The identification of a novel frameshift insertion mutation in the EXT1 gene in a Chinese family with Hereditary Multiple Exostoses

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Abstract

A novel heterozygous mutation (c.325dup) was identified in EXT1 gene from the proband and the affected family members; this mutation was absent in all the unaffected family members. The identification of the novel frameshift insertion mutation (c.325dup) expands the mutation spectrum of HME, which provides new evidence for HME diagnosis.

1. Introduction

Hereditary Multiple Exostoses (HME) is a rare orphan autosomal-dominant pediatric disorder with a prevalence of about 1:50 000 (Wicklund et al., 1995; Schmale et al., 1994). The disease is estimated to occur more frequently in males (male-to-female ratio,1.5:1) (D'Arienzo et al., 2019). HME is characterized by the formation of osteochondromas (non-malignant cartilage-capped bony tumors) or exostoses within the perichondrium next to the growth plates of long bones, ribs, hip and vertebrae in very young and adolescent patients (Ryckx et al., 2013). Osteochondromas can turn into chondrosarcomas or osteosarcomas that can be life threatening in about 2% of the patients (Porter DE and Simpson AHRW,1999; Porter et al., 2004). The current clinical treatment of HME is commonly to resect the symptomatic chondrosarcomas or osteochondromas and to ameliorate the associated skeletal defects. The etiological treatment is not yet available as the evidence on etiological diagnosis of HME is limited.

The identification of likely pathogenic genes associated with HME was reported in the past. Wu *et al.* reported that the majority of the studied patients carried mutations in the exostosin-1 (*EXT1*) and exostosin-2 (*EXT2*) genes (Wu et al., 1994; Wuyts et al., 1996).*EXT1* consists of 11 exons and spans about 312kb at 8q24 (Ludecke et al.,1997), while *EXT2* comprises 16 exons and is located at 11p11.2, spanning about 150kb (Clines et al.,1997). It is known that the genes belong to the *EXT* multigene family, are ubiquitously expressed and act as tumor suppressors. The proteins encoded by*EXT* family genes are involved in the adhesion and/or polymerization of heparin sulfate (HS) chains at HS proteoglycans (HSPG's) (Lind et al.,1998; McCormick et al., 1998; Busse et al.,2007). To date, over 650 mutations in *EXT1* and *EXT2* have been reported, most of which are nonsense, frameshift, or splice site, resulting in the synthesis of truncated EXT proteins with no suppression activity (Jennes et al., 2009; Ciavarella et al., 2013).

In this study, we identified a novel frameshift insertion mutation in the proband, which is absent both in Clinvar or Human Genome database and current clinical reports. The novel frameshift insertion mutation found in the proband was also confirmed in the affected individuals but not in the unaffected individuals; Further, the Polyphen and SIFT analysis were used to evaluate the effect of the frameshift insertion mutation, with a support result of obtaining a dysfunctional protein from the mutated gene. Therefore, we concluded that the genetic variation caused by the frameshift insertion mutation could be associated with

the pathogenicity of HME in this pedigree. The finding could be used as a new support on prenatal diagnosis for preventing the birth defect incidence of HME.

2. 2. Materials and Methods

2.1 Clinical report

The proband (III-3) was a 21-year-old male who was admitted to Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China) due to his leg deformity with limited physical activity. The clinical examination concluded that the proband was diagnosed with HME at orthopedics department. Three generations of the patient family were included in this study with five individuals having HME (**Fig. 1**). In the study, the proband's cousin (III-2) requested a premarital genetic counseling in Tongji Hospital to learn her HME-risk status.

2.2 DNA extraction

Five ml of whole blood sample was collected from the proband (III-3) and the other family members (I-2, II-1, II-2, II-3, II-4, II-5, II-6, III-1 and III-2) individually using EDTA-anticoagulant vacuum blood collection tubes. Genomic DNA was extracted from peripheral blood leukocytes of the collected blood samples using a DNA extraction kit (TianGen, Beijing, China) according to the manufacturer's instructions. And the DNA extracts were stored at -20 for analysis later.

2.3 Mutation screening

The genomic DNA (50 ng) of each sample was enzyme-digested into around 200bp of fragments. The DNA fragments were end-repaired (the 3'end was added one adenine base) and were ligated with barcoded sequencing adaptors. And the ligated fragments about 320bp were captured by XP beads. After PCR amplification, the DNA fragments were hybridized by Nano WES according to the manufacturer's Protocol. The hybridized DNA products were eluted and then subjected to PCR amplification and purification as a DNA library for each sample. Next, the libraries were quantified by qPCR and size distribution were determined using Nano WES (Berry Genomics, China). Finally, Novaseq6000 platform (Illumina, San Diego, USA, with 150 bp pair-end sequencing mode) was used for genomic DNA sequencing. Raw image files were processed using CASAVA v1.82 for base calling and generating raw data. The sequencing reads were aligned to the human reference genome (hg19/GRCh37) using Burrows–Wheeler Aligner tool and PCR duplicates were removed using Picard v1.57 (http://picard.sourceforge.net/). Verita Trekker® Variants Detection System by Berry Genomics and the third-party software GATK (https://software.broadinstitute.org/gatk/) was employed for variant calling. Variant annotation and interpretation were conducted by ANNOVAR (Wang, et al, 2010) and the Enliven® Variants Annotation Interpretation System (a comprehensive tool called Sprinkle was developed and authorized by Berry Genomics) was used for CNV calling. It includes XHMM PCA method (sequencing noise removal), CNV Kit fix module (GC and bias correction), and copy number calculation. The identification of CNV were performed in exons and long segment areas.

Mutation Surveyor Demo software version 4.0 was used to analyze the sample sequences comparing with the reference sequences from the National Center for Biotechnology Information (NCBI) (*EXT1* : NM_ 000127.2; *EXT2* : NM_000401). The detected variants were further evaluated by the Polyphen and SIFT software to determine their associations with the pathogenicity of HME.

3. Results

3.1 Likely genetic variations associated with HME

The exons of the *EXT1, EXT2* and the other 59 genes associated with HME (recommended by American College of Medical Genetics and Genomics, ACMG SF v2.0, shown in **Table 1**) (Kalia et al., 2017) were screened in the proband (III-3) to reveal the possible pathogenic gene variants of HME. No mutations were discovered in the *EXT2* and the other 59 genes. However, a heterozygous frameshift insertion mutation in exon 1 of the *EXT1* gene was detected (c.325dup), which was predicted to cause the early termination of protein translation (**Fig.2A**). We also screened the HME associated genes for the other family members,

including affected individuals (II-1, II-5 and III-1) and unaffected individuals (I-2, II-2, II-3, II-4, II-6 and III-2). The heterozygous frameshift insertion mutation of the proband was also detected in the affected family members but not in the unaffected family members **(Fig.2B and Fig.2C)** .

We examined the reported gene variants of *EXT1* gene in Human Gene Mutation Database (HGMD) (shown in **Table 2**). The heterozygous frameshift insertion mutation identified in our study was absent in HGMD, thus it is recognized as a new gene variant associated with HME**.**

Table 1. 59 genes associated with HME were listed in ACMG SF v2.0

ACTA2	*ACTC1*	*APC*	*APOB*	*ATP7B*	*BMPR1A*	*BRCA1*	*BRCA2*
CACNA1S	*COL3A1*	*DSC2*	*DSG2*	*DSP*	*FBN1*	*GLA*	*KCNH2*
KCNQ1	*LDLR*	*LMNA*	*MEN1*	*MLH1*	*MSH2*	*MSH6*	*MUTYH*
MYBPC3	*MYH11*	*MYH7*	*MYL2*	*MYL3*	*NF2*	*OTC*	*PCSK9*
PKP2	*PMS2*	*PRKAG2*	*PTEN*	*RB1*	*RET*	*RYR1*	*RYR2*
SCN5A	*SDHAF2*	*SDHB*	*SDHC*	*SDHD*	*SMAD3*	*SMAD4*	*STK11*
TGFBR1	*TGFBR2*	*TMEM43*	*TNNI3*	*TNNT2*	*TP53*	*TPM1*	*TSC1*
TSC2	*VHL*	*WT1*					

Table 2. A summary of different *EXT1* gene variants present in the publicly available version of HGMD (Professional Release 2021.12)

Mutation type	**Number of Mutations (publicly available via http://www.hgmd.cf.ac.uk/ac/index.php)**
frameshift	262
nonsense	128
missense	67
canonical-splice	58
Gross deletions/insertions/duplications (> 20 bp)	52
noncoding	10
inframe	9
initiation	2
splice	2
regulatory	1
synonymous	1

3.2 Prediction of protein function

The Polyphen and SIFT analysis showed that the frameshift insertion mutation in *EXT1* gene (c.325dup) was predicted to cause the early termination of protein translation (p.C109Lfs*80). The premature termination of translation could be responsible of loss of the protein function.

3.3 Genetic counseling

The unaffected family member (III-2) requested a premarital genetic counseling in the study to determine her HME-risk status. Since the genetic mode of HME is autosomal dominant inheritance, we suggested that her offspring could be less likely to get the HME associated gene variant from mother according to the Mendelian law when she marries to a healthy male. Differently, when the proband (III-3) and the other affected individuals (III-1) marry to a healthy female, the offspring of them suffering from this disease accounts for 50%.

4. Discussion

Genetic investigations suggested that gene variations in the *EXT1*and *EXT2* genes were often associated with HME, being responsible for 70–95% of the cases. Mutations in *EXT1* account for 56–78% of HME cases, whereas mutations in *EXT2* are detected in 21–44% of cases (Jennes et al., 2009). The *EXT1* gene mutation carriers tend to show more severe symptoms of HME and a greater risk for malignant transformation than the *EXT2* gene mutation carriers did (Francannet et al., 2001; Alvarez et al., 2007). The Human Gene Mutation Database, (HGMD,*http://www.hgmd.org)* stored the*EXT1* gene variants associated with HME that were published in the peer-reviewed literature. To date, there are 592 gene mutation sites in*EXT1* gene presented in (HGMD), which were categorized into 11 categories of mutation type, including frameshift mutation (44.3%), nonsense mutation (21.6%), missense mutation (11.3%), Canonical-splice mutation (9.8%), gross deletions/insertions/duplications mutation (> 20 bp) (account for 8.8%), noncoding mutation (1.7%), inframe mutation (1.5%), initiation mutation (0.3%), splice mutation (0.3%), regulatory mutation (0.2%) and synonymous mutation (0.2%). In this study, we identified a novel frameshift insertion mutation of exon 1 of the *EXT1* gene in a Chinese pedigree with HME. This gene variant was not present in Clinvar/HGMD and current clinical reports.

Our result suggested that the novel frameshift insertion mutation of the*EXT1* gene could cause the early termination of protein translation (p.C109Lfs*80). Strong pathogenicity evidence (PVS1) shows that this mutation could change gene open reading frame, resulting in the loss of protein function; moderate pathogenicity evidence (PM2) of this mutation was undetectable in Shenzhou genome database, Exome Aggregation Consortium (ExAC), 1000 Genomes Project (1000GP) and HGMD; and the pathogenic evidence (PP4) of the identified mutation was consistent with the phenotype of HME. Generally, the combined evidences (PVS1+ PM2+ PP4) supported that this novel mutation was pathogenic (Ahmad et al.,2018; Biesecker et al.,2018; Rajarshi et al.,2018;) but the origin of this pathogenic mutation is unknown.

In WES analysis on other family members, including affected individuals (II-1, II-5 and III-1) and unaffected individuals (I-2, II-2, II-3, II-4, II-6 and III-2), we found that the identified mutation of the proband (III-3) was present in all the affected family members but not present in the unaffected family members. Wu assumed that the pathogenic mutation of the affected individuals (III-1and III-3) was obtained from their fathers ((II-1, II-5), and the mutation of their fathers inherited from the grandfather (I-1) since the genetic type of HME is autosomal dominant inheritance. Based on the current evidence, the offspring of III-1and III-3 will carry this novel mutation with HME incidence of 50% regardless of gender, while the offspring of the unaffected member (III-2) will not carry the HME-risk mutation.

5.Conclusion

In sum, we identified a novel *EXT1*frameshift insertion mutation c.325dup on exon 1 and confirmed that the mutation could have pathogenic effect on gene expression level. Our finding enriches the HME mutation spectrum and provides scientific supports for premarital and prenatal diagnosis in the future.

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Ethics approval

The study was approved by the Research Ethics Committee of Tongji Hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology. Informed consents were obtained from all the participants.

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Conflict of interest

The authors declare that they have no conflict of interest.

Contribution of each author

Wanlu Liu, Conceptualization, Resources, Data Curation, Writing Original Draft

Xinwei Shi, Methodology, Software

Yuqi Li, Formal analysis, Visualization

Fuyuan Qiao, Project administration

Yuanyuan Wu, Supervision, Funding acquisition, Writing - Review & Editing

Consent

The patient and his family provided the written informed consents before the study began.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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