*.

Material And Methods

Patients

A four-generation Chinese kindred was studied with the permission of the family members, 11 out of 22 members were affected with HME (6 female, 5 male, 5 deceased). The proband III2, a 53 years old female, visited the rheumatologic department outpatient clinic for multiple joints pain. The palpable mass near the left knee was first noticed at the age 3 and gradually exostoses were affected both knee. She was born of nonconsanguineous Chinese parents with a family history. Physical examinations revealed exostoses in bilateral knee joints and left waist. The patient IV 3 was a 24 years old male with palpable lesions at the bilateral knees and elbow joints. The diagnosis was made by Multi disciplinary team of rheumatology, orthopedic and radiologic department based on clinical manifestations and radiological images. The other family members' information was recorded due to statements of the proband and family photos.

Genetic test

We did Whole-Exome Sequencing of the proband III2, patient IV 3 on Illumina NovaSeq6000 according to the manufacturer's instruction (Illumina, San Diego, CA, USA). Genomic DNA samples were extracted from

peripheral blood using QIAamp RBlood Mini Kit (Qiagen, Hilden, Germany). The quality of genomic DNA was evaluated by agarose gel electrophoresis analysis, and the quantity was measured by

NanoDrop2000 and Qubit3.0. DNA was sheared with M220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). DNA

target regions were captured by hybridizing the genomic DNA

sample library with the xGen RExome Research Panel v1.0 (IDT,

USA). The captured and amplified DNA samples were then sequenced

using Illumina NovaSeq6000 (Illumina, San Diego, CA, USA) with 150 base-paired end reads. The sequence reads were mapped and aligned to Hg37/GRP. Based on Standards and Guidelines for the Interpretation of Sequence Variants recommended by The American College of Medical Genetics and Genomics (ACMG), we analysed the sequencing data.[17]

The following databases were used to filter the variants, the 1000 Genomes Project (<http://www.1000genomes.org>), Exome Variant Server (<http://evs.gs.washington.edu>), and Exome Aggregation Consortium (<http://exac.broadinstitute.org>). Mutation Taster (<http://www.mutationtaster.org>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>), and SIFT (Sorting Intolerant from Tolerant) human protein (http://sift.jcvi.org/www/SIFT_enst_submit.html) software

programs were used to predict the destructive of amino acid substitution on structure and protein function. Three tools were used to

predict the splice-site variant, namely Human Splicing Finder (<http://www.umd.be/HSF3/HSF.shtml>), GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl), NetGene2(<http://www.cbs.dtu.dk/services/NetGene2/>). Sanger Sequencing was tested to validate the variants.

To figure out the mutation spectrum of EXT1 and EXT2, we studied the mutations that reported in Multiple Osteochondroma Mutation Database(<https://databases.lovd.nl/shared/genes>) and Clinivar(<https://www.ncbi.nlm.nih.gov/clinvar>). The type of mutations was mapped on a linear protein and its function domain (Lollipop plot) by using the online MutationMapper (<https://www.cbioportal.org/visualize>)[18, 19]. The type of variants was also studied.

Results

The pedigree of the HME kindred was showed in Figure IA, According to the clinical classification of Mordenti , these 2 patients were moderate type of IB class[20] . Genetic tests found that the proband was with a novel heterozygous splicing-site mutation in gene *EXT1* (NM_000127:exon5:c.1417+1G>C chr8:118834703). This mutation was further validated by Sanger sequencing (Figure IB) and also tested

in affected and unaffected family members. Affected members had the same mutation and the unaffected ones were negative. The Radiological image of the proband was demonstrated in Figure (IC-D, CT scan and MRI respectively).

EXT1 and *EXT2* are the two causal genes for Multiple Osteochondroma. according to the Multiple Osteochondroma Mutation Database (<https://databases.lovd.nl/> , Date last updated August 18, 2021) [20] and Clinivar (<https://www.ncbi.nlm.nih.gov/clinvar>) , we found that no hotspot for *EXT1* , but for *EXT2* the mutation variants were mainly located in the Exostosin region. (Figure2). Of all the 844 variants in *EXT1* were reported, in them 714 were unique public pathogenic variants (49% substitutions, 38% deletions, 9% duplications in DNA level), 11% were located in splice region and 85% in coding region, and 52% were frameshifts and 28% were stop changes and 16% missense changes in protein level. For gene *EXT2* totally 449 public DNA variants were reported, in them 362 variants were pathogenic (65% substitutions, 26% deletions, 6% duplications in DNA level), and 79% located in coding region and 15% were in Splice region. In protein level 51% were frameshifts and 29% stop changes and 15% missense changes.(details in supplements table1-2)

Discussion

This study indentified a novel splicing-site mutation in *EXT1* from a four-generation HME Chinese family. All 2 affected ones which we did genetic tests were found the same site and the unaffected members were not. For both *EXT1* and *EXT2*, the most common mutation type is substitution, followed by deletion and duplication, located mainly on coding region and more than 10% in splicing region. And unlike *EXT1*, mutation sites of *EXT2* were focused on the exostosin region, and rarely in the glycol-transf-64 domain. Consistent with the previous study suggested that mutation hot-spot region of *EXT2* were Exon 5. [21]

One limitation of this study is that we did not test all the affected patients, this was due to geographic distance and personal will. Both patients complained about pain and shyness in school. A national wide study in the Netherlands showed that multiple hereditary exostoses damaged both physical and psychological well-being. Pain was the major complain, and about half school attendees experienced problems at school and more than a quarter were unable to join the sport activities.[22] HME impact on patients quality of life similar to osteoarthritis in the mental aspect and women responded even worse in the psychological side. [23]

Another limitation is that the diagnosis was based on the family history, clinical manifestations and radiological results, pathologic diagnosis was lacked because family members were moderate type of IB and did not do the surgical removal. Up to now no consensus on the hereditary multiple osteochondromas (HMO) diagnosis criteria was reported. And clinically

The diagnosis was made when patients with more than one osteochondromas of the juxta-epiphyseal region of long bones was confirmed by radiological images. Additionally these patients were with a positive family history and/or genetic tests indicated positive in gene *EXT1* and *EXT2*. [24] Differential diagnosis was made from Dysplasia Epiphysealis Hemimelica, metachondromatosis (MC),

enchondromatosis (Ollier disease and Maffucci syndrome). And the female patient was short-stature, while the other patients were within normal range. Diminished stature was observed in about 37% -53% patients and more severely affected in patients with an EXT1 mutation.[4, 25] The affected patients in this studied showed no sign of malignant degeneration. A international, web-based survey showed that about 2.7% patients underwent malignant transformation at a mean age of 28.6 years. This occurred most commonly on pelvis (8/21)and scapula (4/21) ,followed by proximal femur, rib, skull base, tibia and foot.[6] Whether genetic variants plays a role in the malignant transformation risk is undetermined yet. The molecular basis and characteristic of the HMO patients who had malignant change to chondrosarcoma still need further investigations. A regular doctor visit and yearly physical examination is strongly recommended for the malignant risk.

In conclusion, we found a novel splice-site variant in *EXT1* in one Chinese hereditary multiple osteochondromas family. Additionally by digging the online

Multiple Osteochondroma Mutation Database and clinvar, we present the mutation spectrum and lollipop of variants site on protein domains. Multiple Osteochondromas is a chronic skeletal disease with a certain risk of malignant transformation. Medical managements, includes early diagnosis, genetic counseling, regular medical follow-up help to improve the patients' clinical outcomes. Joint effort of social support and school care are required to help the HMO patients to maintain physical and mental well-being. More cohorts and pathogenesis investigations are needed elucidate the correlation of the genetic basis and clinical manifestations.

Abbreviations

HMO Hereditary Multiple Osteochondromas

HME Hereditary Multiple Exostoses

EXT1 Exostosin Glycosyltransferase 1

EXT1 Exostosin Glycosyltransferase 2

Declarations

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Tongji Hospital and informed written consent was obtained from all the participants and/or their legal guardian(s) for deceased participants. All the methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication: Not Applicable

Availability of Data and Materials: The datasets generated during the current study are available in the following website link

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: WT and YL organized this study and interpreted the patient data. YJL and YHD analysed the data. YHD was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

(a) Family pedigree of the HMO with EXT1 gene mutation. Generation was demonstrated as I to IV. Patient III-2 is the proband. Black and white indicate the family member affected or unaffected respectively by HMO. (b) Whole-exome-sequencing found a novel EXT1 frameshift mutation(NM_000127:exon5:c.1417+1G>C chr8:118834703) in this pedigree. Sanger sequencing shows a heterozygous deletion in proband. (c) CT scan of proband's knee, exostoses are denoted by arrow . Both

distal femur and right proximal tibiae are affected. (d) T1-weighted and T2-weighted MRI scan of proband's left proximal tibia. Arrow indicated the cartilage cap.

Figure 2

(A)(B) Mutations spectrum on a linear protein domain of EXT1 and EXT2 were demonstrated respectively. Missense variants (green), Truncating variants (black), Inframe variants (brown) was labeled

Supplementary Files

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