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Original Article

Analysis of EXT1 gene mutation in hereditary multiple exostosis families

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Abstract: Objective: This study was to investigate the EXT1 gene mutation in 9 patients with hereditary multiple exostosis (HME) and their relatives, and evaluate the relationship between EXT1 gene mutation and clinical characteristics of HME. Methods: The medial record of HME children who received therapy in the Department of Orthopedics of Shanghai Children's Hospital between January 2009 and March 2012 and general information of their relatives were retrospectively reviewed. Blood was collected from these children and their relatives, and RT-PCR and direct sequencing were performed to measure the mutation of EXT1 gene at 11 exons. Bioinformatics analysis was employed to pathogenic risk of corresponding mutations and the correlation between EXT1 gene mutations and clinical characteristics of HME. Results: A total of 9 HME children and 15 relatives (including 9 relatives with HME) were included in the present study, and the median number of tumors was 11 among 18 patients. Seven base changes were identified in 11 exons of EXT1 gene: Exon2 c.1767A>T, Exon3 c.1838C>T, Exon4 c.1987G>A, c.1994G>A, c.2007T>A, Exon9 c.2534G>A and Exon11 c.3168G>A, of which p.Thr332Ser, p.Arg405Lys and p.Trp412Arg were new missense mutations. p.Trp412Arg was closely associated with HME (HumDiv=1.000 and HunVar=0.996). Conclusion: Our study showed a new mutation site of EXT1 gene might be related to the morbidity of HME.

Keywords: Hereditary multiple exostosis, EXT1 gene mutation, pathogenic mutation analysis

Introduction

Hereditary multiple exostoses (HME) is also known as hereditary multiple osteochondromas (HMO), familial multiple exostosis and hereditary teratogenicosteochondroma [1]. It is anautosomal dominant genetic disease and usually characterized by the presence of multiple benign cartilage-capped tumors. EXT1 and EXT2 are two genes which have been widely accepted to be closely related to the pathogenesis of HME [2, 3]. About 80% of HME patients have nonsense mutation, splice site mutation or frameshift mutation, but in a small fraction of patients, the HME cannot be ascribed to the known EXT gene mutation. The symptoms vary between patients and families, and thus it is theoretoally and realistically important to identify the pathogenic mutation sites for the diagnosis and treatment of HME.

In the present study, 24 HME patients and their relatives were recruited, and RT-PCR and direct sequencing were employed for the screening of gene mutations, aiming to identify new mutation sites of EXT1 gene and evaluate the correlation between genotypes and clinical manifestations.

Materials and methods

Patients

Nine HME children and 15 immediate family members were recruited from the Department of Orthopedics of Shanghai Children's Hospital between January 2009 and March 2012. There were 9 affected relatives and 6 healthy relatives. In addition, there were 10 males and 14 females. The tumors were widely distributed except for craniofacial bone: knee: 77.8% (14/18), ankle: 61.1% (11/18), wrist: 44.4% (8/18) and shoulder 27.8% (5/18). Another patient had involvement of the spine (patient 50), causing severe scoliosis. Moreover, 7 patients (38.9%) had more than 10 tumors,

Table 1. Clinical characteristics of 24 subjects included in this study

No	Age of onset (yr)	Sex	Age of diagnosis (yr)	Limb deformity	Affected bone and joint										Influence of height (%)	Number of tumor	History of surgery	Type
					Shoulder	Elbow	Wrist	Hip	Knee	Ankle	Rib	Spine	Hand	Ilium				
10(+)	6	F	7	+	-	-	L	-	-	-	-	-	-	-	-	2	Yes	IIIa
11(+)	5	F	30	-	-	-	L	-	-	L	-	-	-	-	-	2	No	Ia
12(+)	10	M	23	+	-	-	-	-	-	-	-	-	+	+	<10	2	No	IIIa
20(+)	4	F	5	+	-	-	L	-	B	L	-	-	+	-	<10	18	Yes	IIb
30(+)	3	M	3	-	-	-	L	-	B	L	-	-	+	-	<10	8	Yes	IIIa
40(+)	4	F	7	-	-	-	-	-	B	B	+	-	-	-	<10	10	Yes	IIb
41(+)	-	M	34	-	-	-	-	-	R	-	-	-	-	-	-	2	No	Ia
42(-)	-	F	33	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-
50(+)	4	F	7	+	B	-	B	B	B	B	-	+	+	+	>10	51	Yes	IIIb
51(-)	-	F	33	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-
60(+)	6	F	8	-	-	-	-	-	B	R	-	-	-	-	<10	11	Yes	IIb
70(+)	2	M	9	+	L	L	B	R	B	B	-	-	-	-	>10	22	Yes	IIIb
71(-)	-	F	32	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-
72(+)	6	M	35	-	-	-	-	-	R	-	-	-	-	-	<10	2	No	Ia
81(+)	11	M	12	+	B	-	B	B	B	B	-	-	-	-	>10	24	Yes	IIIb
82(-)	-	F	33	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-
83(+)	10	M	35	-	-	-	-	-	R	L	-	-	-	-	<10	2	No	Ia
84(-)	-	M	4	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-
85(+)	6	F	70	-	-	-	-	-	-	L	-	-	-	-	-	2	No	Ia
86(+)	5	F	30	-	-	-	-	-	L	-	-	-	-	-	-	1	No	Ia
87(+)	6	F	8	+	L	-	-	-	L	-	-	-	-	-	<10	7	Yes	IIa
91(+)	6	M	7	+	B	-	B	-	B	B	-	-	+	-	>10	32	Yes	IIIb
92(+)	-	M	36	-	-	-	-	-	B	-	-	-	-	-	-	3	No	Ia
93(-)	-	F	34	-	-	-	-	-	-	-	-	-	-	-	-	0	No	-

limb deformity was found in 8 patients (44.4%; 8/18). In patient 50, 51 tumors were identified. In the present study, the classification system reported by Mordenti et al was employed [4]. Type I HME was found in 38.9% (7/18) of patients, type II in 22.2% (4/18), type III in 38.9% (7/18), and type IIb and others in 50.0% (9/18) (**Table 1**). Informed consent was obtained from each patient or their relatives before study.

Extraction of DNA

Anti-coagulated peripheral venous blood (5 ml) was collected from 24 HME patients and their relatives and stored at -20°C. Total blood DNA was extracted with phenol-chloroform RNA extraction method. The DNA concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm with the DU800 UVspectrophotometer (Beckman, USA).

Sequencing of EXT1 gene and design of primers

Primer was used to design 11 pairs of primers for EXT1 according to the sequence in GeneBnak (GeneBank NG_007455; NM_000127). The primers cover the 11 encoded exons and their side sequence, the proportion of GC was 50%-55% in these primers and the annealing temperature was 57°C. Primers were synthesized in Shanghai Jierui Biotech Co., Ltd (**Table 2**). PCR was performed for EXT1 gene with DNA template and a kit (TaKaRa E Taq with GC Buffer). The mixture used for PCR (25 ul) included 1 ul of template, 1 ul of each primer, 4 ul of dNTP, 0.4 ul of Taq polymerase, 2.5 ul of 10*PCR Buffer, 12.5 ul of 2×GC buffer and 3.6 ul of ddH_2O. The conditions were as follows: pre-denaturation at 95°C for 5 min, a total of 32 cycles of denaturaton at 95°C for 45 s, annealing at 57°C for 45 s and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were stored at 4°C. After detection the concentration and purity of PCR products, these products were subjected to sequencing in Shanghai Saiyin Company. The PCR products after amplification of 11 exons were separated by agarose gel electrophoresis and visualized. The products in different lanes represent distinct exons. The bands were clear and the length was 300-1200 bp,

Table 2. Primers used for PCR of EXT1 gene and anticipated length

Name	Primers	Length (bp)
H-EXT1-1F	5'-GATTGGGAAACTTGGGTGAT-3'	1254
H-EXT1-1R	5'-CCTACTTGGCTCGAGAAGGT-3'	
H-EXT1-2F	5'-TGCCAGAACGATCAGACTTG-3'	800
H-EXT1-2R	5'-CCTTGGAAGAGCTGGATCAT-3'	
H-EXT1-3F	5'-TCCAGGATTCATGCAGTGTC-3'	752
H-EXT1-3R	5'-AGGCAGCTCTTGAGCAATTC-3'	
H-EXT1-4F	5'-GCAGCTGACACTTCTTAAGG-3'	314
H-EXT1-4R	5'-AGCCAAGTGGTCTCACTTAC-3'	
H-EXT1-5F	5'-ACTCTGACTGCCACCATCTT-3'	848
H-EXT1-5R	5'-AGTAGCCGGTGGTAGAGATT-3'	
H-EXT1-6F	5'-AGCGGAGCAAGGAGGAGTAA-3'	353
H-EXT1-6R	5'-GGTGTAACGAGGCAGGATGA-3'	
H-EXT1-7F	5'-CTCCAGCCACCGTAATTCTT-3'	440
H-EXT1-7R	5'-CTCCACAGTGGTTCCACATA-3'	
H-EXT1-8F	5'-GAGATTCCTTCGGTGTTGAG-3'	459
H-EXT1-8R	5'-GAGGAGCCAATTAGCAGAGA-3'	
H-EXT1-9F	5'-GAATTAATGTTTCGCCACAG-3'	866
H-EXT1-9R	5'-GCACATGTCCTCTTGACTTC-3'	
H-EXT1-10F	5'-GCCTTGTAGGCTCCTTATGA-3'	774
H-EXT1-10R	5'-GGACTCTCCAGCCTCTAGAA-3'	
H-EXT1-11F	5'-CCATCTCACCTTGCACTTCT-3'	553
H-EXT1-11R	5'-GGCCATGACAATGATGTCTG-3'	

which were consistent with the anticipated (**Figure 1**).

Statistical analysis

Statistical analysis was performed with SAS 9.3. Continuous variables are expressed as mean ± standard deviation, and t test was employed for comparisons between two groups. Categorical variables were compared with chi square test or Fisher exact test. A value of two sided $P<0.05$ was considered statistically significant.

Results

Sequencing of exons of EXT1 gene

The sequence was compared with that in NCBI by using BLAST, and the mutation sites of exons of EXT1 gene were identified. In addition, the changes in amino acids were obtained by triplet codon analysis. Results showed 7 base changes were found in 11 exons of EXT1 gene: c.1767A>T in exon 2, c.1838C>T in exon 3, c.1987G>A, c.1994G>A and c.2007T>A in exon 4, c.2534G>A in exon 9 and c.3168G>A in exon 11. However, frameshift mutation and deletion mutation were not found, and repeat mutation of large fragments was also not identified (**Figure 2**).

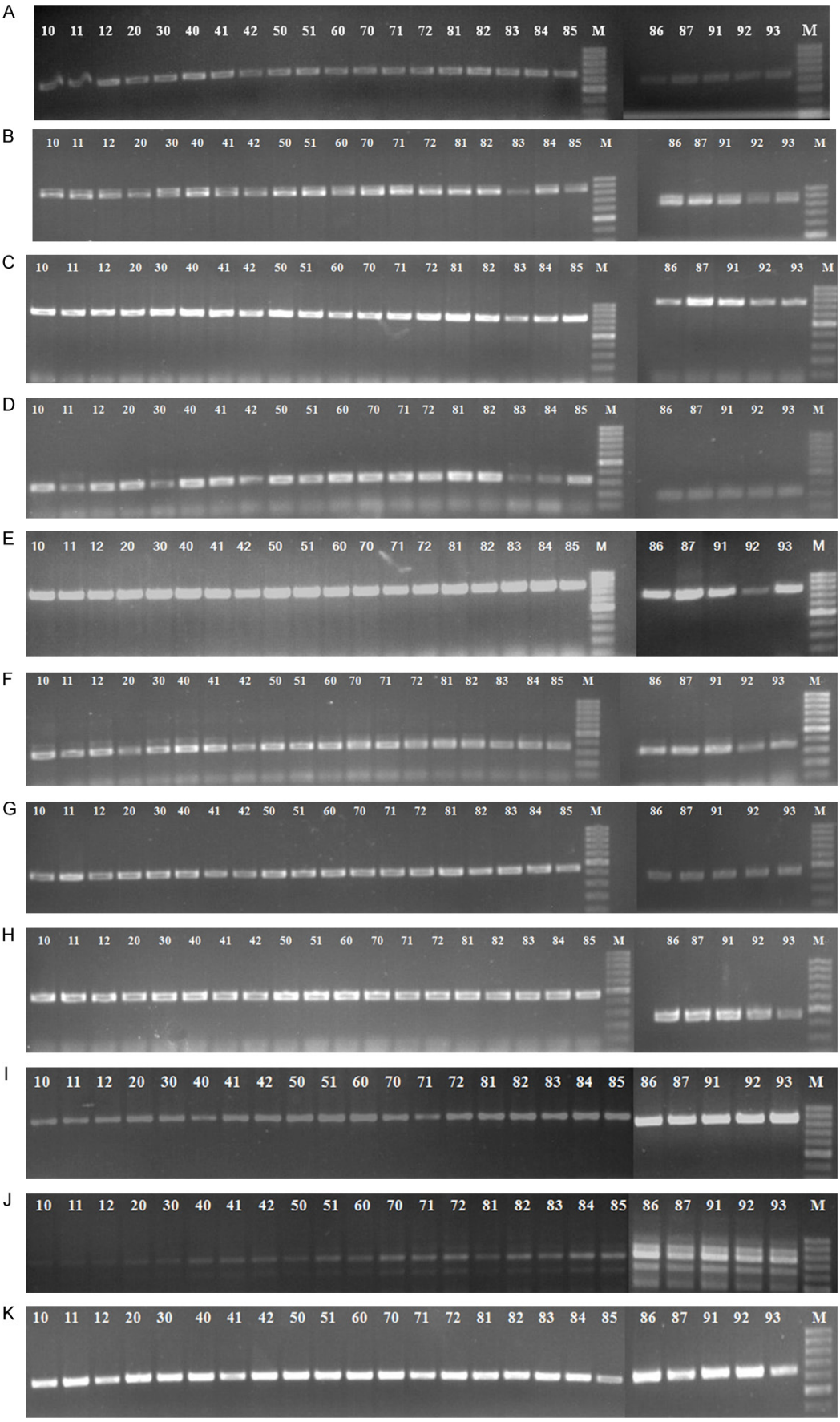
Codon analysis showed these single nucleotide changes corresponding to mutations of 3'UTR: synonymous mutation and missense mutation. Synonymous mutation was found in C→T mutation at 1838 of exon 3 corresponding to cysteine→cysteine at 355, G→A mutation at 1994 of exon 4 corresponding to glutamine→glutamine at 407, and G→A mutation at 2534 of exon 9 corresponding to glutamate→glutamate at 587. Missense mutation was found in A→T mutation at 1767 of exon 2 corresponding to threonine→serine at 332, G→A mutation at 1987 of exon 4 corresponding to arginine→lysine at 405, T→A mutation at 2007 of exon 4 corresponding to tryptophan→arginine at 412 (**Table 3**). Of these mutations, p.Thr→332Ser, p.Arg→405Lys and p.Trp→412Arg had never been reported in dbSNP137, 1000 human genome project and NHLBI database.

Correlation between genotypes and phenotypes

In patient 60, p.Thr332Ser was found, type IIb HME was diagnosed, limb deformity was absent, and ankle and knee were main joints involved. In patient 40, p.Arg405Lys was found, right knee was affected, limb deformity was absent, and type Ia HME was diagnosed. In 5 samples, p.Trp412Arg was found in patients 11, 12, 20, 30 and 50.

In addition, 80% of patients (4/5) with type IIb or higher HME had change in amino acid 412, but the proportion of type IIb or higher HME patients was only 38.5% in remaining 13 patients without change in amino acid 412 (5/13), showing no marked difference between them. Of patients with change in amino acid 412, 60% developed limb deformity, but limb deformity was only found in 26.3% of patients without change in amino acid 412 (5/13), showing no significant difference (**Table 4**).

Deformity was mainly found in the wrist (4/5), ankle (4/5), knee (3/5), shoulder (1/5) and hip (1/5) and had involvement of hand (4/5) and


A
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
B
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
C
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
D
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
E
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
F
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
G
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 M 86 87 91 92 93 M
H
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I
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J
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 86 87 91 92 93 M
K
10 11 12 20 30 40 41 42 50 51 60 70 71 72 81 82 83 84 85 86 87 91 92 93 M

Figure 1. 11 exons of EXT1 gene after amplification by PCR. A: Agarose gel electrophoresis of EXT1-1 after PCR. B: Agarose gel electrophoresis of EXT1-2 after PCR; C: Agarose gel electrophoresis of EXT1-3 after PCR; D: Agarose gel electrophoresis of EXT1-4 after PCR; E: Agarose gel electrophoresis of EXT1-5 after PCR; F: Agarose gel electrophoresis of EXT1-6 after PCR; G: Agarose gel electrophoresis of EXT1-7 after PCR; H: Agarose gel electrophoresis of EXT1-8 after PCR; I: Agarose gel electrophoresis of EXT1-9 after PCR; J: Agarose gel electrophoresis of EXT1-10 after PCR; K: Agarose gel electrophoresis of EXT1-11 after PCR.

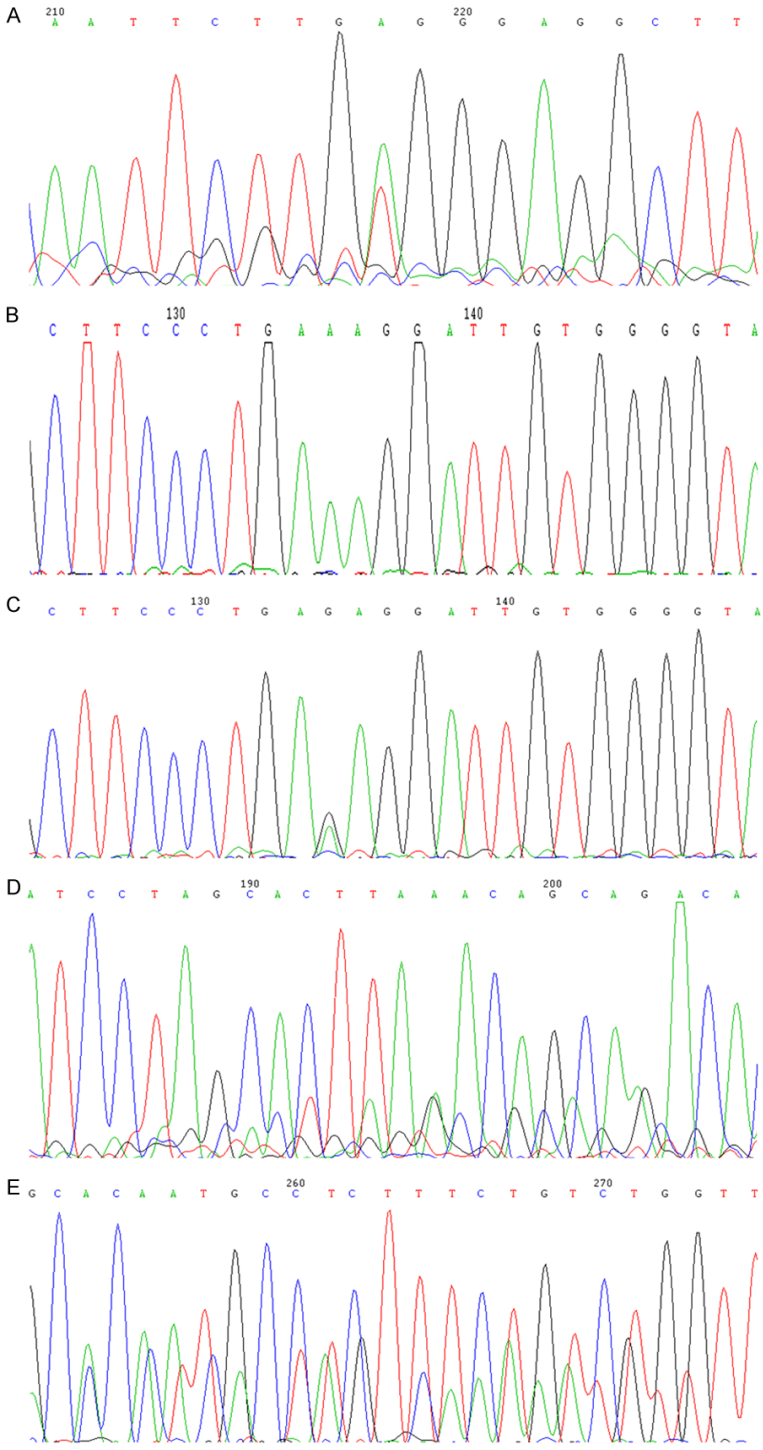

Figure 2. Sequencing of exons of EXT1 gene. A: Patient 12: T→A heterozygous mutation at amino acid 2007 of exon 4; B: Patient 40: G→A homozygous mutation at amino acid 2534 of exon 9; C: Patient 50: G→A heterozygous mutation at amino acid 2534 of exon 9; D: Patient 41: G→A heterozygous mutation at amino acid 1987 of exon 4; E: Patient 60: A→T heterozygous mutation at amino acid 1767 of exon 2.

ilium (2/5). The incidences of wrist deformity (80%) and ankle deformity (80%) in patients with mutations were higher than in patients without mutations (37%) although there were no significant differences (**Table 5**).

Prediction of pathogenic risk

Polyphen-2 Bioinformatics analysis was performed in 3 new mutations of EXT1 gene (http://genetics.bwh.harvard.edu/pph2/) and HumDiv and HumVar were calculated to predict the pathogenic risk. To further explore and confirm the harmfulness of these new mutations of EXT1 gene, SIFT software (http://sift.jcvi.org/www/SIFT_BLink_submit.html) was used to predict the influence of these mutations on the protein functions (**Table 6**).

In the polyphen-2 database, the amino acid 412 of exon 4 of EXT1 gene was highly conservative in different species, but the other two mutations were relatively conservative. The HumDiv and HunVar of c.2007 T>A (p.Trp412Arg) were as high as 1.000 and 0.996, respectively (**Figure 3**).

Both analyses with polyphen-2 software and SIFT

Table 3. Mutation site, corresponding bases and amino acids

No	Patient	Change in base	Change in amino acid	Mutation	Location	Heterozygous/Homozygous
10	Case	-	-	-	-	
11	Case	c.1994G>A	-	Synonymous	Exon 4	
		c.2007T>A	412 Trp-Arg	Missense	Exon 4	
12	Case	c.2007T>A	412 Trp-Arg	Missense	Exon 4	
20	Case	c.2007T>A	412 Trp-Arg	Missense	Exon 4	
30	Case	c.1987G>A	405 Arg-Lys	Missense	Exon 4	
		c.2007T>A	412 Trp-Arg	Missense	Exon 4	Homozygous
40	Case	c.2534G>A	-	Synonymous	Exon 9	Homozygous
41	Case	c.1987G>A	405 Arg-Lys	Missense	Exon 4	
		c.2534G>A	-	Synonymous	Exon 9	
42	Normal	c.2534G>A	-	Synonymous	Exon 9	
50	Case	c.2007T>A	412 Trp-Arg	Missense	Exon 4	
		c.2534G>A	-	Synonymous	Exon 9	
51	Normal	c.2534G>A	-	Synonymous	Exon 9	Homozygous
60	Case	c.1767T>A	332 Thr-Ser	Missense	Exon 2	
		c.1838C>T	-	Synonymous	Exon 3	
70	Case	-	-	-	-	
71	Normal	c.2534G>A	-	Synonymous	Exon 9	
72	Case	-	-	-	-	
81	Case	-	-	-	-	
82	Normal	-	-	-	-	
83	Case	-	-	-	-	
84	Normal	-	-	-	-	
85	Case	c.2534G>A	-	Synonymous	Exon 9	
86	Case	c.2534G>A	-	Synonymous	Exon 9	
87	Case	c.2534G>A	-	Synonymous	Exon 9	
91	Case	c.1838C>T	-	Synonymous	Exon 3	
92	Case	c.2534G>A	-	Synonymous	Exon 9	
93	Normal	c.1838C>T	-	Synonymous	Exon 3	Homozygous
		c.2534G>A	-	Synonymous	Exon 9	

Table 4. Correlation of mutations with clinical types and limb deformity

Amino acid 412	Type IIb or higher		*P*	Limb deformity		*P*
	N	%		N	%	
Mutation	4	80.0%	0.2941	3	60%	0.6078
Wide type	5	38.5%		5	26.3%	

Notes: Fisher exact test.

Table 5. Correlation of mutations with clinical types and limb deformity

	Wrist deformity		*P*	Knee deformity		*P*
	N	%		N	%	
Mutation	4	80.0%	>0.01	4	80.0%	>0.01
Wide type	4	30.7%		7	54.1%	

Table 6. Predictions of pathogenic risk of three new mutations

	Pathogenic risk		Influence on protein function
	HumDiv	HumVar	
p.Thr332Ser	0.828	0.580	0.02
p.Arg405Lys	0.778	0.387	0.02
p.Trp412Arg	1.000	0.996	0.00

software indicated that c.2007 T>A (p. Trp412Arg) was highly correlated with the pathogenesis of HME and the protein function.

Discussion

HME is a genetic heterogenic disease. To date, a total of 6 genes have been identified to be associated with the pathogenesis of HME, in-

Figure 3. Sequences of amino acid 412 in different species.

cluding EXT1, EXT2, EXT3, EXTL1, EXTL2 and EXTL3 which are mapped into 8q24.11-24.31 (OMIM 133700), 11p11-13 (OMIM 1 33701), 19p (OMIM 600209), 1p36, 1p11-p12 and 8p12-p22, respectively and also known as EXT gene family [5, 6]. Of these genes, EXT1 and EXT2 are the widely investigated ones and encode type II transmembrane Exostosin-1 with 746 amino acids and Exostosin-2 with 718 amino acids, respectively. There is 30.9% homology at primary amino acid structure between two proteins. Both proteins localize in Golgi body and endoplasmic reticulum, have the activities of N-acetylglucosaminetransferase and glucuronyltransferase [7] and play important roles in the synthesis of heparin sulfate (HS) [8]. The EXT gene mutation may cause the synthesis of abnormal HS, which leads to the excess proliferation of undifferentiated cartilage cells in the epiphysis, forming growth plate and finally the osteochondroma [8-10]. EXT1 gene mutation may cause severe clinical symptoms [11], 44%-66% of HME is related to the EXT1 gene mutation, but only 27% of HME is associated with EXT2 gene mutation, and 10% of HME is caused by other mutations [12].

In the present study, EXT1 gene mutation was detected, and results showed the clinical symptoms of patients with EXT1 gene mutation were more severe than in those without mutations although significant difference was not observed, which might be ascribed to the small sample size. In available studies, EXT1 gene mutations are mainly found in the first 6 exons, and the following 5 exons are close to the conservative sequence of C terminus and will not develop mutations. Wuyts et al [13] found 29 mutations of EXT1 gene localized in the first 6 exons and mainly affected the amino acid R340 and G339. This indicates that the changes in amino acids R340 and G339 directly affect the structural stability and functions of EXT1 protein and are closely related to the pathogenesis of HME. In a Chinese study, Zhou et al [14] investigated the mutations of EXT1 and EXT2 genes in a Han Chinese HME family of Shaanxi Province, and they found 7 new mutations that had never been reported. Deng et al [15] found a frameshift mutation c.346_356 delins TAT at exon 1 of EXT1 gene and a heterozygous deletion mutation c.2009-2012 del (TCAA) at exon 10 of EXT1 gene, but mutations of EXT2 gene were not identified in 2 HME families.

In the present study, blood was collected from 24 subjects from 9 HME families and subjected to sequencing of EXT1 gene. Results showed three new mutations of EXT1 gene: p.Thr332Ser, p.Arg405Lys and p.Trp412Arg. Missense mutation was mainly found in exons 2 and 4. The correlation between p.Trp412Arg and clinical phenotype of HME was evaluated. Results showed p.Trp412Arg in 5 patients (patients 11 and 12 in family 1, patient 20 in family 2, patient 30 in family 3 and patient 50 in family 5). In addition, 80% of patients with p.Trp412Arg were diagnosed with type IIb or higher HME (4/5), which was higher than in remaining 19 patients without p.Trp412Arg (38.5%; 6/13) although there was no significant difference, which might be associated with the small sample size. Sequencing showed these mutations displayed coseparation with the phenotypes of HME. Thus, we speculate that these mutations may be the pathogenic mutations causing HME. In p.Thr332Ser, tryptophan is a nonpolar amino acid and serine is a polar amino acid. In p.Trp412Arg, tryptophan is a nonpolar amino acid and arginine is a basic amino acid. The changes in amino acid properties after mutation may cause the alteration of space structure of EXT1 protein and the production of abnormal proteins, leading to the occurrence of HME [16, 17]. In the present study, we for the first time reported that p.Trp412Arg was related to the severity of HME symptoms, but he specific role of p.Trp412Arg in the pathogenesis of HME is needed to be further studied.

In previous studies, EXT1 and EXT2 are considered independent pathogenic genes and no studies have identified the mutations of both EXT1 gene and EXT2 gene in the same patient or same family [18]. In our study, EXT1 mutations were not found in 5 patients with severe HME, which might be caused by EXT2 mutation, but the specific pathogenesis of HME should be further investigated in these patients. The investigation of new mutation p.Trp412Arg requires the cloning of EXT1 gene in the blood and tissues of these probands and their relatives and the functional analysis may be performed in animals. We believe that the development of technology, in-depth studies on the EXT genes and the identification of new mutations will deepen the understanding of the genetic heterogeneity of HME and the complexity and diversity of EXT gene mutations.

Disclosure of conflict of interest

None.

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