Whole-exome sequencing identifies a novel mutation of *SLC20A2* (c.C1849T) as a possible cause of hereditary multiple exostoses in a Chinese family

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Abstract. Although the main causative genes for hereditary multiple exostoses (HME) are exostosin (*EXT*)-1 and *EXT-2*, there are numerous patients with HME without *EXT-1* and *EXT-2* mutations. The present study aimed to identify novel candidate genes for the development of HME in patients without *EXT-1* and *EXT-2* mutations. Whole-exome sequencing was performed in a Chinese family with HME and without *EXT-1* and *EXT-2* mutations, followed by a combined bioinformatics pipeline including annotation and filtering processes to identify candidate variants. Candidate variants were then validated using Sanger sequencing. A total of 1,830 original variants were revealed to be heterozygous mutations in three patients with HME which were not present in healthy controls. Two mutations [c.C1849T in solute carrier family 20 member 2 (SLC20A2) and c.G506A in leucine zipper and EF-hand containing transmembrane protein 1 (LETM1)] were identified as possible causative variants for HME through a bioinformatics filtering procedure and harmful prediction. Sanger sequencing results confirmed these two mutations in all patients with HME. A mutation in SLC20A2 (c.C1849T) led to a change in an amino acid (p.R617C), which may be involved in the development of HME by inducing metabolic disorders of phosphate and abnormal proliferation and differentiation in chondrocytes. In conclusion, the present study revealed two mutations [SLC20A2 (c.C1849T) and LETM1 (c.G506A) in a Chinese family with HME. The mutation in SLC20A2 (c.C1849T)] was more likely to be involved in the development of HME.

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Abbreviations: SLC20A2, solute carrier family 20 member 2; HME, hereditary multiple exostoses; *EXT-1*, exostosin-1; *EXT-2*, exostosin-2; HSPG, heparan sulfate proteoglycan; VCF, variant call format; LETM1, leucine zipper and EF-hand containing transmembrane protein

Key words: hereditary multiple exostoses, solute carrier family 20 member 2, mutation, whole-exome sequencing

Introduction

Hereditary multiple exostoses (HME) is an autosomal dominant inheritance disease in children, with an estimated incidence of 1 in 50,000 in the Western population in 1994 (1-3). While the clinical manifestation of HME varies, it is characterised by cartilage-capped tumours (called exostoses or osteochondromas) that occur on endochondral bone, and the most commonly involved bones include the distal femur, proximal tibia, fibula and humerus (1-3).

Currently, the detailed mechanism of HME remains unclear. Numerous previous studies have reported that the development of HME is associated with gene mutations in exostosin (*EXT*)-1 and *EXT-2* (4-6). The *EXT-1* gene is located on chromosome 8 (locus 8q24.1) whereas the *EXT-2* gene is located on chromosome 11 (locus 11p11-13) (4-6). Previous studies have reported that 70-95% of patients with HME display mutations in the *EXT-1* and *EXT-2* genes (6). *EXT* genes encode a protein product of the enzyme glycosyltransferase, which serves an important role in the synthesis of heparan sulfate proteoglycans (HSPGs). As an important component of the extracellular matrix, HSPGs regulate the proliferation and differentiation of chondrocytes (5). Synthesis defects in HSPGs due to mutations in *EXT-1* or *EXT-2* cause the abnormal proliferation and differentiation of chondrocytes and subsequently lead to the development of HME.

Although the main causative genes for HME are *EXT-1* and *EXT-2* (6,7), there are numerous patients with HME without *EXT-1* and *EXT-2* gene mutations (8,9). Therefore, it is crucial to identify other candidate genes in patients with HME. However, few genetic studies have focused on patients with HME without *EXT-1* and *EXT-2* mutations (10,11).

In the present study, whole-exome sequencing was performed in a typical Chinese HME family without *EXT-1* and *EXT-2* mutations. The aim of the present study was to identify novel candidate genes for the development of HME.

Materials and methods

Patients. The patients recruited to the present study were from a family in Guangdong Province, China between January 2016 and September 2016. The proband (age, 4 years; sex, male) sought medical advice at Guangzhou Women and Children's Medical Center due to multiple exostoses at the bilateral distal femur, proximal tibia and fibula, distal tibia, proximal humerus and left scapula (Fig. 1). The proband was diagnosed with HME.

Additionally, the parents were evaluated according to medical record reviews, physical examinations and patient history to obtain detailed information about all family members. As a result, the mother (33 years old) and grandfather (58 years old) of the proband were also diagnosed with HME (Fig. 2). The great-grandfather of the proband was also reported to have multiple exostoses, although physical examination could not be performed to confirm this information as the patient was deceased.

The present study protocol was approved by the Human Ethics Committee of the Guangzhou Women and Children's Medical Center and Guangzhou Medical University (approval no. 2017-320). All patients or their guardians provided written informed consent. The blood samples of all members in the family were obtained.

Library construction, whole-exome sequencing and data analysis. Genomic DNA was extracted from peripheral blood samples using the Genomic DNA Purification kit (Qiagen China Co., Ltd) and DNA concentrations were detected by a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). Sample integrity and purity were detected by agarose gel electrophoresis. Exons of *EXT-1* and *EXT-2* were sequenced by the Guangzhou Institute of Pediatrics at the Guangzhou Women and Children's Medical Center via Sanger sequencing to identify whether the family had mutations in these established HME-associated causal genes (12); however, no mutations were found in these genes. Thus, the results indicated that unknown mutations may be responsible for the development of HME in this family. Therefore, whole-exome sequencing was subsequently performed.

All the processes of whole-exome sequencing were performed by the Beijing Genomics Institute. Briefly, extracted genomic DNA was randomly fragmented to an average size of 250-300 bp on a Covaris s220 sonication machine (Covaris, Inc.). Fragmented DNA was tested by agarose gel electrophoresis and purified using an AxyPrep Mag PCR Clean kit (Axygen; Corning) according to the manufacturer's protocol. The exonic DNA fragments were subjected to end-repair, 3' adenylation, adaptor ligation. Following adaptor ligation, the DNA library was amplified according to a standard Illumina protocols, and the PCR products were recovered using the AxyPrep Mag PCR Clean kit. Then, the library was qualified by an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Inc.) and an ABI StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The qualified libraries were sequenced on the Illumina HiSeq Xten System (Beijing Genomics Institute) with 150 bp end reads. Sequencing image data were then translated to primary raw reads (paired-end reads) using Illumina Base Calling software (CASAVA 1.8; Illumina, Inc). The human reference genome assembly hg19 (GRCh37) was used as a reference sequence in this analysis process.

The detection of variants was performed using a Genome Analysis Toolkit (GATK version 3.3.0; Broad Institute) according to the manufacturer's protocol and all the variants were saved as a variant call format (VCF) file. Tertiary analysis was performed based on VCF files from the secondary analysis using SnpEff software (version 4.1; snpeff.sourceforge.net/index.html), including quality filtering, annotation, data aggregation and functional prediction. According to the phenotype of the family, there were two potential types of inheritance models, including autosomal dominant inheritance and autosomal recessive inheritance. The 1,000 Genomes Project and the Exome Aggregation Consortium database were used to analyse the variants (13). A total of 3 databases [RefGene (14), KnownGene (15) and Ensembl (16)] were used to annotate these variations. All variants were filtered if the variant was annotated as a nonsynonymous, frameshift or splicing mutation in one of these databases. Finally, further harmful prediction was performed for the mutations using common prediction tools [Sorting Intolerant From Tolerant (SIFT; http://provean.jcvi.org), Polyphen2 (genetics.bwh.harvard.edu/pph2), Genomic Evolutionary Rate Profiling (GERP++) (17), Likelihood Ratio Test (LRT; evomics.org/resources/likelihood-ratio-test) and MutationTaster (www.mutationtaster.org)] and the mutations that were predicted to be harmful by ≥3 of these tools and that had no benign predictions were excluded.

Variant validations and selection of the candidate gene. Candidate variants from whole-exome sequencing were validated by Sanger sequencing. Subsequently, the function of the validated gene was evaluated. Genes with a potential ability to influence chondrocyte proliferation and differentiation were more likely to be involved in the development of HME and were determined to be the candidate genes for HME (3).

Alignment of the identified candidate gene. If a gene was determined to be the pathologic candidate gene for HME, alignment of the gene at the locus of mutation among different species (human, mouse, chicken, pig, dog, and zebrafish) was performed using MultAlin (multalin.toulouse.inra.fr/multalin) to investigate whether the mutation was located at a highly conserved region.

Magnetic resonance images (MRI). The cranial MRI examination was performed on the proband to detect pathological change of the brain. In particular, brain calcification was diagnosed as a local lesion with low signal on both T2-weighted imaging and T1 weighted imaging (18).

Positive rate of EXT-1 and EXT-2 mutations. PubMed (pubmed.ncbi.nlm.nih.gov) and Web of Science (www.webofknowledge.com) databases were searched to identify literature that reported the positive rate of *EXT-1* and *EXT-2* mutations in different populations. The rates were extracted and the mean value was calculated.

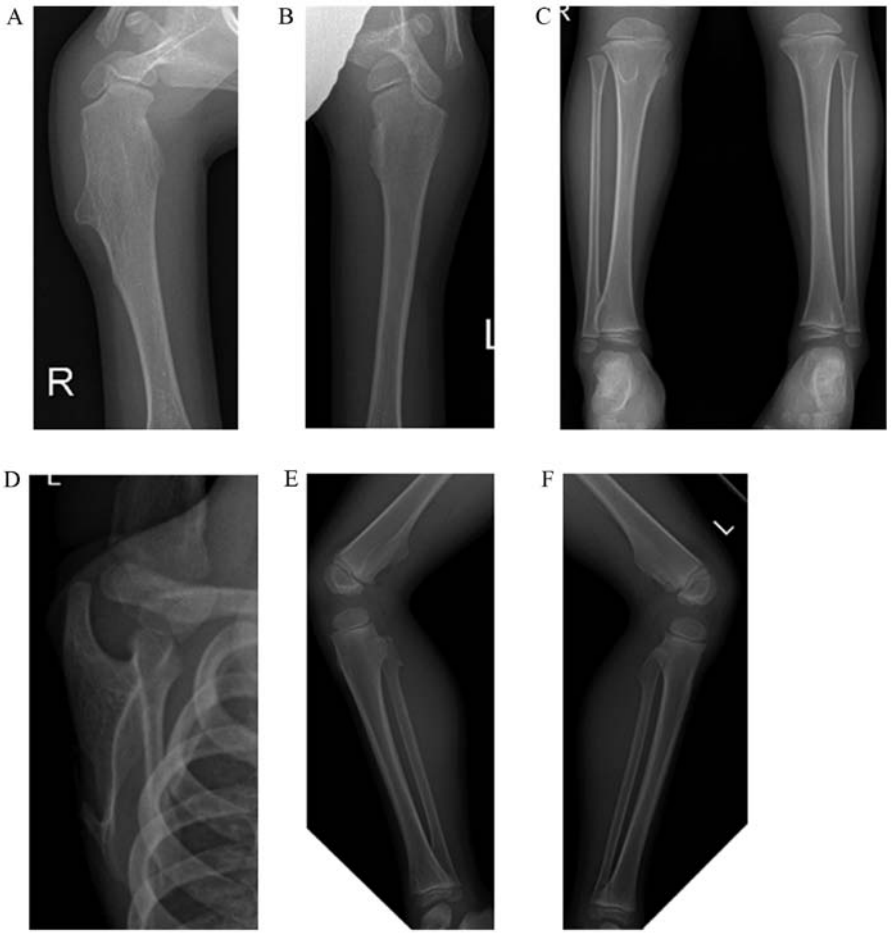

Figure 1. Radiographs of the proband demonstrated multiple exostoses at the bilateral (A and B) proximal humerus, (C) proximal tibia and fibula, distal tibia, (D) left scapula and (E and F) distal femur. R, right; L, left.

Results

Whole-exome sequencing. All samples from the family were successfully sequenced and variants were detected with an average sequencing depth on the target over 100 and 98% coverage of the target region. The high-quality reads obtained from II-1, II-2, II-3, III-1, III-2, III-3 and the proband (IV-1; Fig. 2) were 6,858, 5,668, 5,516, 5,917, 6,049, 5,989 and 5,560 Mb, respectively. VCF files for samples in the family were imported together into SnpEff software for posterior annotation and filtering. According to the phenotype of the family, there were two potential types of inheritance models in this family, including autosomal dominant inheritance and autosomal recessive inheritance.

Under the autosomal dominant inheritance model, the patients with HME had a heterozygous mutation and healthy controls had no variants. A total of 3 databases, including refGene, KnownGene and Ensembl, were used to annotate all the variants. As a result, a total of 1,830 original variants were revealed to be heterozygous mutations in the 3 patients with HME, which were not present in the healthy controls (Data S1, autosomal_dominant.vcf). Of these variants, 182 mutations were nonsynonymous mutations. Mutations located at repeating regions of the genome were excluded, resulting in 145 mutations. Furthermore, variants with mutation frequencies >1% in the Asian population (The 1,000 Genomes Project and the Exome Aggregation Consortium database) were excluded and 28 mutations remained. Among these 28 variants, 2 variants did not result in amino acid polymorphisms and were excluded. Further harmful prediction was performed for these 26 mutations using common prediction tools (SIFT,

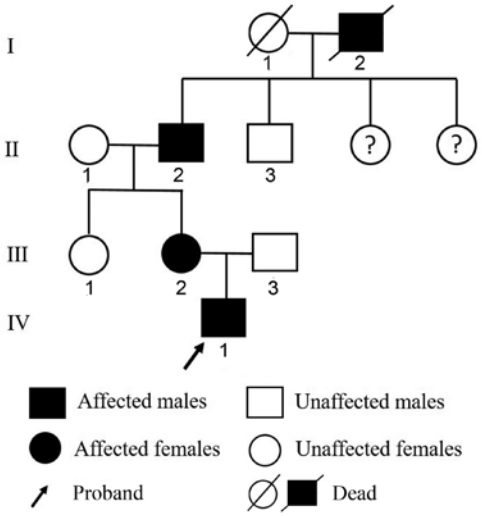

Figure 2. Pedigree of the Chinese family with HME. The arrow indicates the proband. The mother (III-2) and grandfather (II-2) of the proband were also diagnosed with HME. HME, hereditary multiple exostoses.

Table I. Harmful prediction of the 26 gene variations.

Position	Gene	Transcript	Nucleotide change	Amino acid change	Harmful prediction				
					SIFT	Polyphen2	LRT	GERP++	Mutation Taster
Chr1: 228475819	OBSCN	NM_001098623	c.C9869T	p.T3290M	T	D	N	N	N
Chr2: 219870747	CFAP65	NM_194302	c.C4918T	p.H1640Y	T	B	N	N	D
Chr4: 1843162	LETM1	NM_012318	c.G506A	p.R169H	D	D	D	D	D
Chr4: 7026948	TBC1D14	NM_001113363	c.G1135A	p.A379T	T	B	D	D	D
Chr5: 68661455	TAF9	NM_001015892	c.A110G	p.N37S	D	B	D	D	D
Chr5:168093596	SLIT3	NM_001271946	c.C4456T	p.R1486C	T	D	N	D	D
Chr7: 143098436	EPHA1	NM_005232	c.G413A	p.R138Q	T	D	D	D	D
Chr8: 42275431	SLC20A2	NM_001257180	c.C1849T	p.R617C	D	D	D	D	D
Chr8: 67425859	C8orf46	NM_152765	c.G427A	p.V143I	-	B	N	D	N
Chr9: 19347068	DENND4C	NM_017925	c.G4154C	p.S1385T	T	B	N	N	N
Chr9: 100693246	HEMGN	NM_197978	c.A431G	p.Q144R	T	B	N	N	N
Chr9: 116181419	C9orf43	NM_001278629	c.A319G	p.I107V	T	B	N	N	N
Chr12: 88390214	C12orf50	NM_152589	c.A418G	p.M140V	T	B	N	N	N
Chr12: 88505514	CEP290	NM_025114	c.A2174C	p.E725A	T	D	D	D	D
Chr12: 95927927	USP44	NM_001042403	c.G106A	p.E36K	T	D	D	D	D
Chr12: 126004130	TMEM132B	NM_052907	c.G1237A	p.V413I	T	B	N	N	D
Chr14: 32257075	NUBPL	NM_001201574	c.A54G	p.I18M	D	D	D	N	D
Chr14: 51446236	TRIM9	NM_015163	c.A1939G	p.I647V	T	B	N	N	D
Chr15: 44695168	CASC4	NM_138423	c.G1156A	p.V386I	T	D	D	D	D
Chr17: 8048267	PER1	NM_002616	c.G2263A	p.A755T	T	B	N	D	D
Chr17: 10535909	MYH3	NM_002470	c.C4840T	p.R1614W	D	D	-	N	D
Chr18: 21508694	LAMA3	NM_001127718	c.C3406A	p.Q1136K	T	D	-	D	D
Chr18: 22805996	ZNF521	NM_001308225	c.G1226A	p.R409H	T	D	D	D	D
Chr19: 18468286	PGPEP1	NM_001308366	c.C298T	p.R100C	D	B	N	D	D
Chr19: 24309223	ZNF254	NM_001278678	c.G166T	p.G56X	T	-	-	N	D
Chr21: 15884882	SAMSN1	NM_022136	c.G292A	p.G98R	T	B	N	D	N

SIFT, Sorting Intolerant From Tolerant; GERP++, Genomic Evolutionary Rate Profiling; LRT, Likelihood Ratio Test; Chr, chromosome; T, tolerant; D, deleterious; N, non-deleterious; B, benign.

Polyphen2, GERP++, LRT and MutationTaster; Table I) and the mutations that were predicted to be harmful by ≥3 of these tools and that had no benign predictions were excluded. This resulted in a total of 2 mutations, c.C1849T in solute carrier family 20 member 2 (SLC20A2) and c.G506A in leucine zipper and EF-hand containing transmembrane protein 1 (LETM1). Both were nonsynonymous single-nucleotide variant mutations and were selected as the candidate variants.

Under the autosomal recessive inheritance model, patients with HME had homozygous mutations, while healthy controls had heterozygous mutations or no mutations. A total of 3 databases, including refGene, KnownGene and Ensembl, were used to annotate all variants. As a result, a total of 246 original variants were revealed to be consistent with the recessive inheritance model (Data S2, autosomal_recessive.vcf). Of these variants, 19 mutations were nonsynonymous and were not located in repeating regions of the genome. However, all 19 of these variants were excluded due to a mutation frequency >1% in the Asian population (The 1,000 Genomes and the Exome Aggregation Consortium database) (13). Furthermore, harmful prediction using common prediction tools was conducted and all variants were excluded.

Variant validations by Sanger sequencing. The selected candidate variants (c.C1849T in SLC20A2 and c.G506A in LETM1) were validated by Sanger sequencing. Sanger sequencing results confirmed that these two mutations were present in all patients with HME (II-2, III-2 and IV-1), while family members who did not have HME did not have these mutations (Figs. 3 and 4). Of the two genes, SLC20A2 mutation may induce metabolic disturbances of phosphates to regulate chondrocyte proliferation and differentiation, and subsequently lead to the development of HME.

Alignment of the identified candidate gene. SLC20A2 was identified as the pathologic candidate gene. SLC20A2 alignment at the locus of mutation among different species was performed. The mutation locus of SLC20A2 located at a highly conserved region (Fig. 5). Mutations in c.C1849T in SLC20A2 led to a change in an amino acid (p.R617C).

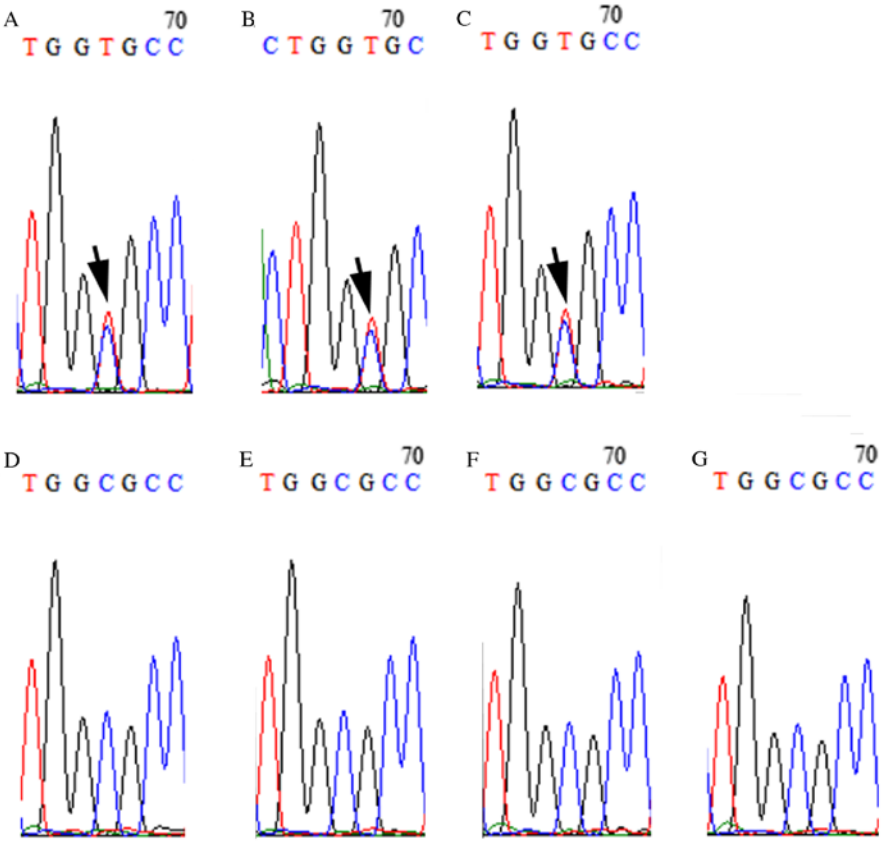

Figure 3. Sanger sequencing confirmed that the mutations of c.C1849T in solute carrier family 20 member 2 were present in all patients with hereditary multiple exostoses. (A-C) Mutations in patients with hereditary multiple exotoses. (D-G) No mutations were identified in healthy controls. The black arrows indicate mutation sites. T, thymine; G, guanine; C, cytosine.

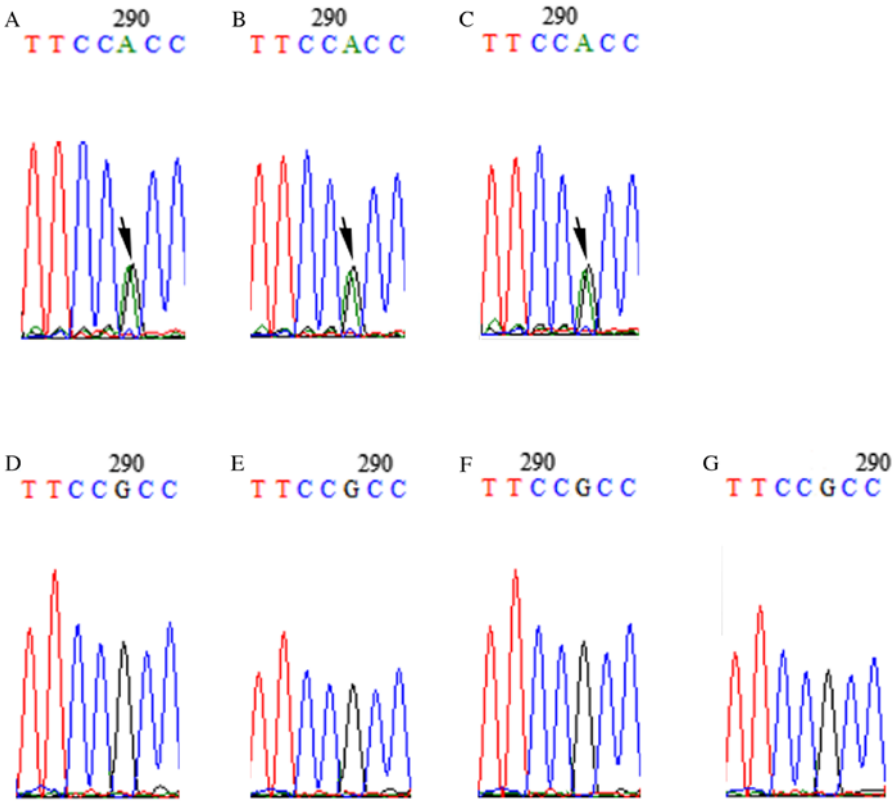

Figure 4. Sanger sequencing confirmed the mutations of c.G506A in leucine zipper and EF-hand containing transmembrane protein 1 were present in all patients with hereditary multiple exostoses. (A-C) Mutations in patients with hereditary multiple exostoses. (D-G) No mutations were identified in healthy controls. The black arrows indicate mutation sites. T, thymine; C, cytosine; A, adenosine; G, guanine.

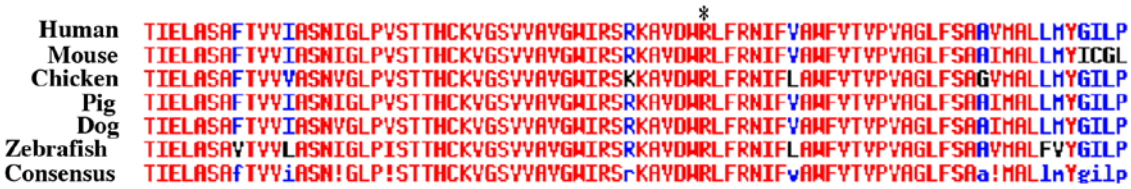

Figure 5. Alignment of solute carrier family 20 member 2 at the locus of mutation among different species.

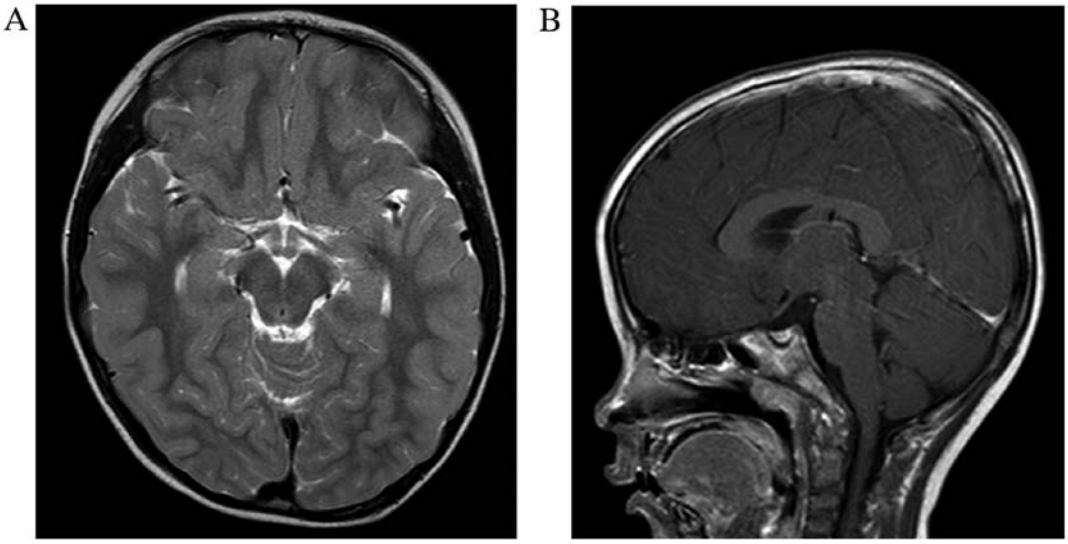

Figure 6. The cerebral magnetic resonance image of the proband did not exhibit brain calcification. The (A) transverse and (B) sagittal sections of the brain.

Results of cranial MRI. Cranial MRI indicated the proband did not exhibit any brain pathologic changes, such brain calcification, tumor or hydrocephalus (Fig. 6).

Reported positive rate of EXT-1 and EXT-2 mutations. A total of 14 papers from PubMed and Web of Science were extracted that reported the positive rate of *EXT-1* and *EXT-2* mutations in populations (Table II) (7-9,19-29). The worldwide prevalence of *EXT-1* and *EXT-2* gene mutations (1999-2018) was 47.2-95.7% in patients with HME. The overall positive rate in patients with HME was 76.8%.

Discussion

Mutations in the *EXT-1* and *EXT-2* genes are considered to be the primary cause of HME (5,7). Numerous studies have identified various mutation sites in *EXT-1* and *EXT-2* (30-32). The reported worldwide prevalence of *EXT-1* and *EXT-2* gene mutations (1999-2018) is 47.2-95.7% in patients with HME (8-10,19-29). However, 23.2% of living patients with HME have no *EXT-1* or *EXT-2* mutations. Presently, few studies have focused on patients with HME without *EXT-1* and *EXT-2* mutations (11,12). Therefore, it is crucial to identify novel candidate genes in patients with HME.

The present study revealed a novel deleterious mutation, c.C1849T in SLC20A2 using SIFT, PolyPhe2, LRT, GERP++ and MutationTaster database analyses. As a member of the SLC20 family of solute carriers (33,34), the SLC20A2 protein is comprised of 652 amino acids with a molecular mass of 7,0392 Da (33,34). The SLC20A2 protein, also known as Pit-2, is a type III sodium-phosphate cotransporter that mediates the transmembrane movement of sodium and phosphate (34-36). SLC20A2 is expressed on the basolateral membranes of polarized epithelial cells and is involved in cellular phosphate homeostasis (33). It is widely expressed in numerous tissues and organs, including the kidneys and intestines (33). Furthermore, previous studies have reported that SLC20A2 is also expressed by chondrocytes (35-37). In the present study, mutations in c.C1849T in SLC20A2 led to a change in an amino acid (p.R617C). Alignments amongst species indicated that this mutation was located at a highly conserved amino acid sequence at which mutations have increased probability to induce structural changes and SLC20 dysfunction, subsequently leading to a disturbance in the uptake of inorganic phosphorus in cells (38). Mutations in SLC20A2 have been increasingly reported in primary familial brain calcification cases (39,40) and, to the best of our knowledge, the present study is the first to report SLC20A2 mutation in patients with HME. Sekine *et al* (38) reported that SLC20A2 variants significantly decreased inorganic phosphate transport activity in endothelial cells induced from induced pluripotent stem cells (iPS-Cs) derived from patients with idiopathic basal ganglia calcification patients compared with control iPS-ECs. Additionally, SLC20A2 is a physiological regulator of tissue mineralization and serves an important role in the determination of bone quality and strength (41). However, cranial magnetic resonance images indicated the proband in the present study did not exhibit brain calcification.

Table II. Positive rates of exostosin (*EXT*)-1 and *EXT-2* genes in patients with hereditary multiple exostoses as reported by various studies.

First author (refs.)	Year	Journal	Country	Positive rate (%)
Xu (19)	1999	Hum Genet	China	47.2 (17/36)
Francannet (20)	2001	J Med Genet	France	85.7 (36/42)
Wuyts (21)	2005	Clin Genet	Belgium	74 (37/50)
Vink (22)	2005	Eur J Hum Genet	Netherlands	63 (22/35)
Lonie (23)	2006	Hum Mutat	United Kingdom	78 (56/72)
Kojima (24)	2008	Genet Test	Japan	54 (23/43)
Jennes (25)	2008	J Mol Diagn	Belgium	68.3 (43/68)
Heinritz (7)	2009	Ann Hum Genet	Germany	95.7 (22/23)
Kang (26)	2012	Gene	China	90 (9/10)
Sarrión (27)	2013	Sci Rep	Span	95 (37/39)
Jamsheer (28)	2014	J Appl Genet	Poland	84.9 (28/33)
Ishimaru (8)	2016	BMC Genet	Japan	66.2 (47/71)
Santos (9)	2018	Mol Genet Genomic Med	Brazil	83 (95/114)
Li (29)	2018	Medicine (Baltimore)	China	93 (68/73)
Total	-	-	-	76.8 (562/732)

SLC20A2 mutation-induced metabolic disturbances of phosphates may be involved in the development of HME (38). Previous studies have demonstrated that phosphate serves an important role in regulating the proliferation and differentiation of chondrocytes (42,43). Zalutskaya *et al* (42) utilized a mouse metatarsal culture model to evaluate the effect of various concentrations of phosphate on endochondral bone formation. The results demonstrated that a phosphate-high concentration (7 nM) significantly decreased the proliferation of chondrocytes and promoted the terminal differentiation of hypertrophic chondrocytes. However, Liu *et al* (44) revealed that low phosphate concentrations inhibited the differentiation of chondrocytes through the parathyroid hormone-related peptide signaling pathway in a mouse metatarsal culture model. Furthermore, Magne *et al* (45) reported similar results. Previous studies have demonstrated that HME is characterized by the abnormal proliferation and differentiation of chondrocytes (46,47). The present study hypothesized that SLC20A2 mutation-induced metabolic disorder of phosphates in chondrocytes may lead to their abnormal proliferation and differentiation and subsequently result in the development of HME.

Additionally, mutations in c.G506A in LETM1 were predicted to be harmful using common prediction tools. LETM1 encodes a protein that is localized to the inner mitochondrial membrane (48). Studies have reported that mutations in this gene cause Wolf-Hirschhorn syndrome (49,50). To the best of our knowledge, no studies concerning the effect of the LETM1 gene on cartilages and bones have been conducted. Thus, further studies are required to evaluate the effect of mutations in c.G506A in LETM1 on the proliferation and differentiation of chondrocytes.

There were limitations in the present study. In the present study, whole-exome sequencing was performed and identified a novel mutation of SLC20A2. Although mutations in SLC20A2 were predicted to lead to disturbance of uptake of inorganic phosphorus, the serum phosphorus levels of the patients was not assessed. Thus, further research is required to investigate the effect of mutation in c.C1849T in SLC20A2 on the expression and function of SLC20A2.

In conclusion, the present study revealed two mutations, c.C1849T in SLC20A2 and c.G506A in LETM1, in a Chinese family with HME. Mutation in c.C1849T in SLC20A2 is more likely to be involved in the development of HME. Considering that the present study was an exploratory study of a family, additional investigations may be required to verify the preliminary results of the present study.

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Availability of data and materials

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

Authors' contributions

YL, XL, HX designed the present study and methodology, and supervised, reviewed and edited the manuscript. YL wrote the original draft of the manuscript and acquired funding. HX and YL provided resources. XL, MZ, FX, JL and ZY performed the experiments. YL and MZ conducted data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the Human Ethics Committee of the GuangZhou Women and Children's Medical Center and GuangZhou Medical University (approval no. 2017-320). Written informed consent to participate was obtained from all patients. In the case of children (<18 years), written informed consent was obtained from the parents.

Patient consent for publication

Written informed consent for publishing data and images was obtained from all patients. In the case of children (<18 years), written informed consent for publishing data and images was obtained from the parents.

Competing interests

The authors declare that they have no competing interests.

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