Mutation Screening of the EXT Genes in Patients with Hereditary Multiple Exostoses in Taiwan

YI-RU SHI,1 JER-YUARN WU,1,3 YU-AN HSU,3 CHENG-CHUN LEE,1,4 CHANG-HAI TSAI,1 and FUU-JEN TSAI1,2,3

ABSTRACT

Hereditary multiple exostoses (HME) is an autosomal dominant disorder characterized by growth of benign bone tumors. This genetically heterozygous disease comprises three chromosomal loci: the EXT1 gene on chromosome 8q23–q24, EXT2 on 11p11–p13, and EXT3 on 19p. Both EXT1 and EXT2 have been cloned and defined as a new family of potential tumor suppressor genes in previous work. However, no studies have been conducted in the Taiwanese population. To determine if previous results can also be applied to the Taiwanese, we analyzed 5 Taiwanese probands with clinical features of HME: 1 of them is a sporadic case, and the others are familial cases. Linkage studies were performed in the familial cases before the mutation analysis to determine to which of the three EXT chromosomes these cases could be assigned. Our results showed that one proband is linked to the EXT1 locus and three are linked to the EXT2 locus; the sporadic case was subsequently found to involve EXT1. We then identified four new mutations that have not been found in other races: two in EXT1—frameshift (K218fsX247) and nonsense (Y468X) mutations and two in EXT2—missense (R223P) and nonsense (Y394X) mutations. Our results indicate that in familial cases, linkage analysis can prove useful for preimplantation genetic diagnosis.

INTRODUCTION

Hereditary multiple exostoses (HME; MIM 133700), the most frequently found skeletal dysplasia, is an autosomal dominant disorder. It is characterized by the presence of multiple exostoses/osteochondromas localized mainly in the juxta-epiphyseal region of long bones (Solomon, 1963).

Three genetic loci for HME have been identified to date: EXT1 (MIM 133700) on chromosome 8q23–q24 (Cook et al., 1993); EXT2 (MIM 133701) on 11p11–p13 (Wu et al., 1994), and EXT3 (MIM 600209) on 19p (Le Merrer et al., 1994). The EXT1 gene is composed of 11 exons and codes for a 2,238-bp cDNA that specifies a protein of 746 amino acids (Ahn et al., 1995). EXT2 is composed of 14 exons that encode protein of 718 amino acids (Stickens et al., 1996; Wuyts et al., 1996). The EXT3 appears to be a minor locus, which is responsible for some 28% of cases (Le Merrer et al., 1994; Legeal-Mallet et al., 1997). Through mutation detection studies, loss of function of either EXT1 or EXT2 appears to be sufficient for tumor formation; it has been suggested that these genes are tumor suppressors (Hecht et al., 1995; Bovee et al., 1999).

In this study, we performed a systematic mutation analysis of EXT genes, including linkage analysis, single-strand conformation polymorphism (SSCP), and DNA sequencing, to determine the EXT chromosomal location and specific mutations responsible for HME cases in Taiwan.

MATERIALS AND METHODS

In this study, multigenerational families with HME were obtained from China Medical College Hospital, Taichung, Taiwan. Family members were evaluated by clinical diagnosis or by review of medical records to document the presence or absence of exostoses. Genomic DNA was prepared from periph-
eral blood lymphocytes by DNA extractor Genomaker DNA extraction kit (Blossom, Taiwan).

**Genetic markers and linkage analysis**

The microsatellite markers, (D8S556, D8S284, D8S514, D8S272 on chromosome 8q, D11S904, D11S935, D11S905, D11S1313 on 11p, and D19S209, D19S216, D19S221, D19S226) on 19p) were designed for human genome linkage analysis and were purchased from the ABI Prism Linkage Mapping Set. The fluorescent labeled PCR products were analyzed on the automated DNA sequencer (ABI Prism 377) and Genescan 672 software (PE Applied Biosystems, USA).

**Mutation analysis**

Primer pairs for exons 1–12 of **EXT1** and for exons 2–14 of **EXT2** were designed, as previously described (Philippe et al., 1997; Wuyts et al., 1998). The PCR products of these exons were subjected to mutation analysis by SSCP, using a GenePhor DNA Electrophoresis System (Pharmacia, Sweden). Exons that exhibited an irregular shift by SSCP were subjected to direct sequencing for mutation identification. Direct sequencing was performed using a Taq DyeDeoxy Terminator sequencing kit (PE Applied Biosystems, USA) with an ABI Prism 377 DNA sequencer. Mutations were confirmed by means of restriction analysis. If necessary, modified primers were designed to create restriction site. The modified primers used in this study were: EXT2-e8M1 (+): 5’-GCATTATTTTTTTATAGGCCCGT-3’, reverse primer EXT2-e8.2 (→): 5’-ACTGGAAATTCACCTTACACA-3’; EXT2-e6M2 (+): 5’-TACTTTCTGTGGTTCTACG-3’. The modified nucleotides in modified primers are underlined. Restriction digestions were performed on PCR amplifications and the fragments were separated on 3% agarose gels.

**RESULTS**

We have examined five probands with the HME phenotype and their relatives. The results of linkage analysis showed that one family is an isolated case, another is linked to the **EXT1** locus (Fig. 1), and the others are linked to the **EXT2** locus (Fig. 2). The four different mutations identified in these families are summarized in Table 1. The missense mutation (R223P) in **EXT2** (Family 1) and the frameshift mutation (K218fsX247) in **EXT1** (Family 2) had been reported before (Shi et al., 2000, 2001). However, the nonsense mutations (Y394X in **EXT2**-linked Families 3 and 4 and Y468X in **EXT1**-linked Family 5) have not yet been reported.

The first new mutation was found in three affected members of Family 3 and two affected members of Family 4. It originates from a substitution of G for A at position 1181 in exon 8 of the **EXT2** gene (Fig. 2B,C). The result was confirmed by BfaI restriction digestion of a 166-bp PCR-amplification product with primers EXT2-e8M1 (+) and EXT2-e8.2 (→). The modified primer EXT2-e8M1 creates a BfaI restriction site (CTAG) that results in digestion producing 25-bp and 141-bp bands in the mutant allele, whereas the wild-type is not digested (results not shown).

The second nonsense mutation is a C → G transversion at position 1,404 in exon 5 of the **EXT1** gene, which was found in the isolated case, Family 5 (Fig. 1B). This mutation results in a premature stop codon at amino acid 468 and it also introduces a BfaI restriction site. The normal band is predicted to have a length of 237 bp and would be expected to be digested.
FIG. 2. Pedigrees of EXT2-linked families and the linkage analysis. The affected allele is represented by a dashed line. (A) Family 1, an EXT2-linked case. (B) A base substitution (c1181G→A) was found in the affected members of Family 4. (C) The affected members of Family 3 had the same mutation (c1181G→A) as Family 4 did.
to produce bands of 175 bp and 62 bp, in the presence of the C1404G mutation (results not shown). Through sequencing analysis, we also found two polymorphisms in EXT2 (Table 2). The first is a T→G substitution at position 966 (exon 6) and the other is an A→C at 79 bp up-stream of intron 4 [IVS4 (−79) A→C] (Fig. 3A, B). Although the T→G transition at nucleotide position 966 in EXT2 would not be expected to cause any protein change (R322R), we designed a primer-EXT2-e6M2 to generate a recognition site for HypCH4 IV restriction digestion to understand the distribution of this polymorphism across the Taiwanese population. The 205-bp ... frequencies of the T/T and T/G genotypes were 0.79 and 0.21, respectively. The second polymorphism [IVS4 (−279) A→C] was detected in 5 out of 9 affected individuals of the three EXT2-linked families. The frequency of the A allele was 0.72 and of the C allele, 0.28, in these three families. Because this polymorphism is not in the coding region, we did not investigate the allelic frequencies in the healthy population.

Through direct sequencing, we also found that six nucleotides of EXT2 gene were different from the sequences obtained through GeneBank (accession numbers, U67357, U67359, U67362) (Table 2). All 9 affected members of the three EXT2-linked families were homozygous at the indicated positions: C/C at IVS4 (−8), T/T at IVS4 (−22), T/T at IVS5 (−14), T/T at IVS6 (−52), C/C at IVS9 (−8), and A/A at IVS10 (+75) (Table 2; Fig. 3C–H). The distribution of above findings is outlined in Fig. 4.

**DISCUSSION**

HME is genetically heterogeneous. From previous studies, it may either be clearly familial or occur sporadically. Our linkage and mutation analyses also showed similar results: we report on one sporadic case, on one EXT1-linked family, and on three EXT2-linked families. This finding also provides additional support for the major roles of EXT1 and EXT2 in HME. However, the most important discovery in our study is the identification of four new mutations in EXT1 and EXT2: a frameshift (K218fsX247) and a nonsense (Y468X) in EXT1; a missense (R223P) and a nonsense (Y394X) in EXT2.

For EXT1, we found an insertion-deletion (651–665delins TT) in exon 1 resulting in a frameshift (K218fsX247) at position 218 and a translational stop 30 codons downstream in the EXT1 protein. According to the functional study by Cheung (2001), 323 amino acid residues of the human EXT1 protein have been evolutionarily conserved among both vertebrate and invertebrate species. These conserved amino acids are essential for EXT1 to function in the polymerization of heparan sulfate. Therefore, we expect that the observed frameshift, which would be expected to result in the loss of 255 amino acids from this conserved region of the EXT1 protein, would result in a drastically truncated protein with probable complete loss of function.

We also found a C→G transition in at position 468 of EXT1. It changes a TAC codon to TAG and produces a premature stop codon. This mutation would result in the loss of 278 amino

<table>
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<td>EXT1/exon5</td>
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<td>Y468X</td>
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</table>

*References: R223P, Shi et al. (2000); K218fsX247, Shi et al. (2001).
FIG. 3. Sequence analysis of EXT2 gene in three EXT2-linked families that were studied. (A) The polymorphism in nucleotide 966 showed a heterozygous status (TG) at this position. (B) A (AC) polymorphism was found at upstream (−79) position of intron 4 [IVS4 (−79)]. (C) A homozygous status (CC) was found at upstream of intron 4 [IVS4 (−8)]. (D) TT was found at IVS4 (−22). (E) TT was found at downstream of intron 5 [IVS5 (+14)]. (F) TT was found at IVS6 (−52). (G) CC was found at IVS9 (−8). (H) AA was found at IVS10 (+75).
acids from the carboxyl terminus of the EXT1 protein. Because the conserved carboxy-terminal region of the EXT1 protein contains a putatively catalytic domain for the synthesis of heparan sulfate (McCormick et al., 2000), the truncated EXT1 protein is probably inactive or degraded rapidly, resulting in a nearly complete loss of function. Therefore, we suspect that the premature stop codon is very likely to be the disease-causing mutation.

In a previous study, mutations found in EXT2 were variable and randomly distributed over the first two-thirds of the coding region, without any real mutation hotspots (Wuyts and Van Hul, 2000). However, several EXT2 mutations, including missense, nonsense, and frameshift mutations, had been detected in exon 4. They are located at position 211 (FS L211), 218 (FS S218), 222 (Y222X) and 227 (D227N) (Philippe et al., 1997; Wuyts et al., 1998; Xu et al., 1999). In our study, we also found an adjacent aa substitution at position 223 (Arg→Pro) in exon 4. This missense mutation did not seem to cause an obviously milder phenotype as compared with mutations leading to premature termination of translation. Therefore, we suspect that amino acids 211–230 might be mutation hotspots for EXT2, and the missense (R223P) mutation might be in a crucial domain of EXT2, which must remain intact for proper function of the EXT2 protein.

Another newly identified nonsense mutation, Y394X, in exon 8 of EXT2, was found in two different families (Families 3 and 4). The premature stop codon appearing at Y394, the middle of the EXT2 protein, leads to a truncated protein missing 324 amino acids from the carboxyl terminus. As observed for EXT1, the protein will be severely truncated when it looses carboxy-terminal end, which is associated with glycosyltransferase activities that catalyze the polymerization of heparan sulfate (Philippe et al., 1997; McCormick et al., 2000; Francannet et al., 2001). Therefore, we suspect this mutation might be the genetic defect responsible for the development of exostoses in these two families.

Besides the above four new mutations, we also found two polymorphisms in EXT2 by direct sequencing; we also noted 6 nucleotides that are different from the EXT2 sequences obtained from the white population reported in GenBank. The first polymorphism was an A→G substitution at upstream of intron 4 [IVS4 (−79)]. The second one was a c966T→G detected in 3 out of 9 affected subjects; the frequencies of these alleles (T and G at position 966) were 0.833 and 0.167, respectively. However, results obtained from healthy (non-HME) individuals were 0.9 and 0.1, respectively. In other words, the frequency of G allele was higher in HME versus non-HME individuals in this study. This result suggests that c966T→G might be used as a single nucleotide polymorphic marker for EXT2 and the marker may be associated with the deficiency of the EXT2 protein. Because there are only three EXT2 families in this study, larger-scale mutation screening would be necessary to prove our inference.

The 6 nucleotides found in EXT2 were different from the data obtained through GenBank. All of the 12 members of our EXT2-linked families were homozygous at these six positions. Hence, we presume that the sequences in Taiwanese Chinese are different from those in the white population (the subjects included in the GenBank database).

Although combining results of mutation detection and linkage analysis can exactly determine the disease-causing mutations/alleles in inherited cases, the whole procedure is costly and time consuming. On the other hand, the linkage analysis used in our study can provide a reliable clue for prenatal diagnosis without mutation detection in such families. The linkage method is relatively low cost and efficient. The microsatellite markers we chose were in the region of chromosome 8q22.3–8q24.3 for EXT1, 11p11.1–11p14.3 for EXT2, and 19p13.3–19p13.2 for EXT3.

The validity of the linkage method was tested in Family 3 (Fig. 2): the baby (III-1) was predicted not to have inherited the disease-causing allele from his father (II-3) and this was confirmed by a subsequent direct sequencing analysis demonstrating the absence of the familial mutation. In the future, in families in which the chromosomal location of the disease-causing mutation/allele is known, the less costly linkage approach might also prove useful for preimplantation genetic diagnosis.

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FIG. 4. The distribution of polymorphisms and nucleotide differences of the EXT2 gene reported in the present study. Gray boxes represent coding regions. Two polymorphisms are drawn above the schematic representation of the EXT2 gene, and the six nucleotide positions are drawn below.


Address reprint requests to:
Dr. Fuu-Jen Tsai
Department of Pediatrics and Medical Genetics
China Medical College Hospital
2 Yuh-Der Road, North District Taichung 404, Taiwan
E-mail: d0704@www.cmch.org.tw

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Address reprint requests to:
Dr. Fuu-Jen Tsai
Department of Pediatrics and Medical Genetics
China Medical College Hospital
2 Yuh-Der Road, North District Taichung 404, Taiwan
E-mail: d0704@www.cmch.org.tw

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