Pathogenic Gene Screening and Mutation Detection in a Chinese Family with Multiple Osteochondroma

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Multiple osteochondroma (MO) is an autosomal dominant disease characterized by abnormal skeleton development: one or more exostoses localized mainly at the end of long bones. Three pathogenic gene loci have been identified and cloned: *EXT1*, 2, and 3. Only *EXT1* and 2 mutations were reported to cause MO. Here, we report on a large Chinese family with MO and a disease-causing mutation in *EXT*. We extracted DNA from peripheral blood samples of 25 family members, 9 with MO. Polymerase chain reaction and direct DNA sequencing of the entire coding regions of *EXT1* and 2 for the nine patients revealed a novel pathogenic mutation, insertion of a T in exon 2 (c.72–73 insT) of *EXT2*. Our results extend the mutational spectrum of MO and can help with genetic counseling and prenatal diagnosis for this family.

Introduction

MULTIPLE OSTEOCHONDROMA (MO), previously called hereditary multiple exostoses, is an autosomal dominant disease with an estimated incidence ranging from 1.3/100,000 (Hennekam, 1991) to 2/100,000 cases (Schmale *et al.*, 1994). Approximately 80% of MO cases are associated with a positive family history, although some cases are sporadic.

The disease is characterized by abnormal skeletal development, with one or more overgrowing exostoses capped by gristle, mainly at the metaphyses of long bones and mostly bilateral limbs. It also affects juxtaepiphyseal flat bones, ribs, and vertebral bones. Diagnosis of MO in patients with a positive family history is during childhood. The bony tumors grow gradually in size and number throughout childhood until the end of puberty, when the epiphyseal plates close and skeletal maturation is completed, which can compress blood vessels or peripheral nerves. Two kinds of complications, skeletal deformity and disproportionate short stature, are common. In general, most exostoses are benign; only a few become malignant; examples are chondrosarcoma or osteosarcoma, which occur in about 2% of patients (Wicklund *et al.*, 1995).

MO is genetically heterogeneous. At least, 3 loci have been isolated by a linkage analysis and positional cloning: *EXT1* at 8 q24.11–q24.13 (Buhler and Malik, 1984; Cook *et al.*, 1993; Ahn *et al.*, 1995), *EXT2* at 11p12–p11 (Wu *et al.*, 1994; Hecht *et al.*, 1995; Stickens *et al.*, 1996; Wuyts *et al.*, 1996), and *EXT3* at 19p (Le Merrer *et al.*, 1994). The EXT family has a putative

tumor suppressor gene function. In addition, 3 other EXT-like genes have been mapped by a homology analysis: *EXTL1* on 1p36 (Wise *et al.*, 1997), *EXTL2* on 1p11–12 (Wuyts *et al.*, 1997), and *EXTL3* on 8p12–22 (Van Hul *et al.*, 1998). EXT family members are homologous in the carboxyterminal region with a coincident expression pattern and encode a signal peptide at the aminoterminalus (Van Hul *et al.*, 1998). MO is mainly associated with mutations in *EXT1* or 2. Neither *EXTL* nor *EXT3* mutations have been detected in MO cases (Wuyts *et al.*, 1998; Francannet *et al.*, 2001).

Here, we describe a Chinese family exhibiting MO (Fig. 1) and our analysis of the pathogenetic mutation by polymerase chain reaction (PCR) and DNA direct sequencing of the complete coding regions of *EXT1* and *EXT2* for 9 of the family members with MO. We found 1 novel mutation, insertion of a T in exon 2 of *EXT2*.

Materials and Methods

MO family

We studied a large Chinese family of 25 members, including 9 with MO (4 males). The proband (III7) was a 37-year-old man with exostoses in the left knee, left foot, right leg, and ribs. The diagnosis of MO in all nine patients was by physical and X-ray examination. Blood samples were obtained from 25 family members with their informed consent and added to ethylenediaminetetraacetic acid. Genomic DNA was extracted from peripheral venous blood by a salting-out method according to standard procedures (Miller *et al.*, 1988).

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FIG. 1. Pedigree of the Chinese family with multiple osteochondroma. ♂: proband; ■: affected male; ●: affected female; ○: healthy female; □: healthy male; Boxes or circles with a crossed line mean that the individual has died. The half-filled circle (III19) is a carrier who carries the genetic mutation, but has a normal phenotype.

Mutation screening

PCR was used to amplify the coding exons of *EXT1* and *EXT2* genes: exons 1 to 11 of *EXT1* and exons 2 to 14 of *EXT2*. The primer pairs for *EXT1* and *EXT2* were designed by use of PRIMER 5 with sequences available from GenBank (Table 1). Exon1 of *EXT1* was separated into 2 overlapping fragments. PCR conditions were predenaturation at 94°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at suitable temperature (Table 1) for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. To detect the concentration of amplified products, PCR products underwent agarose gel electrophoresis and then direct sequencing of amplified products of the coding exons. One of the amplification primers was used as a sequencing primer, and the sequencing results were analyzed by a BLAST search. Amplified products with mutations were ligated to a PMD-18T vector and transformed into the bacteria *Escherichia coli*. Detected mutations were confirmed in another 50 unrelated healthy controls.

Results

Clinical features

The pedigree of the family with MO is in Figure 1. The proband (III7) was a 37-year-old man with exostoses located in the left knee, left foot, right leg, and ribs. Three patients (IV4, IV5, and IV12) had only single exostoses, and for most of the nine patients with MO, the disease was identified during childhood (from 3 to 14 years old). Six patients (III7, III9, III23, IV4, IV6, and IV9) had one or more exostoses located in the long bones of the upper or lower limbs. Exostoses were present in the ribs of four patients (III7, III9, IV5, and IV12) and at the phalanx in only one patient (IV6).

Mutation detection

Screening coding exons by direct DNA sequencing of amplified products revealed no mutations in *EXT1*, but a

pathogenic mutation in exon 2 of *EXT2*: a heterozygous insertion T at c.72–73 in the proband (III7; Fig. 1), which caused a frameshift mutation, thus resulting in a premature translational termination signal at the novel amino acid position 54. All nine patients with MO, but no unaffected family members or unrelated healthy subjects carried this mutation (Fig. 2). The mutation was not reported in the MO Mutation Database (<http://medgen.ua.ac.be/LOVD>). This novel mutation is co-segregated with the phenotype of MO in this pedigree.

Discussion

EXT1 or *EXT2* mutations are responsible for 70% to 95% of MO. *EXT1* mutations are detected in 56% to 78% of families with MO, whereas *EXT2* accounts for 21% to 44% of cases. Mutation analysis of EXTs revealed that *EXT1* mutations are more frequent in the Caucasian population in Europe and North America. However, mutation analysis of EXTs revealed mutations in *EXT2* more frequent than those in *EXT1* in Chinese people. Lei Xu (1999) detected 5 *EXT1* mutations (14%) and 12 *EXT2* mutations (33%) in 36 Chinese families by PCR-single-strand conformation polymorphism (SSCP) and sequencing (Xu *et al.*, 1999). In recent 10 years, *EXT1* or *EXT2* mutations have been reported successively in 29 Chinese families with MO and 4 spontaneous cases affected by MO. *EXT1* mutations accounts for 27% (9/33) of the mutations, whereas *EXT2* accounts for 45% (15/33), much higher than that of *EXT1*. Based on these data, a hypothesis has been put forward: EXT mutations have differences in a large extent between different ethnic populations. Most *EXT2* mutations seem to be private, tending to concentrate in the first two-thirds of the coding region. Especially, mutations in exon 2 of *EXT2* are reported more than are mutations in other exons (Dobson-Stone *et al.*, 2000; Wuyts and Van Hul, 2000; Francannet *et al.*, 2001; Gigante *et al.*, 2001; Seki *et al.*, 2001; Hall *et al.*, 2002; Shi *et al.*, 2002; White *et al.*, 2004; Pedrini *et al.*, 2005; Vink *et al.*, 2005; Lonie *et al.*, 2006; Signori *et al.*,

TABLE 1. PRIMER SEQUENCES OF CODING EXONS FOR *EXT1* AND *EXT2*

Gene and exon	Primer sequence	PCR -product size (bp)	Annealing temperature (°C)
<i>EXT1</i>			
exon1(1)F	5'TGGGAACTTGGGTGATT3'	750	56
R	5' TCAAAGTTGGGTCGGAAG3'		
exon1(2)F	5' ACTTGTGGAACAATGGTAG3'	540	55
R	5' AGGGGAAAGAGGACTGAG3'		
exon2F	5' CAGCAACCCAACCTCCTT3'	316	51
R	5' ATCTGGCTTCGGTCCTCA3'		
exon3F	5'GTCGCTTTCCTCACATT3'	238	55
R	5'CTTTTGGATTTCATCTTCTTT3'		
exon4F	5' AGAAGCCAAATGCTATGA3'	279	50
R	5' AAGGACCAATTATTCAAGC3'		
exon5F	5' AGTGGGAGGGAGGGTAGA3'	302	52
R	5'AGGGTAAACAAGGGCAAC3'		
exon6F	5' GCGGAGCAAGGAGGAGTA3'	350	55
R	5' AGGCAGGATGAATGAAAGG3'		
exon7F	5' GGAGGCATACATAAAATACA 3'	461	54
R	5' TAACAGCAACCCTACAAG 3'		
exon8F	5' CAGGTGAGGATGGGAGAA3'	254	50
R	5ATCGTGCAACATGAGGTG3'		
exon9F	5ATGTTTCGCCACAGTCCC3'	461	52
R	5' ATGCCAAGAGGTTTCACT3'		
exon10F	5' GGGAGTAATAATAGAACCT 3'	483	56
R	5' GAACCACCAGTGAGTGAA 3'		
exon11F	5' ATTTGCCTGACTCCATT 3'	489	58
R	5' ACAGAAGCCAGTGAGGTG 3'		
<i>EXT2</i>			
exon2F	5' GCGGTGGCTTATGCCTATGA 3'	776	56
R	5' CCGGGAGATGGTGTGCTGA 3'		
exon3F	5' CTTTGCATACCTGAGAAGCG 3'	411	50
R	5' AGGACATAAGCCCTAACTGG 3'		
exon4F	5' ACTGGGAAGTAAGGAAAGGG 3'	771	56
R	5' AGAGGCAGGGAAACCACATA 3'		
exon5F	5' TGGAGGTGAAGACTGGTAA 3'	718	57
R	5' CACTGTGGCTCTGTAGGC 3'		
exon6F	5' CAAGGTAGGCTGAGGTAAG 3'	778	58
R	5' CGTAAAGGAAGGGACACG 3'		
exon7F	5' CTTCTTTCCTGGTGTATTCT 3'	839	56
R	5' AGCTCCTGTCCCTCTGTAT 3'		
exon8F	5' TGATAAATGCCAACTTCTG 3'	716	56
R	5' GCACTTAGCACAGTCCCT 3'		
exon9F	5' ACTCCTCCTCTGCTACCC 3'	838	55
R	5' TCCTAAGCCCTCAAACACT 3'		
exon10F	5' GACCTTGGTCGAGTCGTT 3'	716	56
R	5' TAGGGTTTCTCCAGTGCTT 3'		
exon11F	5' ATACAAATCAGGGCAGTT 3'	858	58
R	5' AAGCAGGCTAATGAGAAT 3'		
exon12F	5'ACAATTCATGGGATTTACAG3'	493	50
R	5'AGCAAGTGAGTGGCAGAG3'		
exon13F	5' TGTGAGTTCTGCCGTTGG 3'	669	56
R	5' TCTGGTGGGTTTGGTTTA 3'		
exon14F	5' GCAGACTGTGGCTACTTGA 3'	755	56
R	5' GGTTCCTGAAAGAACTCA 3'		

2007; Jennes *et al.*, 2008). *EXT1* mutations are randomly scattered throughout the gene.

To date, 351 *EXT1* mutations and 166 *EXT2* mutations have been identified by the sequence and quantitative analysis of the complete coding region. Among the *EXT1* mutations, 156 are frameshift mutations, 85 nonsense mutations and 39 splice-site mutations, 39 mis-sense mutations and 19 deletions, and 1 in-frame insertion and 2 translocations, and the

remaining 10 variants have unknown consequence. The unique *EXT2* mutations include 70 frameshift mutations, 37 nonsense mutations, and 21 splice-site mutations, accounting for 77% of mutations. Additionally, 21 mis-sense mutations, 8 deletions, and 9 variants with unknown consequence in *EXT2* have been reported (Jennes *et al.*, 2009). Most of these mutations are predicted to result in premature termination of translation.

FIG. 2. Partial sequencing of exon 2 of *EXT2* in the proband (patient III7). **(A)** Reverse-sequencing result: black arrow shows the insertion site. After this site, the sequencing pattern becomes disorganized. **(B)** Sequencing result of the *Bacilli* polymerase chain reaction product: black arrow shows insertion T at c.72–73. **(C)** Partial sequencing of exon 2 in *EXT2* from unaffected family members and healthy unrelated individuals. Black arrow shows no T insertion.

In this report, we investigated a large Chinese family with MO and performed a sequence analysis of the entire coding regions of both *EXT1* and *EXT2* for all nine patients with MO in the pedigree. We detected a heterozygous insertion of a T in exon 2 of *EXT2* that was present in all nine patients. The insertion results in frameshift mutation and premature translational termination, thus inducing the expression of a predicted truncated EXT protein with only 53 amino acids, 664 amino acids shorter than the wild-type protein. Chinese patients with MO may carry the *EXT2* instead of the *EXT1* mutation, and mutations may be more frequent in exon 2 than other exons of *EXT2*.

Although MO is fully penetrant (Hennekam, 1991; Wicklund *et al.*, 1995), our study of the genotype-phenotype association revealed that the penetrance of EXT is not fully complete in this Chinese family exhibiting MO. One member (III19) is a carrier of the gene mutation; her mother and son have MO, but she does not have any visible exostoses.

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Author Disclosure Statement

No competing financial interests exist.

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