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Mutations in the EXT1 and EXT2 Genes in Hereditary Multiple Exostoses

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Summary

Hereditary multiple exostoses (EXT; MIM 133700) is an autosomal dominant bone disorder characterized by the presence of multiple benign cartilage-capped tumors (exostoses). Besides suffering complications caused by the pressure of these exostoses on the surrounding tissues, EXT patients are at an increased risk for malignant chondrosarcoma, which may develop from an exostosis. EXT is genetically heterogeneous, and three loci have been identified so far: EXT1, on chromosome 8q23-q24; EXT2, on 11p11-p12; and EXT3, on the short arm of chromosome 19. The EXT1 and EXT2 genes were cloned recently, and they were shown to be homologous. We have now analyzed the EXT1 and EXT2 genes, in 26 EXT families originating from nine countries, to identify the underlying disease-causing mutation. Of the 26 families, 10 families had an EXT1 mutation, and 10 had an EXT2 mutation. Twelve of these mutations have never been described before. In addition, we have reviewed all EXT1 and EXT2 mutations reported so far, to determine the nature, frequency, and distribution of mutations that cause EXT. From this analysis, we conclude that mutations in either the EXT1 or the EXT2 gene are responsible for the majority of EXT cases. Most of the mutations in EXT1 and EXT2 cause premature termination of the EXT proteins, whereas missense mutations are rare. The development is thus mainly due to loss of function of the EXT genes, consistent with the hypothesis that the EXT genes have a tumor- suppressor function.

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Introduction

The multiple exostoses (EXT) gene family comprises five homologous genes, including EXT1 (Ahn et al. 1995), EXT2 (Stickens et al. 1996; Wuyts et al. 1996), EXTL1 (Wise et al. 1997), EXTL2 (Wuyts et al. 1997), and EXTL3 (Van Hul et al., in press). Two of these genes, EXT1 and EXT2, have been shown to be involved in the pathogenesis of EXT, as they harbor germline mutations in EXT patients (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). EXT is an autosomal dominant condition with an incidence of ~1/50,000. EXT patients show multiple benign bony outgrowths (exostoses), typically located at the juxtaepiphyseal regions of bones, that develop by enchondral bone formation (Solomon 1964; Hennekam 1991; Schmale et al. 1994). The presence of these bony outgrowths may cause pressure on neighboring tissues, nerves, or blood vessels (Hennekam 1991). The most severe complication is the transformation of an exostosis into a malignant chondrosarcoma, although this complication is observed in only 2%–5% of EXT patients (Solomon 1964; Hennekam 1991; Schmale et al. 1994).

EXT is a genetically heterogeneous disease. In addition to the EXT1 gene on chromosome 8q23-q24 (Cook et al. 1993) and the EXT2 gene on 11p11-p12 (Wu et al. 1994; Wuyts et al. 1995), linkage analysis has indicated the existence of a third gene (EXT3), on chromosome 19p (Le Merrer et al. 1994). However, most EXT families appear to be linked to the EXT1 and EXT2 loci (Cook et al. 1993; Blanton et al. 1996; Legeai-Mallet et al. 1997).

The EXT1 and EXT2 genes are ubiquitously expressed, and they encode proteins of 746 and 718 amino acids, respectively, that show homology to each other, especially at the carboxy terminus (Stickens et al. 1996; Wuyts et al. 1996). Both genes probably have a tumor suppressor function, as chondrosarcomas show loss of heterozygosity in both the EXT1 region on chromosome

Table 1

Summary of EXT Families Analyzed

Family	No. of Patients	Country of Origin	Mutation[a]	EXT1 or EXT2[a]
1	16	Belgium	C514T	EXT2
2	8	Belgium	1173+1G→A	EXT2
3	1	Morocco	1173+1G→T	EXT2
4	2	Morocco	U	U
5	17	The Netherlands	U	EXT2[b]
7	1	Belgium	1469delT	EXT1
10	1	Belgium	U	U
14	4	Italy	C67T	EXT2
15	3	Belgium	1679–1680insC	EXT1
19	2	Germany	2068+2del6	EXT1
24	2	Belgium	U	U
26	2	Italy	G204A	EXT1
27	5	Belgium	A838G	EXT1
28	3	Turkey	C1376G	EXT1
29	5	The Netherlands	1173+1G→A	EXT2
31	1	Belgium	G600A	EXT1
32	2	The Netherlands	U	U
35	1	The Netherlands	624ins5	EXT1
39	1	Belgium	C1018A	EXT1
40	1	Belgium	U	U
42	2	The Netherlands	1173+1G→A	EXT2
49	1	France	C514T	EXT2
60	1	Great Britain	812–814delC	EXT2
64	1	Italy	G599A	EXT1
70	4	USA	1263insAT	EXT2
71	5	USA	649–652delT	EXT2

[a] U = unknown.
[b] Based on linkage analysis (Wu et al. 1994).

8 and the EXT2 region on chromosome 11 (Hecht et al. 1995, 1997; Raskind et al. 1995). Furthermore, an inactivating somatic EXT1 mutation in a chondrosarcoma has been reported (Hecht et al. 1997). Homology searches of dbEST, with the EXT sequences used as query sequences, identified three other genes that display homology to the same carboxyterminal region. These three EXT-like (EXTL) genes have been fully characterized by us and by others. EXTL1 maps to chromosome 1p36 (Wise et al. 1997), EXTL2 to 1p11-p12 (Wuyts et al. 1997), and EXTL3 to 8p12-p22 (Van Hul et al., in press), respectively. None of the EXTL genes has yet been shown to be involved either in EXT or in any other disease.

We have now performed a systematic mutation analysis of the EXT1 and EXT2 genes in EXT families, to estimate the relative proportions of the EXT1 and EXT2 mutations and to study the spectrum and distribution of mutations that lead to EXT.

Subjects, Material, and Methods

EXT Families

Twenty-six families with EXT, including 92 affected patients originating from nine different countries, were studied, to identify mutations in the EXT1 or the EXT2 gene. Seven of the 26 families have been described previously (table 1). Family 1, originating from Belgium, was called "family 2" in the initial report describing the localization of the EXT2 gene (Wu et al. 1994). This family later contributed to the refinement of the EXT2 candidate locus and to the final identification of the EXT2 gene. In both of these reports, the family was referred to as "family 1" (Wuyts et al. 1995, 1996). Family 2, also from Belgium, was reported previously in the paper describing the isolation of the EXT2 gene (Wuyts et al. 1996). Family 5, from the Netherlands, was called "family 1" by Wu et al. (1994). Family 14, reported in an article by Mollica et al. (1984), exhibits brachydactyly and anetoderma in some affected family members. Family 42 was reported previously as "family 3" (Wuyts et al. 1995). Families 70 and 71 are reported as families "HME11" and "HME39" by Raskind et al. (in press). The other 19 families have not been described before (table 1). All EXT diagnoses were based on the results of radiological examination.

Mutation Analysis

All exons of both the EXT1 and the EXT2 genes were amplified separately by PCR; primers are listed in table

Table 2

Primer Sequences Used

EXT1 Primers		
Name	Sequence	Length (bp)
EXT1-ex1a	CAGGCGGGAAGATGGCGGACTGG	212
EXT1-ex1b	*CTCCGGCTGTGGCTCCTCGATGCCC*	
EXT1-ex1c	TGCTCTCAGCTGGCTCTTGTCTCG	201
EXT1-ex1d	*GAATCCTCGTTTTCCAATTGATCCC*	
EXT1-ex1e	CGGAGCCTCTGCGCCCCTTCGTTCC	232
EXT1-ex1f	*CTAGAATGTTTTGGTAACTTTCGGCG*	
EXT1-ex1g	CGTATACCCACAGCAAAAAGGGG	209
EXT1-ex1h	*CATTGTTCCACAAGTGGAGACTCTGC*	
EXT1-ex1i	CCAGTTGTCACCTCAGTATGTGC	168
EXT1-ex1f2	*GGCTTTGGCCAGCATCGCCAGG*	
EXT1-ex1k	CCTGACTACACCGAGGACG	237
EXT1-ex1l	GGTGTCTGATCCTATCCCTG	
EXT1-ex1m	GGTATTCAAGGGGAAGAGGTAC	231
EXT1-ex1j	*ggaccaaggccggcagagccc*	
EXT1-ex2a	ccccacattcgcaatgagtc	225
EXT1-ex2b	*gagaggtgataatgttaaaccc*	
EXT1-ex3a	cgtattggaacagcttcgtctg	224
EXT1-ex3b	*gacgggggcagcaataatctgc*	
EXT1-ex4a	gtgcattctctttgttttacag	235
EXT1-ex4b	*gctgagagaagtgtataaagg*	
EXT1-ex5a	cctttccaaatatcatcagg	237
EXT1-ex5b	*catcttcagggtaaacaagggc*	
EXT1-ex5c	*ccattttgcaatgctctgctctg*	
EXT1-ex6a	gctttccagcgcttcattaggc	210
EXT1-ex6b	*ctggagctggagcaggcagggg*	
EXT1-ex7a	ggcgtacataaatacatcctacccc	189
EXT1-ex7b	*ccaaggctccacagtggttcc*	
EXT1-ex8a	caagactctgaagttacctctttccc	204
EXT1-ex8b	*ggtgactgcctgaacagcccacc*	
EXT1-ex9a	cactgttgattgcttgtttggc	235
EXT1-ex9b	*gtaaagtctgtaagagacatgtcc*	
EXT1-ex10a	cttgtcatcatgtgataatggccc	259
EXT1-ex10b	*gagtgaagcaaggaagaggg*	
EXT1-ex11a	ccttgcacttctctcatattatcc	230
EXT1-ex11b	*CCTCAAAGTCGCTCAATGTCTCGG*	
EXT1-M1	gaatcctcgttttccaatggatc	. . .
EXT1-M2	ggaatctggaaggacccaagccagc	

EXT2 Primers		
Name	Sequence	Length (bp)
EXT2-ex2a	ctctcccctggtgacc	338
EXT2-ex2A8	*CACAGCGATAGACATCAAAACACG*	
EXT2-ex2A26	GACAGTCCCATCCCAGAGCGG	249
EXT2-ex2A25	*GGAGGGAACAAACAGACAGG*	
EXT2-ex2A4	ACTACACTGATGACATCAACCG	176
EXT2-ex2b	*ccctttagttccctgagggcc*	
EXT2-ex3a	gttgacacattaattctccc	184
EXT2-ex3b	*gaacaaaaatgatcttgaaccc*	
EXT2-ex4a	gaataaagtcctttcttctcatcg	205
EXT2-ex4b	*cagtaaaggcacacctggc*	
EXT2-ex5a	gcaattttccaatcacctg	267
EXT2-ex5b	*cctgagcctttgcgagagg*	
EXT2-ex6a	ctagtttgtaatctcttgcctc	222
EXT2-ex6b	*tacgcagaaccactaatgtagag*	
EXT2-ex7a	gggatgtggggctgaaggagg	293
EXT2-ex7b	*ctcctgtccctctgtatccagtc*	
EXT2-ex8a	gcttgctcacttaaaacagc	200
EXT2-ex8b	*gcctcatgtggctagcac*	
EXT2-ex8c	*ttatgctgcccttatcaggccc*	
EXT2-ex9a	cagctgcttttctgacccg	263
EXT2-ex9b	*gatccagctgagagaggcac*	
EXT2-ex10a	cctcacaaaagttaggag	240
EXT2-ex10b	*aaacacactgtgtaaaacc*	
EXT2-ex11a	gaatggttgctgtctgaattggg	235
EXT2-ex11b	*ctcagttttgtcaccttgcc*	
EXT2-ex12a	ccccttatttatcagctaaaggg	220
EXT2-ex12b	*caagtgagtggcagagcc*	
EXT2-ex13a	gtccttgacactgacagccagg	175
EXT2-ex13b	*tagagatcagaggctaaggcgc*	
EXT2-ex14a	caaacccctcctccccacctcctc	318
EXT2-ex14c	*GTGGGTTAGGTGGGTGCATGCC*	
EXT2-M1	ccacctagagagctgggccatcgcat	. . .
EXT2-M2	cagattgaagaaatgcagagccag	
EXT2-M3	catcccaagaatgaagaccaaggac	

NOTE.—Reverse primers are italicized, and modified nucleotides in modified primers are underlined. Primers located in introns are lowercased, whereas primers located in exons are capitalized. The designation "ex" in each primer name is followed by the exon number. EXT2 primers were designed from genomic sequences deposited in GenBank (accession numbers U67356–U67368).

2. Exon 1 of EXT1 and exons 2 and 14 of EXT2 were split into several overlapping fragments, to obtain amplification products that did not exceed 350 bp. PCR was performed in a 20-μl reaction volume, with a final $MgCl_2$ concentration of 1.5 mM. All PCR programs included an initial denaturation of 4 min at 96°C, followed by 25–30 cycles of 30 s at 94°C, 30 s at temperature T_m, and 45 s at 72°C. Finally, an extension at 72°C was performed for 5 min. Temperature T_m was 55°C for all primer combinations, with the exception of primers amplifying exons 7 and 8 of EXT1 and exons 3, 5, and 10 of EXT2. For these primer combinations, temperature T_m was set at 51°C. SSCP analysis of PCR fragments was performed according to standard protocols. PCR fragments that included aberrant fragments were reamplified and purified from agarose gels by use of the Sephaglas Bandprep kit (Pharmacia Biotech). Sequence analysis, based on the dideoxy method, was performed by means of dye terminator chemistry, with *Taq* polymerase. Sequencing reactions were composed of 25 cycles of 30 s at 96°C, 15 s at 52°C, and 4 min at 60°C. After 25 cycles, the reaction products were quickly chilled to 4°C. All PCR products were sequenced directly with one of the amplification primers. Sequences were analyzed on ABI-373 and ABI-377 automatic sequencers (Perkin-Elmer).

Mutations were confirmed by means of restriction analysis, on the basis of creation or destruction of re-

striction sites by the mutation. If necessary, modified primers were designed to create restriction sites. All modified primers used in this study are listed in table 2. Restriction digestions were performed on PCR products in accordance with standard protocols, and the restriction fragments were separated on 3% agarose gels.

Results

We searched for mutations in the EXT1 and EXT2 genes in a set of 26 families with EXT. Mutation analysis was performed by subjecting all exons to SSCP scanning (primers are listed in table 2). All mutations were confirmed by means of restriction assay, sequence analysis, or PAGE analysis. If more patients from the same family were available, segregation of the mutation in the family was confirmed by either restriction analysis or sequence analysis. In the case of missense mutations, 50 healthy control individuals were investigated, to exclude the possibility that the substitution was a polymorphism. Ten different mutations were found in the EXT1 gene, and seven different mutations were found in the EXT2 gene (table 1). In six families, no mutation was detected, although all exons of both the EXT1 and the EXT2 genes were subjected to SSCP analysis.

EXT1 Mutations

The EXT1 gene consists of 11 exons, with a large (1735 bp) first exon (Lüdecke et al. 1997). Because the sensitivity of SSCP analysis is known to decrease with increasing fragment length, we analyzed this exon by means of SSCP analysis of six separate overlapping fragments, all <300 bp. Nucleotide numbering was based on the sequence provided by Ahn et al. (1995), with base 1 corresponding to the first base of the initiation codon. Ten different EXT1 mutations were identified; eight of these have not been reported previously.

Family 26.—The two patients in this Italian family have a nonsense mutation, a substitution of G204 for A in the first exon, that leads to a premature termination of the protein after 67 amino acids. The G204A mutation was confirmed by *Xho*II restriction digestion of a 201-bp PCR-amplification product of primers EXT1-M1 and EXT1-ex1c. The modified primer EXT1-M1 creates a *Xho*II restriction site (GGATCC) that allows digestion in two bands, of 23 bp and 178 bp, respectively, in the wild-type DNA. This restriction site has been lost in the mutant allele.

Family 64.—The mutation in this Italian patient consists of a de novo G599A transition, in the first exon, that substitutes a stop codon for tryptophan 200. The 168-bp amplification product of primers EXT1-ex1i and EXT1-ex1f2 is digested by *Hae*III in bands of 111 bp, 49 bp, and 8 bp in controls. The G599A mutation destroys one GGCC *Hae*III site, resulting in two bands, of 160 bp and 8 bp, in patients carrying this mutation.

Family 31.—The one available patient from this Belgian family has a G600A substitution, in exon 1, that also terminates the EXT1 protein after 199 amino acids. The G600A mutation was confirmed by restriction analysis, as described above for the G599A mutation (family 64).

Family 35.—In this Dutch family, the development of EXT was shown to be caused by the duplication of five nucleotides (TGGGG) following nucleotide 624, in exon 1, which causes a frameshift at F209. The mutation can also be detected by PAGE of the 168-bp amplification product obtained by means of PCR, with primers EXT1-ex1i and EXT1-ex1f2.

Family 27.—In this Belgian family, which includes five EXT patients, we identified an A838G mutation in exon 1. The same mutation has been reported by Raskind et al. (in press) and results in an R280G substitution in the EXT1 protein. The mutation can be detected by *Msp*I restriction digestion, as it creates a novel *Msp*I restriction site. The mutant 231-bp allele amplified with primers EXT1-ex1m and EXT1-ex1j is digested in bands of 184 bp and 47 bp, respectively, whereas the wild type is not digested.

Family 39.—The only available affected member of this Belgian family has a C1018A mutation in exon 2 that results in an R340S substitution. This mutation has never been reported before, but other missense mutations that affect the same arginine at position 340 have been reported previously in three other families (table 3). A modified PCR that uses primers EXT1-M2 and EXT1-ex2a was developed. The 133-bp amplification fragment is digested into fragments of 110 bp and 23 bp, by *Msp*I restriction digestion, in the presence of the C1018A mutation.

Family 28.—In this Turkish family that includes three EXT patients, a C1376G mutation that substitutes a stop codon for a serine at position 459 was identified in exon 5. This mutation introduces a *Sau*3AI restriction site (GATC), which allows mutation detection by PCR, with primers EXT1-ex5a and EXT1-ex5b, followed by *Sau*3AI restriction digestion. The normal band has a length of 237 bp and is digested in bands of 148 bp and 89 bp, in the presence of the C1376G mutation.

Family 19.—A deletion of six nucleotides in the 5′ splice site of exon 5 (1417+2del6) was identified in the two EXT patients in this German family. Although the effect of the 1417+2del6 mutation on the EXT1 splicing has not been studied, it is likely that the 5′ splice site of intron 5 is destroyed. The 1417+2del6 mutation can also be detected by PAGE of the PCR amplification product obtained by PCR, with primers EXT1-ex5a and EXT1-ex5b.

Family 7.—The only available affected member of this

Table 3

Mutations Identified in the EXT1 Gene

	cDNA Change[a]	Exon or Intron	Protein Change[b]	Type of Mutation	Reference(s)
1	118delC	Exon 1	FS H40	Frameshift	Raskind et al. (in press)
2	174–176delC	Exon 1	FS P59	Frameshift	Philippe et al. (1997)
3	G204A	Exon 1	W68X	Nonsense	Present study
4	242–247insC	Exon 1	FS R83	Frameshift	Wells et al. (1997)
5	248–249delG	Exon 1	FS Q84	Frameshift	Wells et al. (1997)
6	C357A	Exon 1	Y119X	Nonsense	Raskind et al. (in press)
7	420ins4	Exon 1	FS S141	Frameshift	Hecht et al. (1997)
8	527del8	Exon 1	FS K177	Frameshift	Hecht et al. (1997)
9	G599A	Exon 1	W200X	Nonsense	Present study
10	G600A	Exon 1	W200X	Nonsense	Present study
11	624ins5	Exon 1	FS F209	Frameshift	Present study
12	713delC	Exon 1	FS S238	Frameshift	Hecht et al. (1997) (two families)
13	A838G	Exon 1	R280G	Missense	Present study; Raskind et al. (in press)
14	G840C	Exon 1	R280S	Missense	Raskind et al. (in press)
15	G1016A	Exon 2	G339D	Missense	Philippe et al. (1997)
16	C1018A	Exon 2	R340S	Missense	Present study
17	C1018T	Exon 2	R340C	Missense	Philippe et al. (1997)
18	G1019T	Exon 2	R340L	Missense	Hecht et al. (1997)
19	G1019A	Exon 2	R340H	Missense	Raskind et al. (in press)
20	1056+1G→A	Intron 2	U	Splice site	Wells et al. (1997)
21	A1057-2G	Intron 2	U	Splice site	Raskind et al. (in press)
22	1091–1093insGG	Exon 3	FS E365	Frameshift	Raskind et al. (in press)
23	G1122A	Exon 3	W374X	Nonsense	Philippe et al. (1997)
24	1203–1204insC	Exon 4	FS L402	Frameshift	Raskind et al. (in press)
25	1215del4	Exon 4	FS R405	Frameshift	Raskind et al. (in press)
26	C1376G	Exon 5	S459X	Nonsense	Present study
27	1417+1G→A	Intron 5	U	Splice site	Philippe et al. (1997)
28	1417+2del6	Intron 5	U	Splice site	Present study
29	1426–1431insC	Exon 6	FS S478	Frameshift	Hecht et al. (1997); Raskind et al. (in press)
30	1431insT	Exon 6	FS S478	Frameshift	Wells et al. (1997)
31	1469delT	Exon 6	FS L490	Frameshift	Present study; Ahn et al. (1995) (two families); Wells et al. (1997); Philippe et al. (1997) (two families)
32	1679–1680insC	Exon 8	FS V561	Frameshift	Present study
33	G1817A	Exon 9	W606X	Nonsense	Wells et al. (1997)
34	1878del3	Exon 9	H627del	1 AA deletion	Raskind et al. (in press)
35	C2053T	Exon 10	Q685X	Nonsense	Raskind et al. (in press)

[a] All mutations were numbered uniformly; the adenosine of the start codon was assigned nucleotide position +1.
[b] U = unknown.

Belgian family has a 1469delT deletion in exon 6, which causes a frameshift, with L490 being the first amino acid substituted. The same mutation has already been reported in six other families (table 3), and it can be confirmed by *Mva*I digestion of the PCR product of primers EXT1-ex6a and EXT1-ex6b. In controls, this results in digestion of the 210-bp amplification product, in three fragments of 90 bp, 70 bp, and 50 bp, respectively. Patients who carry the 1469delT mutation have lost one *Mva*I site, and they exhibit bands of 160 bp and 50 bp, respectively.

Family 15.—Development of EXT in the three affected members of this Belgian family was found to be caused by the insertion of one cytosine in a triplet of cytosines at nucleotide position 1678–1680 in exon 8. The 1678-1680insC mutation causes a frameshift that starts at V561.

EXT2 Mutations

The EXT2 gene consists of 15 exons (GenBank accession numbers U67354–U67368). Exons 1a and 1b were not included in the mutation analysis, as they encode the 5′ UTR. Since exon 2 is 566 bp, it was analyzed

by means of SSCP analysis of three overlapping fragments. The nucleotide numbering of Wuyts et al. (1996) was used, with position +1 given to the adenosine of the start codon. We identified a mutation in the EXT2 gene in seven families. Four of these mutations have never been described before.

Family 14.—This Italian family includes five EXT patients, all of whom have a C67T mutation that creates a stop codon after 22 amino acids. A modified PCR, with primers EXT2-A8 and EXT2-M3, was developed to detect this mutation. The mutation destroys an *Ava*II site created by the modified PCR, which results in the inability of *Ava*II to cut the 249-bp fragment generated by PCR. Controls exhibit a restriction pattern with two bands, of 22 bp and 227 bp, respectively.

Family 49.—A C514T mutation that leads to a premature stop codon at amino acid 172 was identified in a member of this French family. The same mutation was found previously in the large Belgian family 1, and a modified PCR to detect this mutation has been described previously (Wuyts et al. 1996).

Family 71.—In this family from the United States, five EXT patients have a deletion of one thymine in a stretch of four thymines, at position 649-652, in exon 4. The 649-652delT deletion results in a frameshift, with the serine at position 218 being the first amino acid substituted.

Family 60.—In this English patient, we identified a deletion of one cytosine in a triplet of cytosines, at position 812-814, in exon 5. The 812-814delC mutation causes a frameshift, with A271 being the first amino acid substituted.

Family 29.—A 1173+1G→A splice-site mutation, at the +1 position of the 5′ splice site in intron 7, was identified in this Dutch family, which includes five EXT patients. To confirm the mutation, we amplified a 100-bp fragment containing the 5′ splice site, with primers EXT2-ex7a and EXT2-M2. The amplification product was digested by *Scr*FI restriction enzyme. The wild-type PCR fragment contains an *Scr*FI restriction site, whereas the mutant allele has lost this site. Digestion of the amplification product results in fragments of 22 bp and 78 bp, respectively. This mutation was detected previously in the Belgian family 2, and the mutation was shown to cause skipping of exon 7, which leads to a truncated protein (Wuyts et al. 1996).

Family 42.—The two available EXT patients from this Dutch family also have the 1173+1G→A mutation.

Family 3.—The single EXT patient from this Moroccan family has a 1173+1G→T transition at the first nucleotide of intron 7. This mutation can also be detected by *Scr*FI digestion of the EXT2-ex7a and EXT2-M2 amplification products, as described above for the 1173+1G→A mutation (family 29).

Family 70.—The four affected members of this American family have an insertion of two nucleotides at position 1263, in exon 8, which causes a frameshift; A422 is the first amino acid substituted. This 1263insAT mutation can be detected by PAGE of the amplification product of exon 8.

Discussion

We have analyzed 26 EXT families, from nine different countries, for the presence of mutations in the EXT1 and EXT2 genes. For both genes, the intron-exon boundaries have already been determined; this facilitates large-scale mutation analysis at the genomic level. SSCP analysis was chosen as the detection method because of its simplicity and its high detection rate (80%–90%). Ten families were found to have a mutation in the EXT1 gene, and another 10 families were found to have an EXT2 mutation. In six families (23%), we did not detect any mutation. This might be due to the shortcomings of the SSCP screening method. Indeed, large rearrangements (such as the large deletions or translocations, involving the EXT genes, that have been described in the past [Ahn et al. 1995; Lüdecke et al. 1995; Stickens et al. 1996]) or mutational events that result in failure to anneal the primer are not detected by SSCP in a dominant disease, because of the background of the wild-type allele. Also, point mutations that do not cause enough conformational differences during the renaturation process are not detected. This is demonstrated by the 1173+1G→A splice-site mutation in the EXT2 gene, which was initially detected at the cDNA level (Wuyts et al. 1996); in the present study, this mutation appeared to be undetectable by SSCP analysis of genomic DNA. Furthermore, we did not look for mutations either in the 5′ and 3′ UTRs or in the promoter regions. Mutation analysis of the open reading frame of EXT1 has been unsuccessful in several families who demonstrated linkage to the EXT1 locus; this suggests the existence of a minority of mutations outside the EXT1 protein–coding region (Hecht et al. 1997; Wells et al. 1997; Raskind et al., in press). Of our six families without an aberration detectable by SSCP, five were too small to allow us to perform linkage analysis. Only family 5 was suitable for linkage analysis, and this family was previously shown to be linked to the EXT2 locus (Wu et al. 1994). Finally, the majority of families that do not show an EXT1 or an EXT2 aberration may have mutations in other EXT-causing genes. In addition to the EXT3 gene on chromosome 19, the recently identified EXTL1 (Wise et al. 1997), EXTL2 (Wuyts et al. 1997), and EXTL3 (Van Hul et al., in press) genes may be interesting candidates for mutation screening among EXT patients who have neither an EXT1 nor an EXT2 mutation.

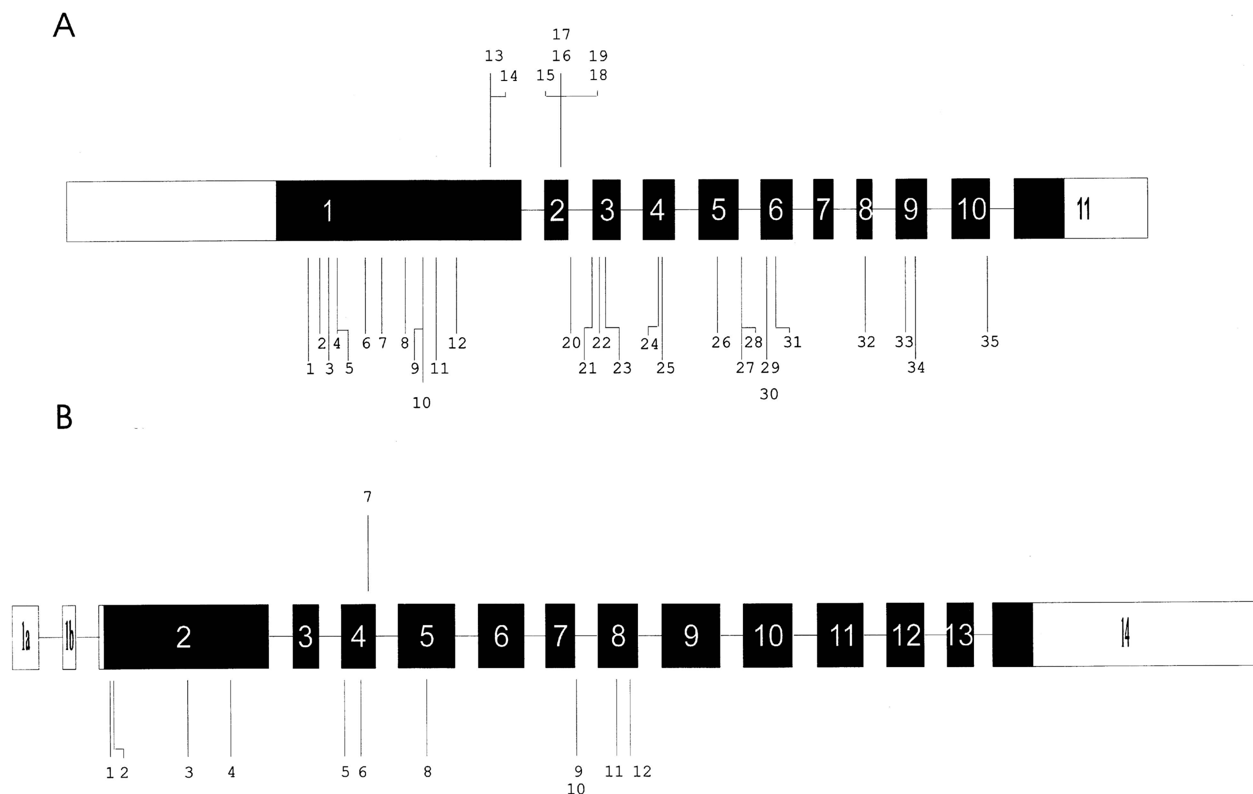

Figure 1 Distribution of mutations in the EXT1 (*A*) and EXT2 (*B*) genes, reported in the present study or in previous studies. Mutations are referred to by numbers, as in tables 3 and 4. Missense mutations are drawn above the schematic representation of the gene, and inactivating mutations are drawn below. Black boxes represent coding regions. Exons have been numbered on the basis of both the genomic structure provided by Lüdecke et al. (1997) for EXT1 and the GenBank accession numbers (U67354–U67368) for EXT2.

EXT1 Mutations

Ten different mutations were identified in the EXT1 gene in our set of 26 EXT families; 8 of the 10 mutations had not been reported before. Thirty-five different EXT1 mutations, summarized in table 3, have been reported, to date. Seventy-seven percent of these mutations are nonsense mutations, splice-site mutations, or frameshifts, which cause premature termination of the EXT1 protein. These truncated EXT1 proteins are probably inactive, and it is likely that they degraded rapidly, resulting in a nearly complete loss of function. Seven different missense mutations have been identified. Interestingly, five of them substitute two adjacent amino acids, G339 and R340. Sequence analysis revealed that these amino acids define part of a potential proteolysis-amidation site (x-G-[RK]-[RK]). Such amidation sites are often found at the C termini of hormone precursors and other active peptides, and they function as active precursor cleavage sites. However, in the EXT1 protein, the amidation site occurs in the middle of the protein, and it seems unlikely that this site is functional. The clustering of missense mutations in these two codons may indicate that these two amino acids, G339 and R340, are crucial for proper function or stability of the EXT1 protein. Both of the other two missense mutations affect arginine 280; this indicates that the R280 position is also essential for functional activity or stability of the EXT1 protein.

Thirty-five different mutations have been described in 43 families (table 1); this implies that most mutations are private mutations that are present in only a single family. However, one mutation, 1469delT, has already been found in six different families, and it probably represents the most recurrent mutation in the EXT1 gene. Analysis of the distribution of mutations over the EXT1 coding region indicates that the carboxyterminal part of the EXT1 genes contains fewer mutations than expected. The distribution of mutations in the EXT1 gene, including those identified in this study and those identified in previous studies, is displayed in figure 1*A*. Only 4 (11%) of the 35 reported EXT1 mutations are located within the 250 carboxyterminal amino acids, which represent 34% of the EXT1 protein. All four of these mutations cause premature termination of the protein, and,

Table 4

Mutations Identified in the EXT2 Gene

	cDNA Change[a]	Exon or Intron	Protein Change	Type of Mutation	Reference(s)
1	C67T	Exon 2	Q23X	Nonsense	Present study
2	77–78insT	Exon 2	FS Y26	Frameshift	Philippe et al. (1997)
3	449del4	Exon 2	FS A150	Frameshift	Stickens et al. (1996)
4	C514T	Exon 2	Q172X	Nonsense	Present study; Wuyts et al. (1996)
5	649–652delT	Exon 4	FS S218	Frameshift	Present study
6	C666G	Exon 4	Y222X	Nonsense	Philippe et al. (1997)
7	G679A	Exon 4	D227N	Missense	Philippe et al. (1997) (two families)
8	812–814delC	Exon 5	FS A271	Frameshift	Present study
9	1173+1G→A	Intron 7	FS R360	Splice site	Present study (two families); Wuyts et al. (1996)
10	1173+1G→T	Intron 7	FS R360	Splice site	Present study
11	C1201T	Exon 8	Q401X	Nonsense	Philippe et al. (1997)
12	1263insAT	Exon 8	FS A422	Frameshift	Present study

[a] All mutations were numbered uniformly; the adenosine of the start codon was assigned nucleotide position +1.

so far, no missense mutations have been identified in this region. Interestingly, this region shows the highest degree of conservation between the several members of the EXT gene family (Van Hul et al., in press; Wuyts et al. 1997).

EXT2 Mutations

Seven different mutations were identified in the EXT2 gene in our set of 26 EXT families (table 1). Four of the seven mutations have not been reported previously. Table 4 summarizes the 12 different EXT2 mutations that have been reported to date.

Family 2, from Belgium, is known to harbor a 1173+1G→A splice-site mutation that results in exon skipping of exon 7 (Wuyts et al. 1996). We identified the same mutation in families 29 and 42 (table 1). All three families with the 1173+1G→A mutation originate from Belgium or the Netherlands, two neighboring countries, and we suspected that these three families share a common ancestor. Therefore, we analyzed several patients from these three families by means of polymorphic markers flanking the EXT2 gene, and a common disease haplotype was indeed found (data not shown). This finding suggests that these families have a common ancestor and that the 1173+1G→A mutation most likely does not represent a recurrent mutation. The distribution of mutations in the EXT2 gene, including those identified in this study and those identified in previous studies, is displayed in figure 1*B*.

Relative Proportion of EXT1 to EXT2 Mutations

Previous estimates of the fractions of EXT cases caused by mutations in EXT1 or in EXT2 were based mainly on linkage studies of EXT families. Cook et al. (1993) provided evidence of linkage to chromosome 8 in 7 of their 11 families. Blanton et al. (1996) mapped 6 of their 12 families to chromosome 8, and they mapped the remaining 6 families to the EXT2 locus at chromosome 11. A recent French study analyzed 29 smaller families and assigned 8 (28%) of them to the EXT1 locus, 5 (17%) to the EXT2 locus, and 3 (10%) to the EXT3 locus on chromosome 19p. However, most of the families were too small to establish definite linkage with one of the EXT loci (Legeai-Mallet et al. 1997). By means of a combination of linkage analysis and mutation analysis of EXT1, Raskind et al. (in press) determined that EXT1 is responsible for the development of exostoses in 66% of the 34 EXT families they analyzed. The present study represents the first study of a larger group of EXT cases in which mutation analyses of both the EXT1 and the EXT2 genes were performed. Of the 26 families examined in this study, 10 (38.5%) had an EXT1 mutation, and 10 (38.5%) had an EXT2 mutation. However, as the three EXT2 families with the 1173+1G→A mutation probably represent one large family, the proportion of EXT1 to EXT2 mutations is 42% to 33%. Recently, Philippe et al. (1997) reported mutations in 12 of 17 EXT families analyzed. Seven (41%) of the mutations were found in the EXT1 gene, and five (29%) were found in the EXT2 gene (Philippe et al. 1997). All of these studies suggest that the majority of mutations that cause EXT are located within the EXT1 and EXT2 genes.

Given the significant number of mutations identified in both the EXT1 and the EXT2 genes, it would be interesting to examine whether phenotypic differences can be observed in patients who harbor mutations in either gene. Unfortunately, because detailed clinical data were not available for all patients in our study, we were

unable to investigate the presence or absence of a possible genotype-phenotype correlation for the EXT genes. Inactivating mutations represent the vast majority of mutations in both the EXT1 (77%) and EXT2 (92%) genes, supporting the hypothesis that both EXT1 and EXT2 have a tumor suppressor function that is lost during the development of exostoses.

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