Identification of mutations in EXT1 and EXT2 genes in six Chinese families with multiple osteochondromas

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Abstract. The aim of the present study was to identify mutations of major causative genes in six unrelated Chinese families with multiple osteochondromas (MO). Radiographic examinations and genetic analyses were performed in 8 patients exhibiting typical features of MO. Analysis was also performed on unaffected members of the six families and 250 healthy volunteers. Radiographies of the patients revealed multiple exostoses in the cartilage of long bones. A total of five different mutations were identified, one in exostosin-1 (EXT1) and four in exostosin-2 (EXT2). Two novel mutations were detected in EXT2; A missense mutation, c.1385G>A, in exon 8, resulting in p.Trp462X; and a splice site mutation, c.725+1G>C, which consisted of a heterozygous guanine-to-cytosine transition at nucleotide 725+1 in intron 3. Three common EXT mutations were also detected: c.1036C>T in exon 5 of EXT2 resulting in p.Gln346X; c.1299C>A in exon 8 of EXT2 resulting in p.Phe433Leu; and c.1038A>T in exon 2 of EXT1 resulting in p.Arg346Ser. In conclusion, the present study identified a novel missense mutation (c.1385G>A) in exon 8 and a splicing mutation (c.725+1G>C) in intron 3 of the EXT2 gene, which are responsible for MO in certain Chinese patients. The findings are useful for expanding the database of known EXT2 mutations and understanding the genetic basis of MO in Chinese patients, which may improve genetic counseling and the prenatal diagnosis of MO.

Introduction

Multiple osteochondromas (MO) is an autosomal dominant disease with a prevalence of 1 in 50,000 and the significant characteristic of the disease is the formation of multiple benign cartilaginous exostoses in the metaphyses of long bones (1,2), and the ratio of male-to-female is ~3:2 (3). MO is accurately diagnosed by radiological observation of exostoses and clinical investigation. In 90% of patients with MO, sessile or pedunculated exostoses are located around the distal femur (1,4). The prevalence of exostoses in other anatomical regions includes 85% in the proximal tibia, 76% in the fibula and 72% in the humerus (2,5,6). The exostoses are formed prior to adolescence, and the size and number increase over time until the closure of the growth plate (7). The major clinical symptoms are localized pain, short stature, restricted joint motion and bone deformities, including discrepancy in leg length, valgus deformities in the knees or ankles, asymmetric pelvis or pectoral region, and bending of the radius bone resulting in carpal subluxation (8). The complications caused by exostoses include pressure on neighboring tissues, nerves or vessels, and the most severe complication is the transformation of a benign osteochondroma into a malignant chondrosarcoma, however the risk of this is 1-2% (2,6). Surgical excision is the critical treatment to prevent progressive deformities and improve functional impairment. Furthermore, certain researchers propose that bisphosphonate therapy may relieve pain in children with MO (9).

MO is a genetically heterogeneous disorder, and linkage analysis has identified at least two loci involved, including exostosin-1 (EXT1) in 8q24.11-q13 and exostosin-2 (EXT2) in 11p11-p13 (10-12). The EXT1 and EXT2 genes have been cloned and validated as disease-causing genes (12). Previous studies have identified EXT1 and EXT2 as tumor suppressor genes that encode glycosyltransferases responsible for heparan sulfate synthesis (13,14). EXT1 and EXT2 contain 11 and 16 exons, respectively, and their protein products are EXT1, with 746 amino acids and EXT2, with 718 amino acids, respectively; there is a 30.9% homology between EXT1 and EXT2 (15). The Multiple Osteochondroma Mutation Database

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Abbreviations: MO, multiple osteochondromas; EXT1, exostosin-1; EXT2, exostosin-2; SNP, single nucleotide polymorphism; HGMD, Human Gene Mutation Database; HS, heparan sulfate

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have been identified in previous studies of different populations, including Chinese, Polish and Taiwanese populations (19-21). In addition to point mutations, which account for 70-75% of MO cases, deletions involving in single or multiple exons of EXT1 or EXT2 result in the pathogenesis of 10% of MO cases (22-25). The remaining 10-15% cases are caused by intronic changes, partial exon deletions, somatic mosaicism, positional changes, including insertion, inversion or translocation not leading to copy number alteration, and alterations affecting EXT1 or EXT2 promoter function (26).

The present study aimed to identify the gene mutations in six unrelated Chinese families with MO to extend the known mutations of EXT1 and EXT2. Identifying more mutations is useful for revealing the genetic basis of MO in Chinese patients and contributes to prenatal counseling and diagnosis.

Patients and methods

Patients. This study was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People’s Hospital (Shanghai, China). Six Chinese families with MO diagnosed using the typical radiological observations of exostoses in the juxta-metaphyseal region of long bones were analyzed in the present study. The pedigrees and radiographies of these families are presented in Figs. 1 and 2, respectively.

In family 1 (Fig. 1A), the proband (II-1) was a 13-year-old boy from Shanghai who complained of the asymmetry of his bilateral lower extremities. The X-rays revealed pelvis tilting, asymmetry of the bilateral lower extremities, and multiple exostoses on the right proximal femur, bilateral distal femurs, right proximal tibia and fibula, and the lower sternum. Multiple exostoses were also identified in the father of the proband. In family 2 (Fig. 1B), the proband (II-1) was a 19-year-old female from Gansu (China). She was admitted to hospital due to deformations of the elbows or hands. In family 3 (Fig. 1C), the proband (II-2) was a 47-year-old female from Shanghai (China). The proband and her 74-year-old mother (I-2) exhibited exostoses on their skulls. In family 4 (Fig. 1D), the proband (II-1) was a 17-year-old boy from Zhejiang (China). The proband exhibited exostoses on the right femur and right proximal tibia and fibula. A 10x8 cm hard lump was observable on the lateral right thigh, with no tenderness. Prior to admission to the clinic, the proband had undergone five operations to remove multiple exostoses at the right axillary fossa, the left knee, ankle, scapula and neck. In family 5 (Fig. 1E), the proband (III-4), a 5-year-old girl from Anhui (China), was referred to the clinic for a deformity of her right digitus annularis and left elbow. X-rays revealed the epiphysis of the right digitus annularis was broken and an exostosis at the left proximal ulna, which resembled Madelung deformity. The proband’s father (II-8) and grandmother (I-2) had similar deformities of the elbows or hands. In family 6 (Fig. 1F), the proband (II-1) was a 19-year-old boy from Zhejiang. The proband complained of asymmetry of the bilateral lower extremities and genu valgum. X-rays revealed multiple exostoses at knees, distal femurs, tibias and fibulas. Prior to the study, the proband had received two operations to remove the exostoses at interior left leg, right crus and both knees.

Methods. Informed consent was obtained from the 6 families and 250 healthy ethnically-matched volunteers prior to blood sampling and DNA analysis. The 250 volunteers (125 males and 125 females; age, 24.7-65.3 years) were recruited from the Department of Osteoporosis of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital. A QuickGene DNA Whole Blood kit (Kurabo Industries Ltd., Osaka, Japan) and a Nucleic Acid Isolation system (QuickGene-610L; AutoGen, Inc., Holliston, MA, USA) were used to extract genomic DNA from 2 ml peripheral blood. The sequences of EXT1 and EXT2 genes were attained from the online database (GenBank accession no. NM_000127 and NC_000011.9). All exons and the exon-intron boundaries of EXT1 and EXT2 genes were amplified via polymerase chain reaction (PCR) using the primers designed by Primer 3 software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences are presented in Table I. The reaction mixture (20 µl) contained 1X GC buffer I (Takara Bio, Inc., Otsu, Japan), 2.5 mM Mg²⁺, 0.2 mM dNTP, 0.2 µM of each primer, 1 unit HotStarTaq polymerase (Takara Bio, Inc.) and 1 µl template DNA. The thermocycling conditions

Figure 1. Pedigrees of six Chinese families with multiple osteochondromas. (A) Family 1, (B) family 2, (C) family 3, (D) family 4, (E) family 5 and (F) family 6. Black symbols represent the affected individuals and open symbols represent the unaffected individuals. Circles and squares indicate females and males, respectively. Arrows identify the probands in the families.
were as follows: Initial denaturation at 95˚C for 2 min followed by 11 cycles at 94˚C for 20 sec, at 64.5˚C for 40 sec, at 72˚C for 1 min, and 24 cycles at 94˚C for 20 sec, at 58˚C for 30 sec, at 72˚C for 1 min and at 72˚C for 2 min. Subsequently, direct sequencing was performed on DNA from 8 patients using the BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.1; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and analyzed the sequences by an ABI Prism 3130 automated sequencer (Thermo Fisher Scientific, Inc.) (18). Subsequently, the identified mutation sites were screened among the unaffected members of six families and 250 healthy volunteers. Single nucleotide mutations were checked using the Polyphred program (droog.gs.washington.edu/polyphred/). Novel mutations were recognized with the Human Gene Mutation Database program (http://www.hgmd.cf.ac.uk/). Disease-causing mutations were predicted using MutationTaster (www.mutationtaster.org).

Results

In total, five mutations were identified in the EXT1 and EXT2 genes in six Chinese families. In F1, a nonsense mutation, p.Gln346X (c.1036C>T), was identified in exon 5 of EXT2 in the proband (II-1) and his father (I-1). In F2 and F3, the
same missense mutation in exon 8 of EXT2, p.Phe433Leu (c.1299C>A), was present in the two probands (II-1 of F2, II-2 of F3). However the proband’s mother in F3, who had similar features to her daughter, harbored no mutations. In F4, a nonsense mutation in exon 8 of EXT2, p.Trp462X (c.1385G>A), was identified in the proband (II-1). In F5, the proband (III-4) had a splice site mutation, c.725+1G>C, which consisted of a heterozygous guanine-to-cytosine transition at nucleotide 725+1 in intron 3 of the EXT2 gene. The proband’s father and grandmother who had similar deformity refused to provide the blood samples. In F6, the missense mutation in exon 2 of EXT1, p.Arg346Ser (c.1038A>T), was present in the proband (II-1). The mutation results are presented in Fig. 3, and the clinical data and mutations identified in EXT1 and EXT2 from patients with MO are summarized in Table II.

The above mutations identified in EXT1 and EXT2 genes were not detected in the unaffected family members or in the 250 healthy volunteers.
Discussion

Previous studies have revealed that EXT1 or EXT2 mutations are detected in majority of patients with MO (14). In general, EXT1 mutations are more frequently reported than EXT2 mutations; however, in Chinese patients, EXT2 mutations have been reported to occur more frequently. The products of EXT genes are heparan sulfate (HS)-synthesizing enzymes, EXT1 and EXT2, which are glycosyltransferases responsible for HS synthesis in the Golgi apparatus. HS is an essential component of cell surface- and matrix-associated proteoglycans (12). HS can regulate the distribution and availability of growth factors and signaling proteins, and influence various critical processes in skeletal growth and morphogenesis. The glycosyltransferase encoded by EXT genes is a heterodimer complex of EXT1 and EXT2, and it only has enzymatic activity as the EXT1/EXT2 complex. The disease-causing mutations of the majority of MO cases are loss-of-function mutations in the EXT1 or EXT2 gene. The mutations result in premature termination of EXT protein translation and premature degradation. EXT2 is a structural component and acts as a chaperone for EXT1 (27). However, EXT1 is more important in controlling cartilage growth, which is a critical regulator of the perichondrium phenotype (28). This may explain why EXT1 mutations are associated with more severe phenotypes of MO compared with EXT2 mutations, including shorter stature, increased skeletal deformities and more severe impairments in forearm rotation, elbow flexion and knee flexion (29,30). In the present study, all the probands were at or before adolescence except the proband of F3. Their clinical symptoms occurred prior to puberty, and the number and size of exostoses increased with age, even when surgical excision had been performed. This is
consistent with previous reports (7,31). The proband of F6 with an EXT1 mutation had asymmetric lower extremities, genu valgum deformity, and at least six exostoses at knees, distal femurs, tibias and fibulas. The other five probands from the five different families with EXT2 mutations also had multiple exostoses in different anatomical regions and had deformities of varying degrees. There was no evident difference of severity in the patients, which may be caused by the limited number of cases. An increased case number is required to observe the different clinical manifestations of patients with MO.

Nonsense, frame shift and splice site mutations constitute a large proportion of the inactivating mutations in EXT genes (14,32). In the present study, one mutation in EXT1 and four mutations in EXT2 were identified, including two nonsense, two missense and one splicing mutation. This corresponds with previous research demonstrating that in Chinese patients with MO, EXT2 mutations may be more frequent than EXT1 mutations (16,17). The detected nonsense mutations led to premature termination at amino acid 346 (p.Gln346X) and amino acid 462 (p.Trp462X) in EXT2, which cosegregated with the disease phenotype in F1 and F4, and the latter had not been previously reported. In the probands of F2 and F3 and F4, a missense mutation (p.Phe433Leu) was identified in exon 8 of EXT2, and in the proband of F6, a missense mutation (p.Arg346Ser) was identified in exon 2 of EXT1. The missense mutations may pinpoint the key domains in EXT1 and EXT2 that contribute to the MO disease mechanism. In proband of F5, a novel splicing mutation (c.725+1G>C) was detected, which consisted of a guanine-to-cytosine transition at nucleotide 725+1 in intron 3 of EXT2. A splicing mutation can cause the alteration of splice sites or formation of new splice sites, and result in alternative transcription and translation. A previous study identified a splice site mutation (c.743+1G>A) in EXT2, which leads to an aberrantly spliced transcript with a premature termination codon and nonsense-mediated decay of mRNA (19). In addition, in F3, no mutations in EXT1 and EXT2 were identified in the mother of the proband, although both of the proband and the mother had exostoses on the skull. As for this mutation negative case, large deletions in single or multiple exons should be taken into account. Large exon deletions, which cannot be detected using direct sequencing, may be present even in the probands. Szuhai et al (34) were the first to identify somatic mosaic large genomic deletions as an underlying mechanism of MO in mutation negative patients using a resolution array-CGH. They reported the presence of a mosaic deletion in ~10-15% of the patient blood cells. In view of this, mutation analysis of the probands and parents to detect the presence of mutations in other tissues and detecting low mutation proportion is important. However, because obtaining other tissues from probands is invasive, the probands and their family members refused to provide their tissues. Due to the limitations in techniques and available samples, the presence of mutations in other tissues and low mutation proportions were not detected.

To conclude, two novel mutations in EXT2 and three known mutations in EXT1 and EXT2 were identified in six MO probands of unrelated Chinese families. Although the majority of MO cases can be easily diagnosed by radiological observations and clinical investigations, the reduced penetrance and intrafamilial variability may cause atypical symptoms. Mutation screening of the EXT genes can be used to confirm the diagnosis in atypical patients. The current results add to the known mutations of EXT2, and contribute to the understanding of the genetic basis of MO in Chinese patients. Furthermore, the mutation screening can be used in genetic counseling and prenatal diagnosis to reduce the burden caused by MO.

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References

Genetic heterogeneity in families with hereditary multiple exostoses.


