Genotype-phenotype correlation in hereditary multiple exostoses

C Francannet, A Cohen-Tanugi, M Le Merrer, A Munnich, J Bonaventure, L Legeai-Mallet

Abstract

Hereditary multiple exostoses (HME) is a genetically heterogeneous autosomal dominant disorder characterised by the development of bony protuberances mainly located on the long bones. Three HME loci have been mapped to chromosomes 8q24 (EXT1), 11p11-13 (EXT2), and 19p (EXT3). The EXT1 and EXT2 genes encode glycosyltransferases involved in biosynthesis of heparan sulphate proteoglycans. Here we report on a clinical survey and mutation analysis of 42 HME French families and show that EXT1 and EXT2 accounted for more than 90% of HME cases in our series. Among them, 27/42 cases were accounted for by EXT1 (64%, four nonsense, 19 frameshift, three missense, and one splice site mutations) and 9/42 cases were accounted for by EXT2 (21%, four nonsense, two frameshift, two missense, and one splice site mutation). Overall, 31/36 mutations were expected to cause loss of protein function (86%). The most severe forms of the disease and malignant transformation of exostoses to chondrosarcomas were associated with EXT1 mutations. These findings provide the first genotype-phenotype correlation in HME and will, it is hoped, facilitate the clinical management of these patients.

(J Med Genet 2001;38:430–434)

Keywords: hereditary multiple exostoses; EXT1; EXT2; chondrosarcoma

Hereditary multiple exostoses (HME, MIM 133700) is an autosomal dominant condition characterised by the development of multiple osteochondromas (exostoses) from the metaphyseal areas of the long bones. The exostoses are either sessile (broad based) or pedunculated and they vary widely in size and number. They can be present at birth and continue to appear and grow throughout childhood and into puberty. Exostoses are usually located in the metaphyseal region of the proximal humerus, distal radius and ulna, proximal tibia, and distal femur, but can also be found on the pelvis and scapula. They can cause multiple and severe complications including pain, restricted range of joint movement, deformities and shortening of the long bones, and nerve or blood vessel compression. The most severe complication is malignant transformation of an exostosis into a chondrosarcoma, which occurs in 0.5–2% of cases. In addition, intra- and interfamilial clinical variability of HME has long been recognised.

HME is genetically heterogeneous as three EXT loci have been identified so far. EXT1 (MIM 133700) has been mapped to chromosome 8q23-24, EXT2 (MIM 133701) to chromosome 11p11-p12, and EXT3 (MIM 600209) to chromosome 19p. Linkage studies have identified EXT1 and EXT2 as the two major disease loci in HME families, while EXT3 appears to be a minor locus. Three additional loci designated EXT1L1, EXT1L2, and EXT3L2 have been identified and mapped to chromosomes 1 (1p36, 1p11-p12) and 8 (8p12). However, no HME family has yet been linked to these loci. Loss of heterozygosity (LOH) at the EXT1, EXT2, and EXT3 loci has been observed among patients with EXT related and unrelated chondrosarcomas suggesting that the EXT genes are tumour suppressors. So far, only two EXT genes have been cloned, namely EXT1 and EXT2. Both genes encode glycosyltransferases harbouring N-acetyl-glucosamine transferase (GlcNac-T) and D-glucuronic acid transferase (GlcA-T) activities that catalyse the biosynthesis of heparan sulphate proteoglycans (HSPG). Recent studies suggest that EXT1 and EXT2 may interact in vivo to form a functional oligomeric complex in the Golgi apparatus where the heparan sulphate polymerisation is thought to occur.

A number of mutations have been reported in HME but the cause of its clinical variability remains unclear. Very few studies have concerned the correlation of genotype to phenotype. We have carried out an extensive mutation analysis of the EXT1 and EXT2 genes in a large series of HME families and investigated whether specific clinical features could be associated with either locus. Here, we show that EXT1 mutations caused the most severe...
forms of the disease and degeneration of exostosis into chondrosarcoma only occurred in EXT1 patients.

**Patients and methods**

**PATIENTS AND CLINICAL STUDY**

A total of 42 HME French families (representing 217 affected subjects) were investigated. In order to document the severity of the disease and intrafamilial variability, a questionnaire was sent to each patient. Patients were asked to fill in the questionnaires with the help of their physicians and orthopaedic surgeons to verify the accuracy of the answers. No discordance between the answers and our observations were noticed. For evaluating the functional rating, a modification of the scale established by the Musculoskeletal Tumor Society was used.22 5

The overall functional grade was based on five factors (including pain) and four categories of deformities, namely brachymetacarpary, bowing of the forearm, shortening of the forearm, varus or valgus angulation of the knees, and shortening of limbs. For an overall functional rating of "fair", at least one of the five factors had to have had that rating (table 1). For each patient, the severity of the disease was evaluated at the time of evaluation, location of the exostoses (vertebral), stature, and functional rating (table 2). For each family, the degree of severity was evaluated from the most affected members.

**GENETIC STUDIES**

Blood samples were obtained with the written consent of patients and unaffected relatives. The whole set of families was tested by using microsatellite DNA markers closely linked to EXT1 (D8S199, D8S198, D8S85), EXT2 (D11S905, D11S903, D11S544), and EXT3 loci (D19S840, D19S413, D19S221). For mutation detection in probands, the whole coding sequence of the EXT1 and EXT2 genes was amplified and analysed by single strand conformation polymorphism (SSCP) either on Hydrolink MDE gels or GeneGel Excel 12.5/24 gels. The Hoefer Automated Gelstainer was used for gel silver staining (Pharmacia Biotech). Amplification products showing abnormal patterns of migration were reamplified and sequenced by using the fluorescent dideoxy terminator method on an automatic sequencer ABI.

**Results**

**GENETIC AND MOLECULAR STUDIES**

Linkage analyses confirmed the existence of at least three EXT loci on chromosomes 8, 11, and 19. Among the 42 families, 29 showed strong linkage to EXT1 (69%), nine to EXT2 (21%), and one to EXT3 (3%). Interestingly, several pedigrees (7%) gave negative lod scores with markers of chromosomes 8, 11, and 19. Taken together, these results suggest that EXT1, EXT2, and EXT3 account for about 90% of cases but that at least one additional locus could account for HME. An abnormal pattern of migration and a mutant genotype was found in 36/38 patients. The mutations identified in EXT1 and EXT2 are summarised in table 3. No mutation was detected in two families linked to EXT1 despite SSCP analysis and extensive sequencing of all EXT1 exons.

Among the 36 mutant alleles reported here, 27 (75%) were found in EXT1. Mutations identified included four nonsense mutations, 19 frameshift deletions or insertions, three missense and one splice site mutation. These mutations were almost randomly distributed over the first nine exons. Most of them were novel, except for five previously reported mutations23 (table 4). Ninety percent of cases but that at least one additional locus could account for HME. An abnormal pattern of migration and a mutant genotype was found in 36/38 patients. The mutations identified in EXT1 and EXT2 are summarised in table 3. No mutation was detected in two families linked to EXT1 despite SSCP analysis and extensive sequencing of all EXT1 exons.

**Clinical study**

Based on the phenotypic expression of the disease, two groups of families were recognised. The first group, which included families with severely affected members, was referred to as group S. In the second group (group M),
Table 4  Summary of EXT gene mutations, clinical data

| Gene | Exon | cDNA change | Protein change | Type of mutation | Mutations reported* | Affecteds | Onset (years) | No of exostoses | Vertebral change | Functional rating | Stature (centile) | Chondrosarcoma | Variable expressivity | Type M/S |
|------|------|-------------|----------------|------------------|---------------------|-----------|--------------|----------------|-----------------|----------------|-----------------|-----------------|----------------|----------------------|---------|
| EXT1 | Exon 1 | 42delG | G15 | Frameshift | A1-1 | 2 | 8 | 6 | Fair | 3 | + | IIIS | S |
| EXT1 | Exon 1 | 248insG | R43 | Frameshift | A1-2 | 9 | 2 | 17 | + | Fair | 50 | − | + | III S |
| EXT1 | Exon 1 | C250T | Q75X | Nonsense | A1-3 | 5 | 1 | 40 | + | Fair | 3 | − | + | IIII/S |
| EXT1 | Exon 1 | 352insC | V118 | Frameshift | A1-4 | 6 | 2 | 10 | − | Good | <3 | − | + | IV S |
| EXT1 | Exon 1 | 458delTC | L153 | Frameshift | A1-5 | 4 | 3 | 13 | − | Fair | 3 | + | + | IV S |
| EXT1 | Exon 1 | 460del2T | F154 | Frameshift | A1-6 | 8 | 9 | 15 | + | Fair | 50 | + | + | III S |
| EXT1 | Exon 1 | 477delTA | D160 | Frameshift | A1-7 | 18 | <1 | >40 | + | Fair | <3 | + | + | IIII/S |
| EXT1 | Exon 1 | 515delA | H172 | Frameshift | A1-8 | 6 | 1 | 20 | − | Fair | 25 | − | + | I S |
| EXT1 | Exon 1 | 549delGT | S180 | Frameshift | A1-9 | 5 | 4 | 30 | − | Fair | 25 | − | + | II S |
| EXT1 | Exon 1 | 679delE | R227 | Frameshift | A1-10 | 9 | 2 | 20 | − | Fair | 25 | − | + | I S |
| EXT1 | Exon 1 | 712delT | S238 | Frameshift | A1-11 | 6 | 2 | 30 | − | Fair | 3 | + | + | IV S |
| EXT1 | Exon 1 | 712delT | S238 | Frameshift | A1-12 | 6 | 3 | 17 | + | Fair | 50 | − | + | IIIS |
| EXT1 | Exon 1 | C1018T | R340C | Missense | A2-12 | 9 | 4 | <10 | − | Fair | 50 | − | + | IIIS |
| EXT1 | Exon 1 | C1018T | R340C | Missense | A2-13 | 7 | 5 | 17 | + | Fair | 75 | − | − | I S |
| EXT1 | Exon 1 | C1018T | R340C | Missense | A2-14 | 9 | 4 | <10 | − | Fair | 50 | − | + | IIIS |
| EXT1 | Exon 1 | C1018T | R340C | Missense | A2-15 | 2 | 5 | 10 | − | Fair | 50 | + | + | III S |
| EXT1 | Exon 1 | C1018T | R340C | Missense | A2-16 | 5 | 3 | 20 | − | Fair | 75 | + | − | I S |
| EXT1 | Intron 5 | G1417A | Splice site | A5-17 | 7 | 1 | 12 | + | Fair | 50 | − | + | IIIS |
| EXT1 | Exon 6 | 1431insT | S478 | Frameshift | A6-18 | 7 | 1 | 12 | + | Fair | 50 | − | + | III S |
| EXT1 | Exon 6 | 1469delT | L490 | Frameshift | A6-19 | 3 | 1 | 22 | − | Fair | 50 | − | − | I S |
| EXT1 | Exon 6 | 1469delT | L490 | Frameshift | A6-20 | 2 | 6 | 10 | − | Good | 25 | − | + | M |
| EXT1 | Exon 7 | 1568delT | L523 | Frameshift | A6-21 | 7 | 2 | 15 | − | Fair | 25 | − | + | I S |
| EXT1 | Exon 8 | 1642delA | S548 | Frameshift | A8-22 | 2 | <1 | 40 | − | Fair | 25 | − | + | II S |
| EXT1 | Exon 8 | G1754A | W582X | Nonsense | A9-23 | 13 | <1 | >20 | + | Fair | 25 | − | + | III S |
| EXT1 | Exon 9 | G1744A | W582X | Nonsense | A10-24 | 12 | <1 | 30 | + | Fair | 25 | + | + | IIIS |
| EXT1 | Exon 9 | G1745A | W582X | Nonsense | A10-25 | 5 | 2 | 10 | − | Fair | 10 | − | + | I S |
| EXT1 | Exon 9 | G1776A | Y592X | Nonsense | A10-26 | 4 | 2 | 10 | − | Fair | 75 | − | − | I S |
| EXT1 | Exon 9 | G1744A | W582X | Nonsense | A10-27 | 2 | 2 | 30 | − | Fair | 30 | + | + | IIIS |
| EXT1 | Exon 10 | 44delA | L161 | Frameshift | A10-28 | 9 | 4 | <10 | − | Fair | 50 | − | − | M |
| EXT1 | Exon 10 | 44delA | L161 | Frameshift | A10-29 | 3 | 10 | 10 | − | Good | 25 | − | + | M |
| EXT1 | Exon 10 | 44delA | L161 | Frameshift | A10-30 | 4 | 10 | 10 | − | Good | 25 | − | + | M |

Un = unknown, NI = none identified.

*Mutations previously reported.
†A = EXT1 locus, B = EXT2 locus, C = EXT3 locus, D = unknown locus. The first number corresponds to exon and the second number refers to family.

affected members had a moderate phenotype. Group S could be subdivided into four distinct clinical subgroups (table 3). In type IS, the number of exostoses ranged from 10 to 25 with no vertebral location and a height above the 10th centile. Type IIS was associated with a larger number of exostoses (>25). Patients in the type IIS group had vertebral exostoses and patients belonging to type IVS group had very short stature (equal to or below the 3rd centile).

Clinical data were available for 42 pedigrees and 110 questionnaires from affected subjects were collected (table 4). Among the 42 families, seven belonged to group M (type M). The 35 other families belonged to group S. Among them, 10 had the characteristics of type IS, five of type IIS, eight of type IIIS, and six families could not be assigned a specific type, as the patients exhibited characteristics of several types. Interestingly, chondrosarcoma was observed in nine patients from seven families, which all belong to group S.

Intrafamilial variability was observed in most families, supporting the previously reported variable expressivity of HME.7 In four families (A1-12, A6-19, B3-32, B3-33), clinical assessment of the affected subjects was in favour of anticipation. The disease showed a gradient of severity ranging from a mild phenotype in the grandparents (one to two exostoses) to a severe form in the probands (17-30 exostoses).

GENOTYPE-PHENOTYPE CORRELATION IN HME PATIENTS

The relationship between genotypes and clinical features are shown in fig 1. Most of the families with a moderate phenotype (type M) were associated with EXT2 mutations, while all type IIS, IIS, or IVS families except one (B3-32) were associated with EXT1 mutations (table 4). A statistical analysis of the data confirmed that type S (IS, IIS, IIIS, IVS) was significantly associated with EXT1 mutations while type M was associated with EXT2 mutations (p=0.0129, Fischer's exact test). Interestingly, the type IS phenotype was associated with either EXT1 or EXT2 mutations, without hotspots or a specific type of mutation in one or the other gene. No correlation was found between the type IIS or type IIIS phenotypes and location of the mutations in the EXT1 gene. By contrast, mutations associated with type IVS (patients with very short stature) were consistently located in the first EXT1 exon. It is worth noting that chondrosarcomas were restricted to patients carrying EXT1 mutations.

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The phenotypic variability of HME was noted in 2/3 of the familial forms caused by EXT1 mutations. By contrast, members of families harbouring an EXT2 mutation showed clinical homogeneity of the disease except for two families. The family linked to EXT3 belonged to group M. Interestingly, incomplete penetrance was noted in that family, especially in females. This was confirmed by x ray analysis of all family members. No specific phenotype was observed in three families unlinked to the three EXT loci.

Discussion
In order to determine the respective contribution of the three EXT loci in HME, we investigated their distribution among 42 families. Thirty nine families (93%) proved to be linked to one of the three loci but three families showed no linkage to any of them. No linkage to one of the three EXT-like loci has been reported so far in HME and no mutation has been identified. All these observations support the existence of at least one additional (EXT4) locus in HME.

Mutation analysis showed that EXT1 mutations were involved in 64% of HME families while EXT2 mutations occurred in 21% of families. These findings differ from previous studies in white patients. This may be because of a more efficient detection of mutations which allowed us to detect 95% of them. The failure to detect mutations in two EXT1 linked families suggests that mutations might be located either within the promoter or elsewhere outside the coding region.

Mutations within the EXT1 gene were found to be evenly distributed over the first nine exons, while the last exon, encoding part of the conserved carboxy-terminal domain, harboured significantly fewer mutations. Most EXT2 mutations appeared to be novel and randomly distributed over the first eight exons. As observed in EXT1, no mutation was identified in the carboxy-terminal region of the protein. The EXT mutations reported here are expected to cause premature translation termination and loss of function as mutated gene products lack one of their catalytic domains putatively located at the C-terminal end of the protein.

It is worth noting that most EXT1 mutations reported here (19/27) were novel mutations, but the common EXT1 mutations were also detected. Hence, the 1 bp deletion (1469delT) previously detected in seven families was also identified in two of our families. This deletion occurred at the end of a polycytosine tract, known to be a mutation hot spot. Similarly, the previously reported R340C mutation was found in three patients. This amino acid substitution is likely to alter proper functioning of the EXT1 protein. Remarkably, the corresponding arginine residue was substituted for lysine in a Chinese hamster ovary (CHO) cell mutant lacking glycosyltransferase activity. Furthermore, McCormick et al have shown that an EXT1 construct containing the R340C mutation was enzymatically inactive in vitro.

The genotype-phenotype analyses reported here suggested a strong correlation between EXT1 mutations and the severity of HME phenotypes. Statistical analyses firmly established a significant correlation between EXT1 mutations and the severe phenotypes (type S). Hence, large numbers of exostoses, short stature, and/or vertebral location were consistently associated with EXT1 mutations. These particularly severe cases were not accounted for by a cluster of specific mutations. Moreover, malignant degeneration of exostoses into chondrosarcomas was also observed in families carrying EXT1 mutations. In keeping with this, it has recently been shown that hereditary osteochondromas and secondary chondrosarcomas are associated with LOH at the EXT1 locus. By contrast to a previous report, we did not observe loss of the second EXT allele in the somatic DNA of the benign osteochondromas that we have studied. According to the tumour suppressor model, we speculate that single EXT1 germline mutations would be the first hit causing multiple benign osteochondromas while inactivation of the second allele would trigger malignant transformation. Our preliminary results on somatic DNA from two secondary chondrosarcomas also indicate LOH at the EXT1 locus (not shown).

Similarly, moderate phenotypes were frequently associated with EXT2 mutations, while the group of severe type IS phenotype resulted from mutations in either gene. The intrafamilial clinical variability that we have observed remains unclear at present. One could speculate that mutations or polymorphisms in a modifier gene may modulate the disease phenotype.

Recent studies have shown that the EXT1 and EXT2 gene products exert both GlcA-T and GlcNAc-T activities in vitro and biochemical analyses have shown that the two EXT proteins can interact to form homo/hetero-oligomers in vivo. Indeed, formation of a stable EXT1-EXT2 complex in vitro is required to elicit maximal GlcA-T activity. Recent experiments indicate that EXT1 and EXT2 cannot substitute for each other in transfected cells. However, while EXT1 alone exhibited significant GlcA-T and GlcNAc-T activity in transfected cells, EXT2 alone had no glycosyltransferase activity and was unable to compensate for the abrogation of GlcA-T activity caused by a R340C mutation in EXT1.

![Figure 1](www.jmg.bmj.com)

Figure 1 Genotype-phenotype correlation. Distribution of EXT1 and EXT2 mutations among moderate (M) and severe (IS, IIS, IIIS, IVS) types of HME (data from only one person (proband) in each family were used).
Whether a functional EXT1 protein could partially restore GlcA-T activity in EXT2 mutant cells is still unknown. Such difference in the glycosyltransferase activities of the two EXT enzymes could explain the lesser severity of HME phenotypes caused by EXT2 mutations. Alternatively, EXT2 may play a non-essential role in heparan sulphate synthesis, acting as a chaperone for EXT1.

In conclusion, our study provides what we believe to be the first evidence of genotype-phenotype correlation in HME, as EXT1 mutations are more likely to trigger malignant degeneration of exostoses. It is hoped that this study will improve the management of patients.

Accession numbers and URLs for data in this article are as follows: GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html for EXT1 genomic sequence (139949) and for EXT2 genomic sequence (134921), Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim for EXT1 (MIM 135700), EXT2 (MIM 135701), and EXT (MIM 600209).

We are deeply grateful to the HME patients and their families following physicians for collaboration: P Calvas, M P Cordier, V V. We are deeply grateful to the HME patients and their families and for statistical analyses and W Van Hul for providing EXT primers. This work was supported by a grant from Margaritte-Jeannin for statistical analyses and W Van Hul for hereditary multiple exostoses.

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doi: 10.1136/jmg.38.7.430

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