

## Mutation Screening of the EXT1 and EXT2 Genes in Patients with Hereditary Multiple Exostoses

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### Summary

Hereditary multiple exostoses (HME), the most frequent of all skeletal dysplasias, is an autosomal dominant disorder characterized by the presence of multiple exostoses localized mainly at the end of long bones. HME is genetically heterogeneous, with at least three loci, on 8q24.1 (EXT1), 11p11-p13 (EXT2), and 19p (EXT3). Both the EXT1 and EXT2 genes have been cloned recently and define a new family of potential tumor suppressor genes. This is the first study in which mutation screening has been performed for both the EXT1 and EXT2 genes prior to any linkage analysis. We have screened 17 probands with the HME phenotype, for alterations in all translated exons and flanking intronic sequences, in the EXT1 and EXT2 genes, by conformation-sensitive gel electrophoresis. We found the disease-causing mutation in 12 families (70%), 7 (41%) of which have EXT1 mutations and 5 (29%) EXT2 mutations. Together with the previously described 1-bp deletion in exon 6, which is present in 2 of our families, we report five new mutations in EXT1. Two are missense mutations in exon 2 (G339D and R340C), and the other three alterations (a nonsense mutation, a frameshift, and a splicing mutation) are likely to result in truncated non-functional proteins. Four new mutations are described in EXT2. A missense mutation (D227N) was found in 2 different families; the other three alterations (two nonsense mutations and one frameshift mutation) lead directly or indirectly to premature stop codons. The missense mutations in EXT1 and EXT2 may pinpoint crucial domains in both proteins and therefore give clues for the understanding of the pathophysiology of this skeletal disorder.

### Introduction

Hereditary multiple exostoses (HME), or diaphyseal aclasis (MIM 133700 and 133701), is an autosomal dominant disorder characterized by the presence of multiple exostoses (osteochondromas) localized mainly in the juxtaepiphyseal region of long bones. HME is the most common of all skeletal dysplasias, with estimates of prevalence ranging from 1.3/100,000 (Hennekam 1991) to 2/100,000 cases in a population (Schmale et al. 1994). Approximately 80% of cases are considered to be familial. Although some authors report a penetrance as low as 66% (Sugiura et al. 1976), more recent reviews tend to indicate that the condition is fully penetrant (Hennekam 1991; Luckert-Wicklund et al. 1995). More than 80% of individuals with HME are diagnosed during the first decade of life (Solomon 1963). Exostoses increase in size and number until closure of the growth plate, at the end of puberty. The exostoses can result in skeletal deformity and mild short stature or can cause vascular or peripheral nerve compression. The most serious complication of HME is the malignant degeneration of an exostosis to a chondrosarcoma or an osteosarcoma, which occurs in ~2% of patients with HME (Luckert-Wicklund et al. 1995).

HME is genetically heterogeneous, and linkage analysis has identified at least three loci, on chromosomes 8q24.1 (EXT1; Cook et al. 1993), 11p11-p13 (EXT2; Wuyts et al. 1995), and 19p (EXT3; Le Merrer et al. 1994; Legeai-Mallet et al. 1997). Multiple exostoses are seen in contiguous-gene syndromes mapped to chromosomes 8q24.1 (Langer-Giedion syndrome, MIM 150230) and 11p11-p12 (defect 11 syndrome, MIM 601224; Bartsch et al. 1996). Sporadic exostoses are histopathologically indistinguishable from those seen in HME or in contiguous-gene syndromes.

Chromosome abnormalities observed in patients with sporadic or inherited exostoses (Mertens et al. 1994; Lüdecke et al. 1995) and with loss of heterozygosity (LOH) on chromosomes 8, 11, and 19, reported in EXT-related and non-EXT-related chondrosarcomas (Hecht et al. 1995; Raskind et al. 1995; Hogue et al. 1996; Hecht et al. 1997), suggest that EXT1, EXT2, and EXT3 may act as tumor suppressor genes. According to the

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generally accepted two-hit hypothesis of tumorigenesis, proposed by Knudson (1971), mutation of one copy of an EXT gene (the inherited germ-line mutation) is insufficient to cause the development of an exostosis. A single chondrocyte will only become the stem cell for an exostosis when its normal second EXT copy also is inactivated by a somatic mutation. This is consistent with the fact that, as with other tumor-suppressor gene syndromes, there is earlier development of exostoses in inherited cases. Sarcomatous degeneration in an exostosis then would require additional somatic mutation events in a multistep process (Hecht et al. 1997), as illustrated by the adenoma-carcinoma sequence of carcinogenesis in colorectal cancer (Kinzler and Vogelstein 1996). This is supported by the observation that chondrosarcomas exhibiting only LOH for chromosomes 8 and/or 11 are biologically less aggressive than tumors with LOH for EXT loci and for the p53 gene located at 17p13 (Raskind et al. 1995).

The EXT1 and EXT2 genes recently have been cloned by means of positional cloning strategies (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996; Lüdecke et al. 1997), and they define a new family of related proteins with putative tumor-suppressor activity. A third member of the EXT-gene family, named "EXT-like" ("EXT-L"), has been identified by means of similarity searches of the database for expressed sequence tags (dbEST) (Stickens et al. 1996) and has been mapped to chromosome 1p36.1 (Wise et al. 1997), excluding it as a candidate in EXT3-linked pedigrees.

A few reports in the literature describe mutations in HME patients, either in EXT1 (Ahn et al. 1995; Raskind et al. 1996; Hecht et al. 1997; Wells et al. 1997) or in EXT2 (Hogue et al. 1996; Stickens et al. 1996; Wuyts et al. 1996), but none of them reports mutation screening of both the EXT1 and EXT2 genes simultaneously. This study is, therefore, the first mutation analysis of isolated cases or of HME families not previously linked to either locus, in which both the EXT1 and EXT2 genes have been screened for mutations. Mutation scanning was performed by conformation-sensitive gel electrophoresis (CSGE), a heteroduplex method described by Ganguly et al. (1993). In a total of 17 familial or isolated HME cases screened, we found 7 cases (41%) with EXT1 mutations and 5 (29%) with EXT2 mutations.

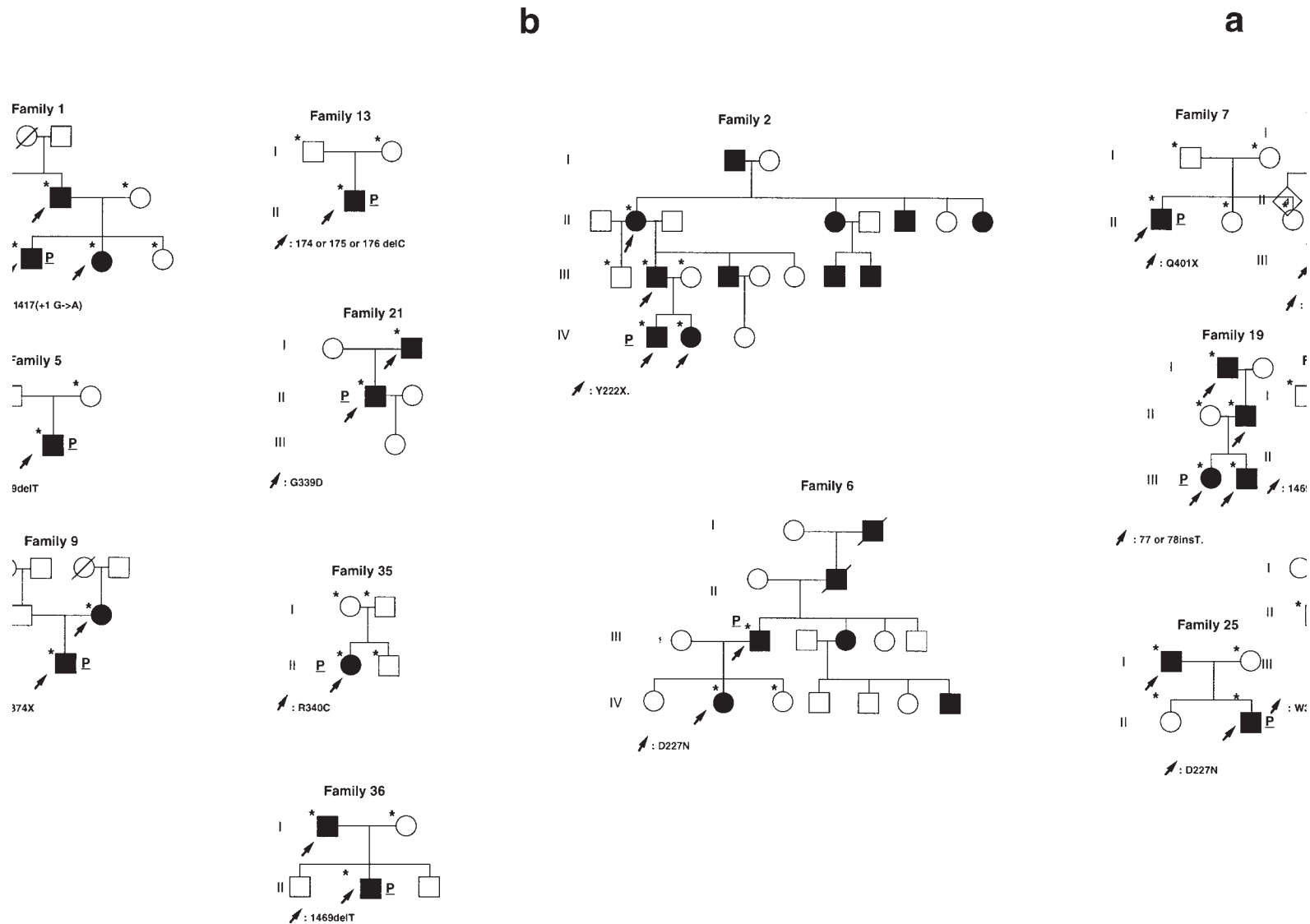
### Subjects, Material, and Methods

The 17 families studied here were collected in the Oxford region and are of English origin, except for family 9, which is of Philippine origin. To obtain informed consent, we followed protocols consistent with the guidelines of the Royal College of Physicians and approved by the Central Oxford Research Ethics Committee. In figure 1, we present the pedigrees for all families in which we found the disease-causing mutation.

Genomic DNA was extracted from peripheral blood lymphocytes, by use of the Nucleon DNA extraction kit (Scotlab), or from buccal smears, a noninvasive technique that is more comfortable for young patients. This protocol was adapted from Bowtell (1987) and Jeanpierre (1987). Mouth swabs were collected by the firm rubbing of a cotton bud backwards and forwards between the cheek and gum, for 20 s (10 cotton buds were used per individual), and were collected in a 50-ml Falcon tube containing 5 ml of transport buffer (100 mM NaCl, 10 mM Tris, pH 8, 25 mM EDTA, pH 8, and 0.5% SDS). The buds were incubated in transport buffer for 2–3 h at 65°C (or overnight at 37°C), with 200 µg proteinase K/ml. Subsequently, guanidine HCl and ammonium acetate were added, to a final concentration of 0.9 M and 0.6 M, respectively. The mix was incubated for 1 h at 60°C in a shaking incubator. Buds then were placed inside a 20-ml syringe; the 20-ml syringe was placed into a 50-ml Falcon tube and was spun at 1,000 rpm for 5 min, in order to dry the buds. Two milliliters of cold chloroform were added, and the tubes were shaken for 10 min, were left to stand for 1 min, and were spun down at 2,500 rpm for 5 min. DNA was precipitated finally with 2 vol of cold ethanol, at –20°C over at least 2 h, and was spun down at 2,500 rpm for 30 min at 4°C. The pellet was washed twice in 70% ethanol and was resuspended in a 500-µl buffer of 10 mM Tris and 0.1 mM EDTA.

For the proband of each family, exons 11 and 13 and the flanking intronic regions of the EXT1 and EXT2 genes, respectively, were PCR amplified by use of specific primer pairs (table 1). The primer pairs for exons 2–14 of EXT2 were designed by use of sequences available through GenBank (U67356–U67368). We did not screen exons 1, 1a, and 1b, which belong to the 5' UTR of the EXT2 gene. The PCR conditions consisted of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, the annealing temperature (table 1) for 30 s, and 72°C for 30 s, and ended with a final elongation step at 72°C for 5 min. PCR reactions were performed in an MJ Research PTC-225 thermal cycler. Heteroduplex analysis was performed subsequently by means of CSGE analysis, by means of a modified version of the protocol described by Ganguly et al (1993). The 0.8 mm-thick gel contained 10% polyacrylamide:bis(acryloyl)piperazine (99:1), 15% formamide, 10% ethylene glycol made with a glycerol-tolerant buffer of 89 mM Tris, 7 mM Taurine, and 0.1 mM EDTA. PCR products were heated to 98°C for 5 min, followed by incubation at 68°C for 1 h, to allow formation of heteroduplexes. Samples then were subjected to electrophoresis at 500 V for 16 h at room temperature. The gel was stained subsequently with ethidium bromide (1 µg/ml) for 15 min, and DNA was visualized by UV light.

When a CSGE variant was detected in the proband



**Figure 1** Pedigrees of the HME families with a mutation in either the EXT1 or the EXT2 gene. Individuals with HME are indicated by blackened symbols and non-HME individuals by unblackened symbols. Initial CSGE analysis was performed only on the proband (indicated by a "P") from each HME family. An asterisk (\*) indicates the individuals from each pedigree who were screened, by CSGE, for a mutation and who subsequently were analyzed at the sequence level. Individuals heterozygous for the mutation are indicated with an arrow. *a*, Families with mutation in EXT1. *b*, Families with mutation in EXT2.

**Table 1****Primers Used to Amplify Exons from EXT1 and EXT2**

| Gene, Exon, and Primer <sup>a</sup> | Primer Sequence                | PCR-Product Size (bp) | Annealing Temperature/<br>MgCl <sub>2</sub> Concentration (°C/mM) |
|-------------------------------------|--------------------------------|-----------------------|---|
| EXT1:                               |                                |                       |   |
| 1(1):                               |                                |                       |   |
| 1 Forward                           | TGG.AAA.CTT.GGG.TGA.TTC        | 523                   | 52/1.5  |
| 210                                 | GAC.AAA.GAG.GCA.CGC.CTG        |                       |   |
| 1(2):                               |                                |                       |   |
| 109                                 | GTT.ACC.AAA.ACA.TTC.TAG.CGG.C  | 614                   | 58/1.5  |
| 208                                 | CAA.GGC.TGA.CTC.CCA.AAG.AC     |                       |   |
| EXT2:                               |                                |                       |   |
| 2(1):                               |                                |                       |   |
| 2(1) Forward                        | AAG.TGT.CAT.TTG.CCA.TCC.TA     | 436                   | 54/1.5  |
| 2(1) Reverse                        | TCC.ACG.TAC.TTT.TTC.AGA.GC     |                       |   |
| 2(2):                               |                                |                       |   |
| 2(2) Forward                        | TGA.CTG.GAA.TGT.AGA.GAA.GC     | 442                   | 53/1.5  |
| 2(2) Reverse                        | TTC.CCT.TTA.GTT.CCC.TGA.GG     |                       |   |
| 3:                                  |                                |                       |   |
| 3 Forward                           | GAT.GTG.CTG.TTG.GGA.TTT.C      | 331                   | 50/1.5  |
| 3 Reverse                           | AGT.ATG.TGG.TAG.ATT.TAG.GA     |                       |   |
| 4:                                  |                                |                       |   |
| 4 Forward                           | GTA.ATT.CCT.GTT.CCT.CTC.CAC    | 283                   | 57/1.5  |
| 4 Reverse                           | TTG.CTT.TGA.TGC.CCA.CCA.C      |                       |   |
| 5:                                  |                                |                       |   |
| 5 Forward                           | TGA.AGA.CTG.GTA.AGG.AAC.AC     | 404                   | 54/1.5  |
| 5 Reverse                           | TTG.CAT.GCT.GAA.AAA.CAA.CTC    |                       |   |
| 6:                                  |                                |                       |   |
| 6 Forward                           | ATG.CCT.CAG.TAT.TGC.TTG.G      | 325                   | 53/1.5  |
| 6 Reverse                           | ACC.TGA.GGA.GAT.TTT.ATT.TGT.A  |                       |   |
| 7:                                  |                                |                       |   |
| 7 Forward                           | GAT.GTT.GTT.TCT.GCT.TGT.G      | 339                   | 50/2.5  |
| 7 Reverse                           | CAT.TAA.TTT.CCT.TTC.GTT.T      |                       |   |
| 8:                                  |                                |                       |   |
| 8 Forward                           | TCT.AGT.TTT.CCC.ACT.CTG.TCT.C  | 253                   | 59/2.5  |
| 8 Reverse                           | TTC.CTT.CCA.CCC.ACC.CTG.AC     |                       |   |
| 9:                                  |                                |                       |   |
| 9 Forward                           | GTC.AGC.CAT.ATT.GTT.ACA.GC     | 358                   | 54/2.5  |
| 9 Reverse                           | TCA.GAA.ATG.GTA.TTG.CTA.TTG.A  |                       |   |
| 10:                                 |                                |                       |   |
| 10 Forward                          | TCA.TGC.TTT.TAC.TAC.TTT.ATC.TC | 370                   | 56/2.5  |
| 10 Reverse                          | CAC.CTT.TTG.GAC.TCT.ACT.GGA.   |                       |   |
| 11:                                 |                                |                       |   |
| 11 Forward                          | TCT.GTT.GAT.ACC.TGT.TTG.GAT.A  | 270                   | 56/5  |
| 11 Reverse                          | AAT.TGG.GTC.AAA.TAA.AAT.GTG    |                       |   |
| 12:                                 |                                |                       |   |
| 12 Forward                          | CTA.AAG.GGA.ACT.GCT.ATT.TTT.G  | 334                   | 54/1.5  |
| 12 Reverse                          | AAT.GTG.ACC.GCA.TCA.ATC.ATA    |                       |   |
| 13:                                 |                                |                       |   |
| 13 Forward                          | GCA.TGA.TTT.TAT.TGT.CCT.TGA.C  | 262                   | 56/1.5  |
| 13 Reverse                          | GAG.GTG.ACT.ATG.GCT.ACC.AG     |                       |   |
| 14:                                 |                                |                       |   |
| 14 Forward                          | TTG.CTG.TTA.TCT.CTC.AAC.CTC    | 341                   | 56/1.5  |
| 14 Reverse                          | CAC.CCT.TAA.CCT.ACT.ACT.CTG    |                       |   |

NOTE.—Sequences for exons 2–11 of EXT1 are reported in the article by Wells et al. (1997).

<sup>a</sup> Since exon 1 of EXT1 and exon 2 of EXT2 are so large (963 bp and 566 bp, respectively), we used two sets of overlapping PCR primers to screen for mutations, by CSGE.

**Table 2****Characteristics of EXT1 and EXT2 Gene Defects in Patients with Multiple Exostoses**

| Family   | Location of Mutation | Mutation           | Consequence      | Name of Mutation <sup>a</sup> |
|--|----------------------|--------------------|------------------|-------------------------------|
| Familial cases:                                |                      |                    |                  |                               |
| 1  | EXT1/Intron 5        | TAGgta→TAGata      | Altered splicing | 1417 (+1G→A)                  |
| 2  | EXT2/Exon 4          | TAC→TAG (Tyr→Stop) | Nonsense         | Y222X                         |
| 6  | EXT2/Exon 4          | GAT→AAT (Asp→Asn)  | Missense         | D227N                         |
| 9  | EXT1/Exon 3          | TGG→TGA (Trp→Stop) | Nonsense         | W374X                         |
| 19   | EXT2/Exon 2          | TATATC→TATTATC     | Frameshift       | 77- or 78insT                 |
| 21   | EXT1/Exon2           | GGT→GAT (Gly→Asp)  | Missense         | G339D                         |
| 25   | EXT2/Exon4           | GAT→AAT (Asp→Asn)  | Missense         | D227N                         |
| 36   | EXT1/Exon 6          | CCCCCTGG→CCCCCGG   | Frameshift       | 1469delT <sup>b</sup>         |
| Isolated cases<br>( <i>de novo</i> mutations): |                      |                    |                  |                               |
| 5  | EXT1/Exon 6          | CCCCCTGG→CCCCCGG   | Frameshift       | 1469delT <sup>b</sup>         |
| 7  | EXT2/ Exon 8         | CAG→TAG (Glu→Stop) | Nonsense         | Q401X                         |
| 13   | EXT1/Exon 1          | GCTTCCCGG→GCTTCCGG | Frameshift       | 174-, 175- or 176delC         |
| 35   | EXT1/Exon 2          | CGC→TGC (Arg→Cys)  | Missense         | R340C                         |

<sup>a</sup> According to the nomenclature defined by Beaudet and Tsui (1993). For both EXT1 and EXT2, base no. 1 corresponds to the first base of the initiation codon in the cDNA sequences (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996).

<sup>b</sup> Already reported by Ahn et al. (1995) and by Wells et al. (1997).

(indicated in fig. 1 by a “P”), for a particular exon, a second PCR reaction was performed, for all available members (affected and unaffected; indicated in fig. 1 by an asterisk [\*]) of the corresponding pedigree, and the PCR products were sequenced subsequently, without cloning in the forward and reverse orientations, with the same primers used during the PCR reaction. The PCR products were purified (QIAquick PCR purification kit; Qiagen); were cycle sequenced, by use of dye-terminator chemistry, with the AmpliTaq FS enzyme (Applied Biosystem); and were run on an ABI 373 DNA sequencer. The sequences were analyzed visually by comparison of the mutated sequence with a sequence from a normal control. For each family, individuals heterozygous for the mutation are indicated with an arrow (fig. 1).

## Results

In this study, we report the results of mutation screening for the EXT1 and EXT2 genes in 17 HME families, of which 8 include isolated cases of HME corresponding to *de novo* mutations. For a scanning method, we chose CSGE, a heteroduplex method, because it is easy to use and nonradioactive and because it allowed the screening of up to 70 samples per gel. It is reported elsewhere that CSGE detects 88% of mutations (Ganguly et al. 1993), a sensitivity that is acceptable for this mutation analysis. We found 7 (41%) EXT1-linked families and 5 (29%) EXT2-linked families. Table 2 summarizes the 10 different mutations found in 12 families, during the course of this study, and the corresponding pedigrees are de-

picted in figure 1. In addition to these mutations, we also report several probable polymorphisms (table 3).

### EXT1 Mutations

A 1-bp thymidine deletion in exon 6 (1469delT) was found twice, in families 5 and 36. The five other alterations in EXT1 consisted of one frameshift mutation in exon 1 (174-, 175-, or 176delC; family 13), one nonsense mutation in exon 3 (W374X; family 9), two missense mutations in exon 2 (G339D in family 21 and R340C in family 35) (fig. 2C), and a splice-site mutation affecting intron 5 (1417[+1G→A]; family 1) (fig. 2A). We examined 72 unaffected, unrelated individuals (144 chromosomes), by direct sequencing of exon 2, and never found the two amino acid (aa) substitutions reported in families 21 and 35. The Gly→Asp substitution at position 339 changes a small neutral polar aa to an acidic aa, and the R340C mutation replaces a basic aa with a polar aa. In family 21, we only were able to obtain DNA samples for the affected individuals I-2 and II-1. The introduction of a Cys at position 340 potentially allows the formation of a new disulfide linkage. Gly at position 339 is conserved in EXT2, whereas Arg340 is replaced by an Ala.

### EXT2 Mutations

Four mutations were found in EXT2. Two were nonsense mutations (Y222X in family 2 and Q401X in family 7); one was a frameshift mutation (77- or 78insT; family 19); and a missense mutation in exon 4 was found in two different families (D227N; families 6 and 25) (fig. 2B). The missense mutation D227N changes an

**Table 3****Benign Polymorphisms of EXT1 and EXT2 Genes**

| Location of Polymorphism | Polymorphism                                   | Name of Polymorphism <sup>a</sup> | Frequency in HME Patients ( <i>n</i> = 18) <sup>b</sup> |
|--------------------------|--|-----------------------------------|---|
| EXT1:                    |  |                                   |   |
| Exon 6                   | CCC→CCT (Pro→Pro)                              | P477P                             | .16   |
| Exon 9                   | GAG→GAA (Glu→Glu)                              | E587E                             | .19   |
| EXT2:                    |  |                                   |   |
| Exon 2                   | CGG→AGG (Arg→Arg)                              | R10R                              | .03   |
| Exon 3                   | C→T, 84 nucleotides before the start of exon 3 | 537 (-84C→T)                      | .03   |

<sup>a</sup> According to the nomenclature defined by Beaudet and Tsui (1993). For EXT2, base no. 1 corresponds to the first base of the initiation codon in the cDNA sequence (Wuyts et al. 1996).

<sup>b</sup> *n* = the no. of patients, or one-half the no. of alleles, tested.

acidic aa for a neutral polar aa, and it affects an aa in a small domain conserved between the EXT1 and EXT2 proteins (DVSIP at positions 227–231 in the EXT2 protein). Direct sequencing of 120 control chromosomes from unrelated unaffected individuals has never revealed this aa substitution at position 227.

### Discussion

Our findings provide additional support for the EXT1 and EXT2 genes being involved in HME. However, we did not find any mutation in the EXT1 or the EXT2 gene, in five families, but this can be explained by several reasons. First, CSGE is only 88% effective (Ganguly et al. 1993) and is unable to detect gross rearrangements, such as partial or complete gene deletions or any chromosomal abnormalities involving EXT1 or EXT2. Furthermore, we did not look for mutations in the promoter regions of the EXT1 and EXT2 genes or in the 5' UTR of EXT2. Lastly, a few families of our panel could be linked to the EXT3 locus on chromosome 19 or to the EXT-L gene on chromosome 1p36. In fact, the existence of a few families in which EXT1, EXT2, and EXT3 loci have been excluded suggests that there is another, yet-unidentified EXT locus responsible for HME (Hecht et al. 1997).

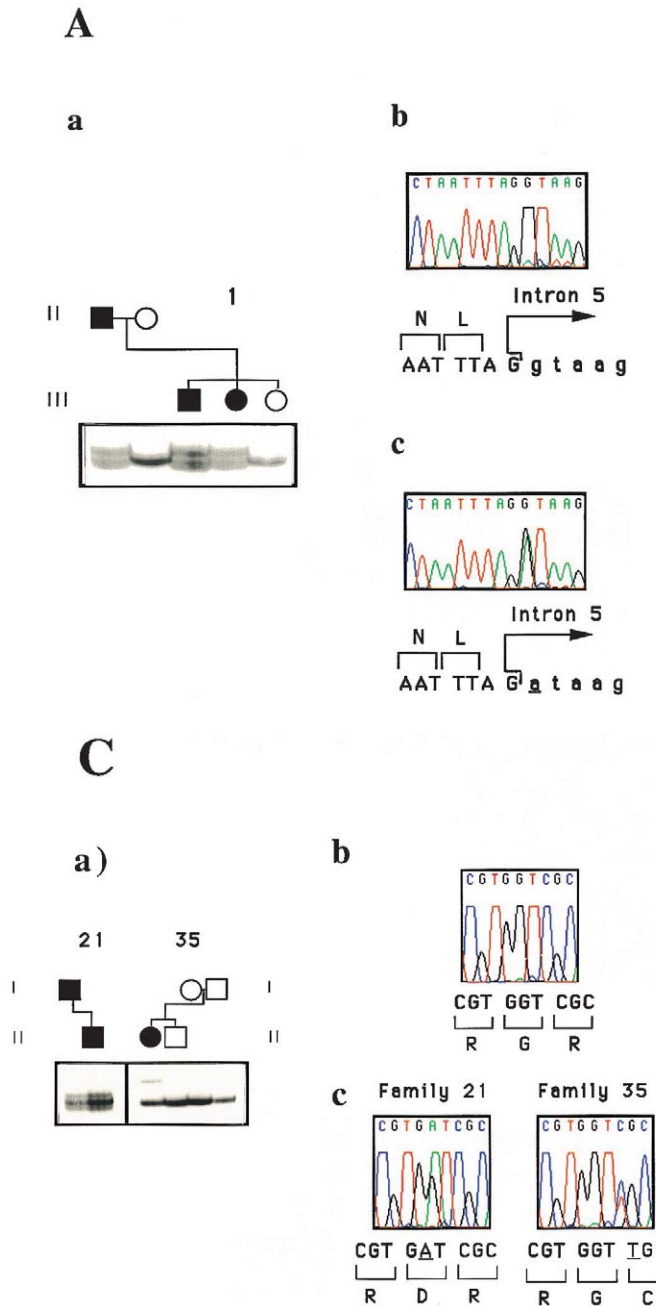
The 1-bp deletion found in families 5 and 36, in exon 6 of EXT1, has been reported elsewhere (Ahn et al. 1995; Wells et al., in press). This deletion immediately follows a polycytosine tract, and such polypyrimidine tracts are known to be deletion hot spots (Lohmann et al. 1994). There are two additional tracts of six cytosine residues, in EXT1 (one in exon 1 and one in exon 6), but they do not appear to be mutation hot spots, in our families. The nine other genetic alterations in EXT1 or in EXT2 that are reported in this study are new mutations.

The nonsense and frameshift mutations lead directly or indirectly, respectively, to premature stop codons, which, upon translation, may result in drastically trun-

cated proteins. A nonmutational polymorphic stop codon in the human tumor suppressor gene BRCA2 has been reported (Mazoyer et al. 1996). However, this polymorphic stop codon was located at the carboxy-terminal part of the BRCA2 protein, leading to a truncated protein missing only 92 aa's of a protein of >3,400 aa's. In our families, the premature stop codons are all located in the amino-terminal part of the EXT1 or the EXT2 protein. We therefore think that it is highly probable that these premature stop codons are the disease-causing mutations. In some rare cases, premature stop codons have been shown to induce exon skipping (Dietz et al. 1993; Gibson et al. 1993); moreover, there is evidence that nonsense codons reduce mRNA abundance (Maquat 1996), decreasing the amount of truncated protein produced by the translational machinery.

Of interest, the two missense mutations in exon 2 of EXT1, found in families 21 and 35 (fig. 2C), involve two adjacent aa's (Gly and Arg at positions 339 and 340, respectively). Moreover, another aa substitution at Arg340 (R340H) also has been reported by Raskind et al. (1996). This strongly suggests that this region needs to be intact, for proper functioning of the EXT1 protein. A ProSite search revealed that the two missense mutations in exon 2 occur in a potential 3-aa proteolysis/amidation site ( $\alpha$ -G-[RK]-[RK], in which  $\alpha$  is the amidation site [ProSite PDOC00009]). So far, such proteolysis/amidation sites have been described in the C-terminal part of the precursors of hormones and other active peptides, since neutral hydrophobic residues are good substrates for the amidation site. In the EXT1 protein, a potential tumor suppressor, this potential proteolysis/amidation site occurs in the middle of the protein, and the amidation site consists of an Arg, a charged residue that is less reactive. We cannot firmly exclude this site as being active in the EXT1-derived protein, although this possibility is unlikely.

The EXT1 mutation found in family 1 is a splicing



**Figure 2** CSGE and sequence analysis of the splice-site and missense mutations reported in this study. *A*, Splicing defect 1417(+1G→A) at the conserved GT position of the donor splice-site of intron 5 of EXT1, in family 1. *B*, D227N aa substitution located within exon 4 of EXT2, in families 6 and 25. *C*, G339D and R340C at exon 2 of EXT1, in families 21 and 35, respectively. *a*, CSGE variant. *b*, Normal sequence. *c*, Sequence of the mutated allele; the nucleotide change is underlined. For mutation D227N, the reverse sequence obtained with primer 4R is shown, because it is easier to see the heterozygous C→T transition. Nevertheless, the sequence obtained with primer 4F unambiguously confirms the alteration at nucleotide 681, in families 6 and 25.

mutation characterized by a 1417(+1G→A) transversion (fig. 2A) at the conserved GT position of the donor splice site (Nakai and Sakamoto 1994). Splice-site mutations can result in one or a combination of the following types of abnormal pre-mRNA processing: exon skipping, cryptic splice-site usage, or intron retention. For family 1, we cannot predict the effect of the splice-site mutation, but exon skipping appears to be a more frequent phenomenon than cryptic splice-site usage (Krawczak et al. 1992). In any case, the abnormal mRNA is likely to contain a premature stop codon, therefore leading to the synthesis of a nonfunctional truncated protein.

The missense mutation D227N, in exon 4 of EXT2, was found in two different families (families 6 and 25). In family 6, this mutation was found in two affected members but not in one unaffected individual, who was 15 years of age. It was not possible to obtain DNA samples from more-distant affected members of this multigenerational family, who live in New Zealand. The same aa substitution also is present in family 25, but, surprisingly, the father, who also is heterozygous for the G→A transversion, initially was reported as unaffected. According to several authors (Hennekam 1991; Luckert-Wicklund et al. 1995), reduced penetrance in HME fam-

ilies remains unproven; indeed, only a very careful radiographical exam of individuals who are carriers of a mutation in one EXT gene could rule out the presence of small or nonpalpable exostoses in any part of the skeleton. When we found the D227N mutation in the father of family 25, we undertook a careful clinical examination and found five tiny, but still palpable, exostoses (sites—both distal radii, both proximal tibiae, and the left distal tibia). It appears, therefore, that intrafamilial variable expressivity is likely to account for most cases of clinical nonpenetrance, in HME pedigrees.

Most mutations leading to haploinsufficiency (nonsense mutations, frameshifts, and altered splicing) reported in this study and by other groups appear to be private; they do not cluster in any particular region of the EXT1 and EXT2 genes. Nevertheless, none are located at the extreme carboxy-terminal region of the protein. This might be expected, since a protein will be more severely truncated when premature termination of translation occurs at the amino-terminal extremity. The high proportion (8 of 12) of null mutations found by our mutation analysis gives additional support to the hypothesis that EXT genes define a new family of tumor suppressor genes. Two missense mutations affecting two consecutive aa's in exon 2 of EXT1 and one missense mutation found in two different families, in exon 4 of EXT2, seem to pinpoint crucial domains in both proteins. In our series, missense mutations did not seem to cause obviously milder phenotypes, when compared with mutations leading to premature termination of translation. This would support the contention that missense mutations in exon 2 of EXT1 and in exon 4 of EXT2 pinpoint crucial domains that must remain intact for proper functioning of the EXT-derived proteins.

We plan to extend this strategy of EXT1 and EXT2 gene screening to a much larger data set. The results will assist in the generation of protocols for clinical identification and management of HME families. In our panel of 12 HME families with mutation in the EXT1 or the EXT2 gene, we found 50% of the mutations in only three different exons (exons 2 and 6 of EXT1 and exon 4 of EXT2) from a total of 11 and of 13 translated exons screened in the EXT1 and EXT2 genes, respectively. Mutation screening in HME patients, therefore, should start with these three exons, together with exon 1 of EXT1, which contains two tracts of six cytosines.

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## References

- Ahn J, Ludecke H, Lindow S, Horton W, Lee B, Wagner M, Horsthemke B, et al (1995) Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). *Nat Genet* 11:137–143
- Bartsch O, Wuyts W, Van Hul W, Hecht JT, Meinecke P, Hogue D, Werner W, et al (1996) Delineation of a contiguous gene syndrome with multiple exostoses, enlarged parietal foramina, craniofacial dysostosis, and mental retardation, caused by deletions on the short arm of chromosome 11. *Am J Hum Genet* 58:734–742
- Beaudet A, Tsui L (1993) A suggested nomenclature for designated mutations. *Hum Mutat* 2:245–248
- Bowtell D (1987) Rapid isolation of eukaryotic DNA. *Anal Biochem* 162:463–465
- Cook A, Raskind W, Blanton SH, Pauli RM, Gregg RG, Francomano CA, Puffenberger E, et al (1993) Genetic heterogeneity in families with hereditary multiple exostoses. *Am J Hum Genet* 53:71–79
- Dietz H, Valle D, Francomano C, Kendzior R, Pyeritz R, Cutting G (1993) The skipping of constitutive exons in vivo induced by nonsense mutations. *Science* 259:680–683
- Ganguly A, Rock M, Prockop D (1993) Conformation-sensitive gel electrophoresis for rapid detection of single base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90:10325–10329
- Gibson R, Hajianpour A, Murer-Orlando A, Buchwald M, Mathew C (1993) A nonsense mutation and exon skipping in the Fanconi anaemia group C gene. *Hum Mol Genet* 2:797–799
- Hecht JT, Hogue D, Strong LC, Hansen MF, Blanton SH, Wagner M (1995) Hereditary multiple exostosis and chondrosarcomas: linkage to chromosome 11 and loss of heterozygosity for EXT-linked markers on chromosomes 11 and 8. *Am J Hum Genet* 56:1125–1131
- Hecht JT, Hogue D, Wang Y, Blanton SH, Wagner M, Strong LC, Raskind W, et al (1997) Hereditary multiple exostoses (EXT): mutational studies of familial EXT1 cases and EXT-associated malignancies. *Am J Hum Genet* 60:80–86
- Hennekam R (1991) Hereditary multiple exostoses. *J Med Genet* 28:262–266
- Hogue DA, Clines G, Lovett M, Hansen MF, Hecht JT (1996) Mutational analysis of hereditary multiple exostoses-2 (EXT2). *Am J Hum Genet Suppl* 59:A263
- Jeanpierre M (1987) A rapid method for the purification of DNA from blood. *Nucleic Acids Res* 15:9611
- Kinzler K, Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* 87:159–170
- Knudson A Jr (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 68:820–823
- Krawczak M, Reiss J, Cooper D (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 90:41–54



- Le Merrer M, Legeai-Mallet L, Jeannin P, Horsthemke B, Schinzel A, Plauchu H, Toutain A, et al (1994) A gene for hereditary multiple exostoses maps to chromosome 19p. *Hum Mol Genet* 3:717–722
- Legeai-Mallet L, Margaritte-Jeannin P, Lemdani M, Le Merrer M, Plauchu H, Marotiaux P, Munnich A, et al (1997) An extension of the admixture test for the study of genetic heterogeneity in hereditary multiple exostoses. *Hum Genet* 99:298–302
- Lohmann D, Brandt B, Hopping W, Passarge E, Horsthemke B (1994) Spectrum of small length germline mutations in the RB1 gene. *Hum Mol Genet* 3:2187–2193
- Luckert-Wicklund C, Pauli R, Johnston D, Hecht J (1995) Natural history study of hereditary multiple exostoses. *Am J Med Genet* 55:43–46
- Lüdecke H, Ahn J, Lin X, Hill A, Wagner M, Schomburg L, Horsthemke B, et al (1997) Genomic organization and promoter structure of the human EXT1 gene. *Genomics* 40:351–354
- Lüdecke H, Wagner M, Nardmann J, La Pillo B, Parrish J, Willems P, Haan E, et al (1995) Molecular dissection of a contiguous gene syndrome: localization of the genes involved in the Langer-Giedion syndrome. *Hum Mol Genet* 4:31–36
- Maquat LE (1996) Defects in RNA splicing and the consequence of shortened translational reading frames. *Am J Hum Genet* 59:279–286
- Mazoyer S, Dunning A, Serova O, Dearden J, Puget N, Healey C, Gayther S, et al (1996) A polymorphic stop codon in BRCA2. *Nat Genet* 14:253–254
- Mertens F, Rydholm A, Kreicbergs A, Willen H, Jonsson K, Heim S, Mitelman F, et al (1994) Loss of chromosome band 8q24 in sporadic osteochondilaginous exostoses. *Genes Chromosom Cancer* 9:8–12
- Nakai K, Sakamoto H (1994) Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene* 141:171–177
- Raskind WH, Conrad EU, Chansky H, Matsushita M (1995) Loss of heterozygosity in chondrosarcomas for markers linked to hereditary multiple exostoses loci on chromosomes 8 and 11. *Am J Hum Genet* 56:1132–1139
- Raskind WH, Matsushita M, Conrad EU, Wells D, Sandell L, Wagner M, Houck J (1996) Spectrum of EXT1 mutations in hereditary multiple exostoses. *Am J Hum Genet Suppl* 59:A280
- Schmale G, Conrad E, Raskind W (1994) The natural history of hereditary multiple exostoses. *J Bone Joint Surg Am* 76:986–992
- Solomon L (1963) Hereditary multiple exostosis. *J Bone Joint Surg* 45:292–304
- Stickens D, Clines G, Burbee D, Ramos P, Thomas S, Hogue D, Hecht J, et al (1996) The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. *Nat Genet* 14:25–32
- Sugiura Y, Sugiura I, Iwata H (1976) Hereditary multiple exostoses: diaphyseal aclasis. *Jpn J Hum Genet* 21:149–167
- Wells DE, Hill A, Lin X, Ahn J, Brown N, Wagner JE (1997) Identification of novel mutations in the human EXT1 tumor suppressor gene. *Hum Genet* 99:612–615
- Wise C, Clines G, Massa H, Trask B, Lovett M (1997) Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family. *Genome Res* 7:10–16
- Wuyts W, Ramlakhan S, Van Hul W, Hecht J, van den Ouweland AMW, Raskind WH, Hofstede FC, et al (1995) Refinement of the multiple exostoses locus (EXT2) to a 3-cM interval on chromosome 11. *Am J Hum Genet* 57:382–387
- Wuyts W, Van Hul W, Wauters J, Nemtsova M, Reyniers E, Van Hul E, De Boule K, et al (1996) Positional cloning of a gene involved in hereditary multiple exostoses. *Hum Mol Genet* 5:1547–1557