Novel *EXT1* mutation identified in a pedigree with hereditary multiple exostoses

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Abstract. Hereditary multiple exostoses (HME) is an autosomal dominant bone disorder characterized by the presence of multiple benign cartilage-capped tumors. *EXT1* located on chromosome 8q23-q24 and *EXT2* located on 11p11-p12 are the main disease-causing genes which are responsible for ~90% of HME cases. Mutations of *EXT1* or *EXT2* result in insufficient heparan sulfate biosynthesis, which facilitates chondrocyte proliferation, boosts abnormal bone growth of neighboring regions, causes multiple exostoses, and ultimately leads to possible malignant transformation. A family who displayed typical features of HME was enrolled in the present study. Mutation screening by Sanger sequencing identified a novel heterozygous nonsense mutation c.1902C>A (p.Tyr634X) in the *EXT1* gene exclusively in all 3 patients, which is located in the glycosyltransferase domain and results in the truncation of 112 amino acids at the C-terminus of the EXT1 protein. Thus, the present study identified a novel disease-causing *EXT1* mutation in a pedigree with HME, which provides additional evidence for developing quick and accurate genetic tools for HME diagnosis.

Introduction

Hereditary multiple exostoses (HME) is an autosomal dominant disease characterized by multiple cartilage-capped outgrowths at the growth plates of long bones. HME usually presents early in life with 80% of patients diagnosed before the age of 10 years (1). The prevalence of HME is estimated to be ~1 in 100,000 in the European population (2) and at least 1 in 50,000 in the state of Washington (3). HME leads to serious complications, such as compression of peripheral nerves and vessels, bone deformity, short stature and interference with joint movement (3). Each of these complications may necessitate surgical removal of the exostosis. However, the possibility of the recurrence of exostosis is high, and patients usually have multiple surgeries in the same area.

Genetic studies have identified the link between HME and 3 loci (4-7): *EXT1*, which maps to 8q24.1, *EXT2*, which maps to 11p13 and *EXT3*, which is located on the short arm of chromosome 19, while its exact position has not been successfully mapped. It has been estimated that 60-70% of HME patients have mutations in *EXT1*, as well as 30-40% in *EXT2*. To date, over 650 mutations have been found in these 2 genes, most of which are nonsense, frame shift, or splice-site mutations (8,9), which result in truncation, premature termination, premature degradation and nearly complete loss of function of *EXT* proteins (10). *EXT* family members have been identified as tumor-suppressor genes, and share a homologous C-terminus glycosyltransferase domain which plays a key role in heparan sulfate (HS) biosynthesis.

Here, we identified a three-generation Chinese kindred with HME and discovered a novel nonsense mutation c.1902C>A (p.Tyr634X) in the *EXT1* gene exclusively in the patients resulting in a truncated glycosyltransferase domain at the C-terminus of the EXT1 protein.

Materials and methods

Patients and clinical data. A three-generation Chinese kindred ZJ-H001 from Zhejiang Province, China was identified (Fig. 1). Diagnosis of HME was based on radiological examinations which indicated at least 2 osteochondromas in the juxta-epiphyseal region of long bones, and was further confirmed by joint and long bone palpitations (Fig. 2). There were 3 patients with HME (1 male and 2 females) in the kindred, inherited from generation to generation (Fig. 1 and Table I), and all 3 patients and 3 unaffected family members (I2, II4 and III2) were enrolled in the present study. All 3 patients showed multiple exostoses, arising from the humerus, ulna, hands, hips, femurs, tibia, fibula and knee joints. Shorter limb lesions were observed in all 3 patients, limb deformity was noted in 2 patients and hip and knee valgus or joint activity was only found in 1 patient. None of the 3 patients had serious complications. The only clinical manifestation was a painless

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Table I. Clinical data of the 3 patients with HME.

ID	Gender	Age of onset (years)	Age at present study (years)	Height (cm)	Localization	Classification
II2	Female	5	40	156.4	Ulna, hips, femurs, tibia, fibula	IB
II3	Male	7	39	160.2	Humerus, hips, femurs, tibia, fibula	IIB
III1	Female	3	19	150.5	Ulna, hands, hips, femurs, tibia, fibula	IIIB

HME; hereditary multiple exostoses.

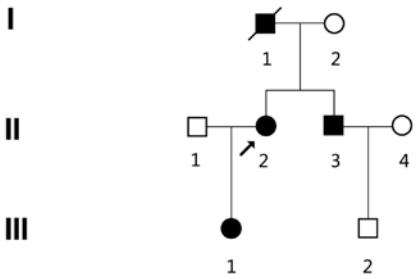

Figure 1. Pedigree of the Chinese family with HME. The filled symbols represent the affected individuals, while the empty symbols indicate the unaffected family members. The proband (II2) is noted with an arrow. There is no consanguinity in this family. HME, hereditary multiple exostoses.

lump on the bone and occasionally pain (mean VAS score 5.7) with functional limitations near knees or hips. The medical history was obtained by using a questionnaire regarding the subjective degree of HME, age at onset, evolution and other relevant clinical manifestations (Table I). The median age of the HME patients was 29.5 years. All 3 patients presented with osteochondromas before 10 years of age (median age of onset, 5), and none of the patients had any surgical removal of osteochondromas prior to the present study. The average height (160.2 cm for males and 153.5 cm for females) was much lower than the average height of the same age group reported by the 2010 National Physique Monitoring Bulletin Administration of Sports of China. In addition to the kindred ZJ-H001, 200 ethnically unrelated healthy individuals (107 males and 93 females, aged from 22 to 40 years) were included in the present study. All study procedures were approved by the Ethics Review Committee of the Zhejiang Provincial People's Hospital and were carried out following written informed consent obtained from each individual and/or parents of the children.

Molecular analysis. Genomic DNA was purified from peripheral blood leukocytes using an AxyPrep Nucleic Acid Purification kit (Axygen, Union City, CA, USA) according

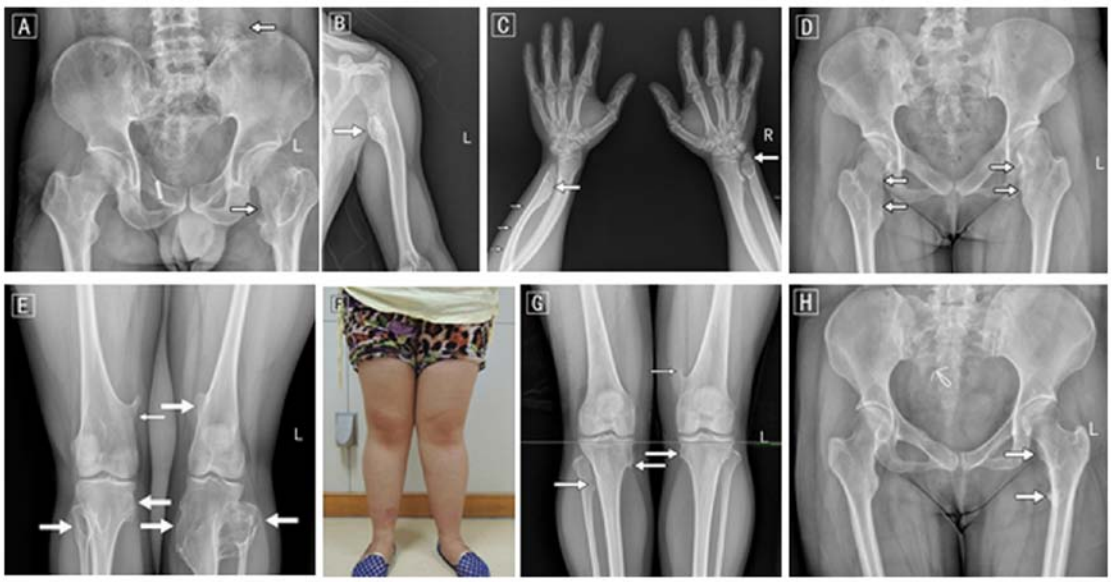

Figure 2. Radiographs and clinical images of patients II2 (A-C), II3 (D-F) and III1 (G and H). (A) Osteochondromas on the posterior superior iliac spine and left proximal femur (white arrows). A pseudoarthrosis formed between the osteochondroma and transverse process of the fifth lumbar vertebra. (B) Single osteochondroma with bowing humerus (white arrows). (C) Distal ulnar osteochondromas (thick arrows) with left ulnar shortening and bowing deformity (thin arrows). Abnormal distal radioulnar joint is also present. (D) Osteochondromas in bilateral proximal medial femurs and coxa valga. Osteochondromas in the medial femur may affect its development, resulting in acetabular dysplasia. (E) A large number of sessile osteochondromas (thick arrows) and pedunculated osteochondromas (thin arrows) pointing away from the joint. A large tibiofibular osteochondroma is evident on the proximal side of the left knee. (F) Clinical image displaying left genu varum. (G and H) Multiple osteochondromas are noted around the bilateral knee and on the proximal side of the left femurs (white arrows).

Table II. Primer sequences used for sequencing the *EXT1* and *EXT2* genes.

Primers	Reverse	Forward
EXT1 gene		
Exon 1.1	5'-CCCTTCGGTCTTTCATCTTT-3'	5'-GACGTGACGCTCGGCCAAT-3'
Exon 1.2	5'-GGAGTTGGCATCTCGCTTCT-3'	5'-GCAAGGCAATCACTTCGTC-3'
Exon 1.3	5'-AAGGGGAAAGAGGACTGAGG-3'	5'-GGAGAGAAGAACACAGCGGTAG-3'
Exon 2	5'-CCTCCACCCCTCACTTGTCA-3'	5'-GCAACCCAACCTCCTTCCTC-3'
Exon 3	5'-TCTGGTTATTGAAAGGGGTGGA-3'	5'-GCAGTGTCAAAAATGCCAGTCA-3'
Exon 4	5'-TTTGTGGAGTTTGTCAGGAATG-3'	5'-GAAGCCAAATGCTATGAAGAAT-3'
Exon 5	5'-CAATGCAGGGTGTTAGATGGA-3'	5'-TAAAGTGGGAGGGAGGGTAGA-3'
Exon 6	5'-GTAACGAGGCAGGATGAATGA-3'	5'-AAATCATCCAGGAGGGAACAT-3'
Exon 7	5'-CTGATTTGAAAGCCTATTGTGG-3'	5'-AATGTTCTGAGGTTGTGTGGGA-3'
Exon 8	5'-GTGCTAACAGGAATCGGGCT-3'	5'-TGAGATTCCTTCGGTGTTGAG-3'
Exon 9	5'-CCAACTGAAAATGTTACTCTACC-3'	5'-ATTATGAATTAGTGGGGAGAAGG-3'
Exon 10	5'-GCACCAATCATACACTCTTTTCTA-3'	5'-ATGGGTATGTGTTTTCTGTCTCA-3'
Exon 11	5'-TTTCCACGAAGTTTGAGCTTTT-3'	5'-GCTCATTTGCCTGACTCCATT-3'
EXT2 gene		
Exon 1	5'-GCAGGAGTGGAAATCGGAG-3'	5'-ATTGCCCTCCAGGAATGTTA-3'
Exon 2	5'-ACCAACTCAAGAGCAGAAGCA-3'	5'-GGCGTGGTGGTCACAGTTAC-3'
Exon 3	5'-TGCCAGGACATAAGCCCTAACT-3'	5'-CTGTTGGGATTTCCAGGAGTTT-3'
Exon 4	5'-AAACAAAGGAGAGAACGGAGT-3'	5'-TGATTCAAGGATAGAACGCAG-3'
Exon 5	5'-CACAAGACACCAGACATCCAAG-3'	5'-GTGGAGGTGAAGACTGGTAAGG-3'
Exon 6	5'-CCTTGGTTTGTGAACTGCTCT-3'	5'-GGCGTCAACCCTTGTAGAAAC-3'
Exon 7	5'-AAGTAAACCCCACTCAGGCATT-3'	5'-GAAGGAGGTTTGGGATGTTGTT-3'
Exon 8	5'-ACTGCTGAAACCCTGCTGTG-3'	5'-AAGTGTGCCTGGTTGGAGTG-3'
Exon 9	5'-CCCAAGTATAAAAAGCACACTCTC-3'	5'-TAAAGGAATTAGCCTAACCTGGAG-3'
Exon 10	5'-GTATGCCAGGGCTTGGAGTT-3'	5'-GTAAAAGCCACCAAGCCTGC-3'
Exon 11	5'-CCCACACTCTTACGCACACCT-3'	5'-CTTTGGATTTGATGAGAGCCG-3'
Exon 12	5'-ATGGTTATCTCGAAGTGACAGGA-3'	5'-TCTCCAGAATCCCATTATGACCT-3'
Exon 13	5'-GACCGCATCAATCATAGAACCT-3'	5'-GCCTCCTTTTACCCTTCCTATT-3'
Exon 14	5'-GACCCTTCCAGCCATTACAAA-3'	5'-CTTGTGAGTTCTGCCGTTGG-3'
Exon 15.1	5'-AGGAATTGGTGTTTAAGACACAG-3'	5'-TTTGCTGTTATCTCTCAACCTCT-3'
Exon 15.2	5'-ACGCTGACTGGCAAAACAACTA-3'	5'-TACATCAATGAGTTCTTTCAGGGA-3'

to the manufacturer's instructions. Sanger direct sequencing DNA sequence analysis of the EXT1 and EXT2 coding region including the 100-bp flanking intron-exon junctions by polymerase chain reaction (PCR) of genomic DNA followed by sequencing reactions with Sanger sequencing chemistry using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., Foster City, CA, USA) on an ABI 3730XL automated sequencer. Primer sequences are listed in Table II. Sequence data were analyzed by Sequencer Demo 3.0 and Mutation Surveyor Demo V4.0 using the reference sequences from NCBI (NM_000127.2 for EXT1, NM_001178083.1 for EXT2).

In silico analysis. In the present study, we used Mutation Taster software (11) to determine possible changes in protein structure that may affect phenotype.

Evolutionary conservation and structural prediction. Clustal X1.83 was used to compare human wild-type EXT1 protein sequence (NP_000118) with the orthologs from mouse, *Drosophila*, *Danio*, *Caenorhabditis* and *Xenopus* (sequences obtained from http://www.ensembl.org/). We used PredictProtein (https://www.predictprotein.org/) to predict the possible structural changes caused by the nonsense mutation.

Results

DNA sequencing identifies a novel EXT1 mutation in all of the HME patients. We sequenced each exon of *EXT1* and *EXT2* genes in all 3 patients (II2, II3 and III1) and the 3 unaffected family members (I2, II4 and III2) in the kindred ZJ-H001. As previously reported by others, the present study also identified 3 SNPs, c.28C>A, c.1065C>T and c.1761G>A, which are located in exons 1, 3 and 9 of the *EXT1* gene, respectively (Table III). All patients were carriers for genetic lesions of EXT1. Moreover, we identified a novel nonsense mutation in the *EXT1* gene c.1902 C>C/A (amino acid 634Y>X) in all 3 HME patients (Fig. 3), and 3 novel synonymous mutations in the unaffected family

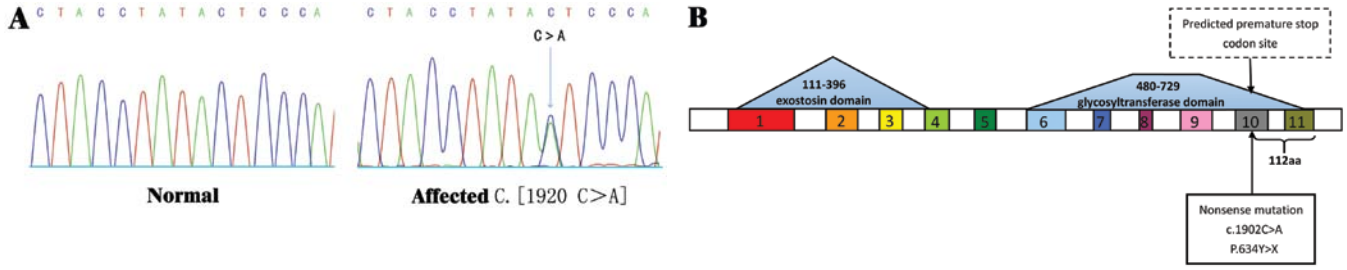

Figure 3. DNA sequencing identifies a novel mutation in the *EXT1* gene. (A) Heterozygous mutation c.1902C>A (p.Tyr634X) in the *EXT1* gene was detected exclusively in the HME patients (right panel) but not in the health controls (left panel). (B) The mutation, c.(1902C>A), located in exon 10, was predicted to introduce a premature stop codon at position 634, truncating 112 amino acids at the C-terminus of the EXT1 protein. HME, hereditary multiple exostoses.

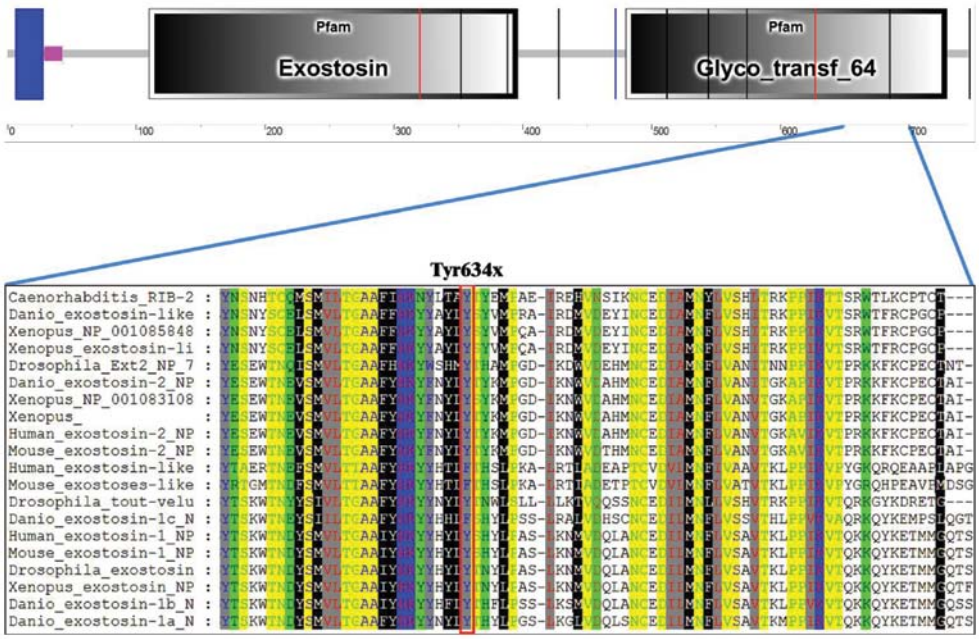

Figure 4. Sequence alignment of EXT1 orthologs.

Table III. SNPs identified in the *EXT1* gene.

ID	Nucleotide change	Trivial name	Location	Reported
II3	c.28C>A (het)	p.10R>R	Exon 1	Y
III1	c.28C>A (het)	p.10R>R	Exon 1	Y
III2	c.28C>A (het)	p.10R>R	Exon 1	Y
I2	c.1065C>T (het)	p.C355C	Exon 3	Y
II2	c.1065C>T (het)	p.C355C	Exon 3	Y
II3	c.1065C>T (hom)	p.C355C	Exon 3	Y
III1	c.1065C>T (het)	p.C355C	Exon 3	Y
I2	c.1761G>A (het)	587E>E	Exon 9	Y
II3	c.1761G>A (het)	587E>E	Exon 9	Y
III1	c.1761G>A (het)	587E>E	Exon 9	Y
III2	c.1761G>A (het)	587E>E	Exon 9	Y

members c.1005C>C/T, c.1761G>A and c.1839G>A/G (amino acid 335C>C, 587E>E and 613T>T, respectively) (Table IV). Of note, this novel 634Y>X mutation was exclusively identified in all 3 affected patients, but not in the unaffected family members. To assess the possibility that this novel *EXT1* mutation is a disease-causing mutation, we further sequenced 200 ethnically unrelated healthy individuals and confirmed that none of these 200 healthy donors carried the 634Y>X mutation. Thus, our data suggest that the novel 634Y>X nonsense mutation in the *EXT1* gene was a disease-causing mutation in the Chinese pedigree (ZJ-H001) with HME.

In silico analyses indicate the critical impact of the Y634X mutation on EXT1 function. To understand the potential impact of the 634Y>X nonsense mutation on EXT1 function, we further performed *in silico* analyses. EXT1 protein is composed of 746 amino acids with 2 critical domains: the exostosin domain (aa110-396) and the glycosyltransferase domain (aa480-729) (Fig. 3B). Try634 is located in the glycosyltransferase domain, which is highly conserved among many species (Fig. 4), indicating the potential conserved role of Try634 in EXT1 function. The 634Y>X nonsense mutation results in the premature termination of EXT1 protein translation and generates a truncated EXT1 protein lacking 112 amino acids at the C-terminus (Fig. 3B). Since the glycosyltransferase domain plays an essential role in HS biosynthesis, loosing the C-terminus of the glycosyltransferase domain may have

Table IV. Changes in the *EXT1* gene detected in the patients by direct sequencing.

ID	Location	Nucleotide change	Amino acid change	Mutation type	Category
I2	Exon 3	1005C>CT	335C>C	Synonymous	SNV
I2	Exon 9	1761G>A	587E>E	Synonymous	SNV
II2	Exon 10	1902C>CA	634Y>X	Nonsense	SNV
II3	Exon 10	1902C>CA	634Y>X	Nonsense	SNV
III1	Exon 10	1902C>CA	634Y>X	Nonsense	SNV
III2	Exon 9	1839G>AG	613T>T	Synonymous	SNV

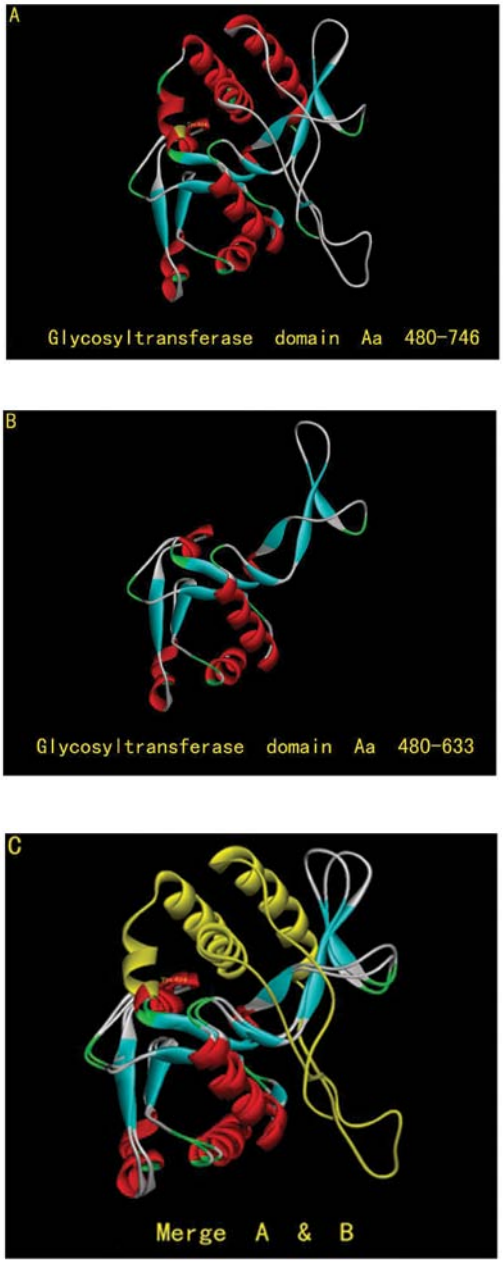

Figure 5. 3-D structure modeling predicts the impact of the novel heterozygous nonsense mutation c.1902C>A (p.Tyr634X) by PredictProtein. Compared to the structure of the glycosyltransferase domain in wild-type (A) EXT1 protein, the truncated (B) EXT1 protein caused by the Y634X mutation lost 4 α-helices and 6 β-pleated sheets, which may have resulted in the drastic alteration of the entire protein structure and even loss of glycosyltransferase activity and other EXT1 functions. (C) The merge of normal EXT1 protein with mutant (Tyr634X) EXT1.

a significant impact on EXT1 function, particularly on HS biosynthesis. Consistently, by online prediction tool, Mutation Taster, it was predicted that substitution of the Y634 to the terminator codon affects protein function and causes diseases (probability score, 1). Finally, we used the PredictProtein tool (12) to predict the possible structural changes caused by the 634Y>X mutation, and found that the 634Y>X mutation may affect the secondary structure of the EXT1 protein by changing the numbers of α-helices and β-pleated sheets in addition to truncating the 112 amino acids at the C-terminus of EXT1 (Fig. 5).

Discussion

The EXT family of genes consists of tumor-suppressor genes whose loss of function or dysregulation causes hereditary multiple exostoses. The EXT proteins are endoplasmic reticulum-localized type II transmembrane proteins comprising an N-terminal cytoplasmic tail, a transmembrane domain, a stalk, and a large globular domain (13). EXT1 and EXT2 form heteroligomeric glycosyltransferases in Golgi apparatus, and are tightly associated with glycosyltransferase activities involved in the polymerization of heparan sulfate (HS) via alternating addition of GlcAc and GlcNAc residues to lengthen HS chains. The EXT1/EXT2 complex possesses substantially higher glycosyltransferase activity than either EXT1 or EXT2 alone (14,15). HS proteoglycans are known to be necessary for signaling of fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) and are involved in the gradient formation of morphogens such as hedgehog or bone morphogenetic proteins (BMPs) (16). Mutations in the glycosyltransferase genes, usually by causing a frame-shift in protein elongation or missense in amino acid code, creates truncated forms of the enzymes that these genes encode, leading to lower enzyme activity and less HS chain synthesis (10,17) Critical loss of full length HS chains results in deranged ligand-receptor interactions as well as abnormal ligand diffusion gradients for a number of signaling pathways (Ihh, BMPs, FGF, Wnt), resulting in the development of osteochondroma (18-20). Mice lacking *EXT1* or *EXT2* were found to be embryonic lethal, and the embryos failed to undergo gastrulation, possibly as a result of a disruption of several signaling pathways critical to mesoderm development and formation of extra-embryonic structures. Heterozygous null $EXT2^{+/-}$ mice showed growth plate disturbances similar to those in their $EXT1^{+/-}$ counterparts, with a disorganized proliferative zone and changes in Ihh expression domains (21).

Generally, EXT1 mutations are correlated to more severe presentations of the disease; male patients usually exhibit more severe symptoms when compared with female patients which is hypothesized to be caused by a later growth plate closure, allowing more time for exostosis formation. Accordingly, patients with a greater number of exostoses (>20) usually have more disabilities and deformities (22-24). In our HME family, all patients carred the heterozygous mutation of EXT1 while no EXT2 mutation was noted. Compared with the second generation, patient III1 showed an increasing severity of the disease with larger tumor volume, larger tumor quantity, more onset positions and deformity, which may be due to the fact that the growth plate of patients II2 and II3 had been closed for a long time or due to the degeneration of osteochondromas. This finding supports the variation in the expressivity of HME that is in favor of genetic anticipation in this disease (22). Future studies using a larger patient population and applying the whole exome sequencing technology to identify more disease-causing mutations or investigating the epigenetic mechanisms contributing to disease progression should enhance insights regarding the link between genotype and disease phenotype as well as the molecular and pathophysiological mechanisms of HME.

Multiple osteochondromas usually increase in size and number during both childhood and adolescence. Although they have a predilection for the juxta-epiphyseal region of the long bones, in fact, they can be present on any bone of the body including short bones, flat bones and irregular bones. It has been shown that many HME patients do not present any clinical symptoms in the early onset or even in later stages. There is strong evidence to suggest that rare mutations in a set of key genes are responsible for a substantial portion of complex HME disease (25). HME is also known to be characterized by a wide clinical heterogeneity. Evolutionary forces generate vast genetic heterogeneity by introducing many new variants in each generation. Current sequencing technologies offer the possibility of finding rare disease-causing mutations and mutation-associated genes. The present study identified a novel disease-causing *EXT1* mutation exclusively in all patients in a Chinese pedigree with HME, which not only highlights the critical pathophysiological role of the *EXT1* gene in HME, but also supports the high value of developing rapid and accurate genetic tools for HME diagnosis.

Our study of a Chinese HME kindred identified one novel disease-causing mutation c.1902C>A (p.Tyr634X) in the *EXT1* gene, and *in silico* analysis revealed the significant impact of this variation on the glycosyltransferase activity of EXT1. This offers new clinical and genetic data for better understanding the pathogenesis of this disease. Moreover, our findings also provide important information for genetic counseling and designing optimal strategies for the quick and accurate molecular diagnosis of HME.

Acknowledgements

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