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Hereditary Multiple Exostoses (EXT): Mutational Studies of Familial EXT1 Cases and EXT-Associated Malignancies

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Summary

Hereditary multiple exostoses (EXT) is an autosomal dominant disorder characterized by the formation of cartilage-capped prominences that develop from the growth centers of the long bones. EXT is genetically heterogeneous, with three loci, currently identified on chromosomes 8q24.1, 11p13, and 19q. The EXT1 gene, located on chromosome 8q24.1, has been cloned and is encoded by a 3.4-kb cDNA. Five mutations in the EXT1 gene have been identified—four germ-line mutations, including two unrelated families with the same mutation, and one somatic mutation in a patient with chondrosarcoma. Four of the mutations identified resulted in frameshifts and premature termination codons, while the fifth mutation resulted in a substitution of leucine for arginine. Loss of heterozygosity (LOH) analysis of chondrosarcomas and chondroblastomas revealed multiple LOH events at loci on chromosomes 3q, 8q, 10q, and 19q. One sporadic chondrosarcoma demonstrated LOH for EXT1 and EXT3, while a second underwent LOH for EXT2 and chromosome 10. A third chondrosarcoma underwent LOH for EXT1 and chromosome 3q. These results agree with previous findings that mutations at EXT1 and multiple genetic events that include LOH at other loci may be required for the development of chondrosarcoma.

Introduction

Hereditary multiple exostoses (EXT) is an autosomal dominant condition that is associated with excessive bony growth (exostoses) at the ends of the long bones (Jaffe 1943; Solomon 1963). Decreased range of motion and pain are among the common complications of EXT (Schmale et al. 1994; Luckert-Wicklund et al. 1995). Malignancy is estimated to occur in <5% of EXT cases and commonly develops from a pelvic exostosis (Schmale et al. 1994; Luckert-Wicklund et al. 1995). Chondrosarcoma is the most frequently observed tumor, but osteosarcoma also is reported (Schmale et al. 1994; Luckert-Wicklund et al. 1995). No other tumors have been associated with EXT, and the overall health of individuals with EXT is good (Schmale et al. 1994; Luckert-Wicklund et al. 1995).

Three genetic loci have been identified, on chromosomes 8q24.1, 11p13, and 19q, which are designated "EXT1," "EXT2," and "EXT3," respectively (Cook et al. 1993; LeMerrer et al. 1994; Wu et al. 1994). The EXT1 gene recently has been cloned and sequenced, and the coding region is composed of 3.4 kb of cDNA encoded in 11 exons (Ahn et al. 1995). This gene has no homology to any other described gene (Ahn et al. 1995).

The EXT1 protein is ubiquitously expressed in many tissues, and the function of the protein is not known (Ahn et al. 1995). The only known effect of a mutated or inactivated EXT1 gene appears to be specific to actively growing bone. It has been postulated that mutations in this gene allow inappropriate bone growth to be juxtaposed to the growth plate. As the bone continues to grow, the exostoses appear to migrate toward the diaphysis, and new exostoses may arise from the growth plate until puberty. At puberty, with growth-plate fusion, linear growth ceases and new exostoses no longer develop.

Previous studies have demonstrated loss of heterozygosity (LOH) for EXT1 and EXT2 loci in chondrosarcoma tissue, and a multistep model of tumorigenesis has been suggested (Hecht et al. 1995). In this study, we have evaluated several EXT1-linked families for mutations in the EXT1 gene and have tested a series of chondrosarcomas and chondroblastomas for LOH and for mutations in the EXT1 gene.

Material and Methods

The intron/exon boundaries of EXT1 were determined in previous studies (Ahn et al. 1995). The primer

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Table 1

Primer Sequences for EXT1 Exons with Mutations

Exon	Primer Sequence
1:	
107-Forward	5′ tttatattccggcacttggc 3′
207-Reverse	5′ ccttctccatggactgtccct 3′
109-F	5′ ggtaccaaaacattctagcggc 3′
209-R	5′ aggctgggttgaaacta 3′
122-F	5′ gcttcgatttcacccttgc 3′
222-R	5′ aaatataaggccgtgaaccg 3′
2:	
114-F	5′ cccacattcgcaatgagtcttc 3′
214-R	5′ cttcggtctaattcacccaa 3′
6:	
106-F	5′ ctttccagcgcttcattagg 3′
206-R	5′ tctctgaggcgggaggaatg 3′

sequences used to amplify EXT1 exons 1, 2, and 6 are listed in table 1. The sequences for the remaining exons and the conditions for amplification of all exons are to be found in the work of Wells et al. (in press).

Six families demonstrating evidence of linkage to the EXT1 locus on chromosome 8 were described elsewhere in an EXT-linkage study (Blanton et al. 1996). These families are shown in figure 1. Their probabilities of linkage to the EXT1 locus are listed in table 2 (Blanton et al. 1996). One affected individual from each family was selected for mutation testing. If a mutation was identified in the affected individual, then all family members were tested to determine whether the mutation cosegregated with the disease phenotype.

Mutation testing was done by the sequencing of all exons, following PCR amplification, by use of the Sequenase PCR product–sequencing kit (United States Biochemical [USB]). To detect insertion/deletion mutations, the amplified exons were cloned into the pGEM-T vector (Promega Biochem) by following the manufacturer's instructions. Individual clones were then picked, and DNA was isolated and electrophoresed on denaturing polyacrylamide gels (Sambrook et al. 1989). The DNA from clones exhibiting a size variation were then sequenced by use of the Sequenase protocol (USB). Mutations were numbered according to the EXT1-cDNA numbering in the report by Ahn et al. (1995).

LOH was tested in two sporadic chondrosarcomas (individuals 9 and 10; table 3) and in two sporadic chondroblastomas (individuals 7 and 8; table 3). The short-tandem-repeat polymorphisms used were located on chromosomes 3q, 8q24.1, 10, 11p13, and 19q (table 4). In addition, the polymorphic loci from chromosomes 3q and 10 also were used to test for LOH in six previously ascertained and characterized chondrosarcoma samples (individuals 1–6; table 3), including two chondrosarcomas that had come from individuals with EXT (individuals 4 and 6; table 3) (Hecht et al. 1995). These six chondrosarcomas had been tested previously for LOH for chromosomes 8 and 11, and two of the individuals (2 and 6; table 3) had demonstrated LOH for chromosomes 8 and 11, respectively (Hecht et al. 1995).

Sequencing of EXT1 exons 1–11 was done for both the constitutional and tumor DNA from each of the patients in the LOH study. In addition, sequencing of EXT1 exons 1–11 was done on an EXT individual (individual 11; table 3) from a family that had been shown to have linkage to the EXT2 locus and whose chondrosarcoma previously had been shown to undergo LOH for both EXT1 and EXT2 (Hecht et al. 1995).

Results

Mutational Analysis

Four of the six families examined had mutations in the EXT1 gene, and all of these mutations segregated with the disease phenotype (table 2). A 1-bp deletion, 1364delC, in exon 1 was found in both family 2 and family 8 (fig. 2, *top*), which resulted in a frameshift and premature stop codon at nucleotide 1403. Family 5 had a 4-bp insertion, 1035ins4, also in exon 1, which resulted in a frameshift and premature stop codon at nucleotide 1213 (fig. 2, *middle*). Family 6 had a G1635T transversion in exon 2, which resulted in a missense Arg339Leu substitution (fig. 2, *bottom*). Complete sequencing of exons 1–11 of the EXT1 gene in families 3 and 13 did not reveal any mutation.

Mutations in EXT1 also were identified in two individuals with chondrosarcoma. A mutation in exon 6, 2077–2082insC, in the constitutional DNA was found in individual 6 (constitutional DNA), which resulted in a frameshift and premature stop codon at nucleotide 2208 (fig. 3). Interestingly, in a previous study, this tumor had been shown to undergo LOH for chromosome 8q24.1 (Hecht et al. 1995), but when the tumor DNA was analyzed, it was found to have retained the wild-type EXT1 allele. Analysis of other polymorphic loci confirmed that the tumor and the constitutional DNA were derived from the same individual.

The final EXT1 mutation was identified in the chondrosarcoma of individual 10. An 8-bp deletion, 1178del8, in exon 1 resulted in a frameshift and premature stop codon at nucleotide 1213 (fig. 4). This mutation did not appear in the constitutional DNA, which suggests that it was somatic in origin. No mutations in exons 1–11 of the EXT1 gene were detected in the other samples.

LOH analysis

Ten paired constitutional/tumor DNA samples were examined for LOH. The tumor from individual 10 was found to have undergone LOH for chromosomes 8q and

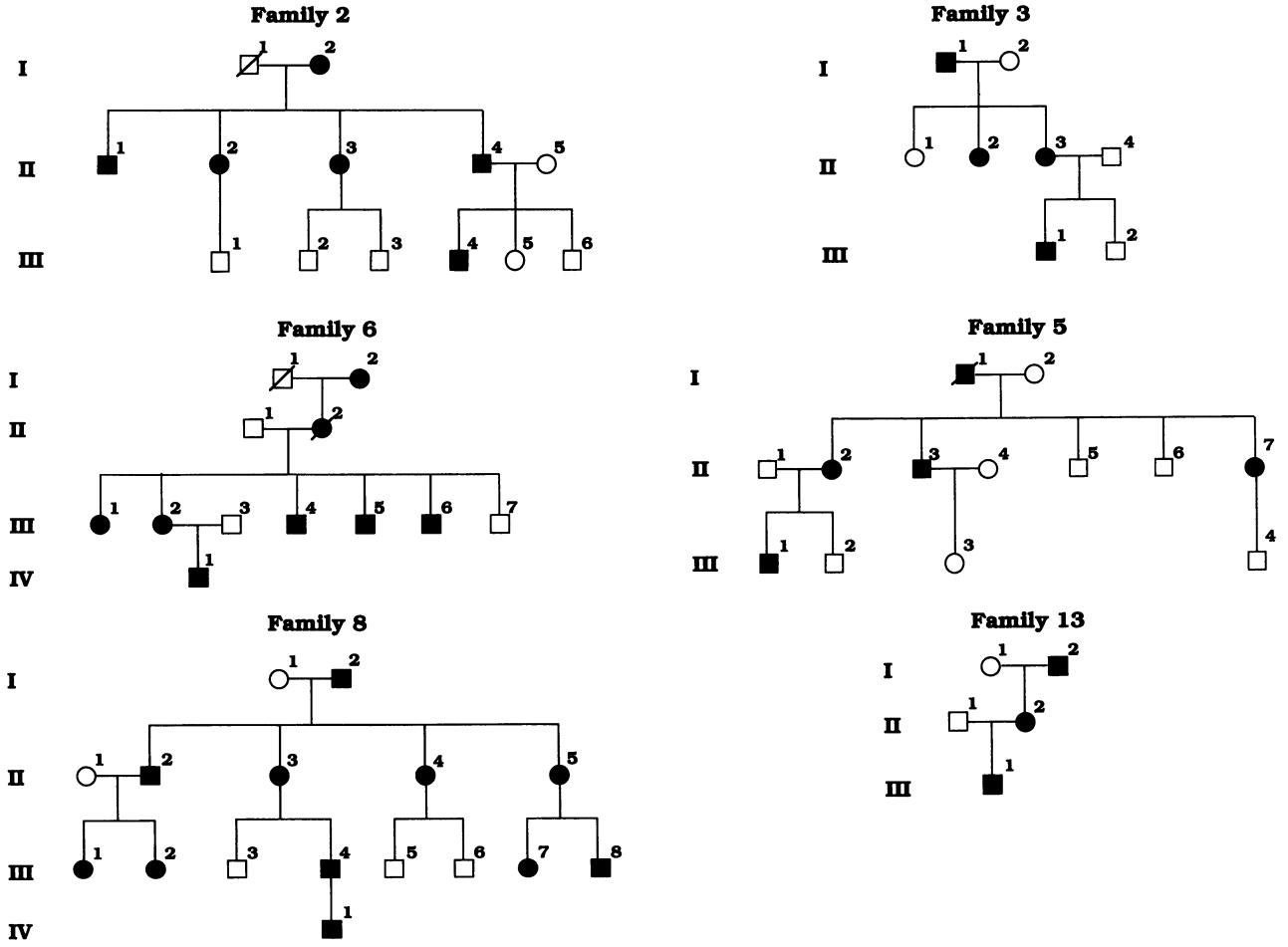

Figure 1 Pedigrees of EXT1-linked families that were studied. All blackened circles and squares indicate confirmed affected status.

19q (fig. 5). The tumor from individual 6 (chondrosarcoma DNA) was found to have undergone LOH for chromosome 3q (fig. 6). As discussed, this same individual previously had been found to have undergone LOH for chromosome 8q (Hecht et al. 1995) and had retained the wild-type allele at EXT1. The tumor from individual 2, which previously had been shown to have a homozygous deletion of the chromosomal region containing EXT2, also was found to have undergone LOH for the entire chromosome 10 (fig. 7). LOH was not observed in any of the other tumor samples.

Table 2

Familial Mutation Results, with Probability of Linkage to EXT1 Gene

Family	Probability of Linkage	Exon	Mutation	Amino Acid Substitution
2	1.00	1	1364delC	...
3	.99	...	...	...
5	1.00	1	1071ins4	...
6	.91	2	G1670T	Arg339Leu
8	1.00	1	1364delC	...
13	.99	...	...	...

Discussion

Germ-line mutations were identified in four families that demonstrated linkage to the EXT1 locus (Blanton et al. 1996), including two unrelated families that were found to have the same mutation. A fourth germ-line mutation was identified in an individual with a chondrosarcoma and a family history of EXT. A fifth mutation was found only in the tumor of a chondrosarcoma patient with no family history of EXT.

Four of the mutations identified resulted in frameshifts and premature termination codons, whereas the fifth mutation resulted in a substitution of leucine for arginine. A previous report had identified a 1-bp deletion that resulted in a frameshift and premature termination codon in exon 6 (Ahn et al. 1995). Thus, the

Table 3

Constitutional and Tumor DNA Mutational Results

Individual	FH[a]	Tissue[b]	Chromosomes Showing LOH	Exon	Mutation
1	−	CS			
2	−	CS	10, 11[c]		
3	−	CS			
4	+	CS			
5	−	CS			
6	+	CS	3, 8[d]		
6	+	C		6	2077–2082insC
7	−	CB			
8	−	CB			
9	−	CS			
10	−	CS	8, 19	1	1178del8
11	+	CS	8, 11[e]		

[a] FH = family history of EXT; a plus sign (+) denotes presence of EXT; and a minus sign (−) denotes absence of EXT.
[b] C = constitutional; CS = chondrosarcoma; and CB = chondroblastoma.
[c] Homozygous loss of chromosome 11 polymorphic markers from previous study by Hecht et al. (1995).
[d] LOH in chromosome 8 polymorphic markers from previous study by Hecht et al. (1995).
[e] Deletion D11S903 and LOH in chromosomes 8 and 11.

Table 4

Polymorphic PCR Markers and Allele Sizes

Chromosome and Marker	Allele Size (bp)
3q:	
D3S1246	110–128
D3S1282	140–154
D3S121	193–200
D3S1243	177–185
8q24.1:	
D8S85	74–84
D8S527	272–284
D8S522	203–217
D8S199	204–230
10:	
D10S249	118–134
D10S220	267–291
D10S109	82–98
D10S212	189–201
11p13:	
D11S905	208–228
D11S90	272–284
D11S554	203–217
D11S1313	204–230
19q:	
D19S216	179–191
D19S413	69–91
D19S221	191–211
D19S226	235–263

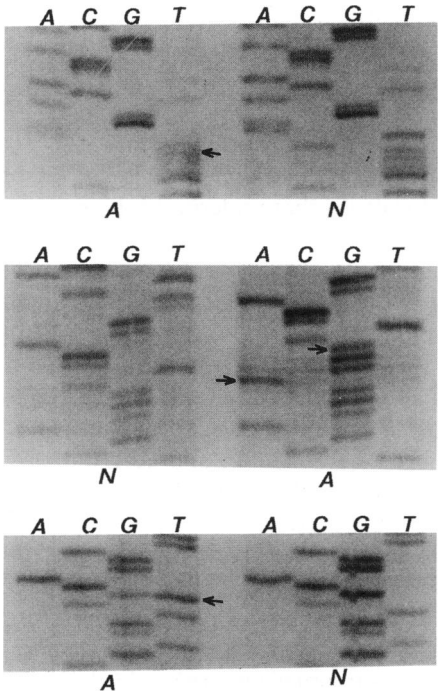

Figure 2 Familial mutations shown with cloned normal, *N*, and mutant, *A*, sequences from the same individual, for comparison. *Top*, 1329delC from family 2. The same mutation was identified in family 8. *Middle*, 1035ins4 in family 5. *Bottom*, G1635T substitution in family 6.

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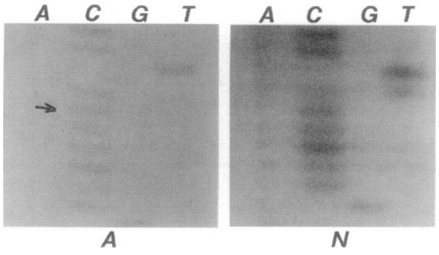

Figure 3 Cloned constitutional DNA sequence demonstrating insertion of a cytosine into one allele, *A*, and the normal allele sequence, *N*, for comparison.

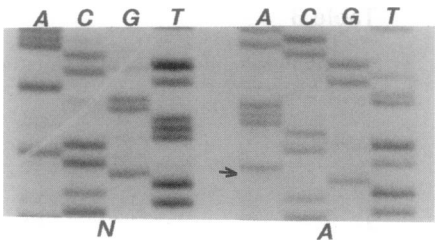

Figure 4 Normal constitutional DNA sequence, *N*, and chondrosarcoma demonstrating 8-bp deletion in exon 1, *A*, both from individual 10.

majority of identified mutations resulted in truncation of the protein product and could result in haploinsufficiency.

Mutations in families 3 and 13 were not detected in exons 1–11 of EXT1. Recombinants, in both families, excluded linkage to both the EXT2 locus and the EXT3 locus (Blanton et al. 1996). Family 13 provided only a very small LOD score for each chromosome 8q locus tested; however, this was due to small family size. LOD scores of >1 but <2 were obtained for family 3. Thus, in both families, the exclusion of EXT2 and EXT3 linkage and small positive scores for EXT1 created a high probability of linkage to EXT1. It is possible that the mutations in these families may lie within the promoter or may affect splice sites in the mRNA. Alternatively, a fourth, unidentified EXT locus may exist. Other analyses have identified EXT families in which all three known EXT loci have been excluded, which suggests that there is another, unidentified EXT locus (W. Raskind, unpublished data).

LOH studies revealed that there are at least five chromosomal locations that undergo LOH in chondrosarcomas and osteosarcomas. This finding is consistent with the location of tumor-suppressor genes that play a role in tumorigenesis. In previous studies, we demonstrated frequent LOH for chromosomes 8q and 11p (Hecht et al. 1995; Raskind et al. 1995). Our new data have demonstrated additional LOH events, on chromosomes 3q, 10, and 19q. LOH involving chromosomes 8q, 10p, and 11p has been described in sporadic and EXT-related chondrosarcomas (Hecht et al. 1995; Raskind et al. 1995). LOH for chromosome 3q has been described in sporadic osteosarcoma (Yamaguchi et al. 1992; Hecht et al. 1995; Raskind et al. 1995; R. P. Kruzelock, E. C. Murphy, L. C. Strong, and M. F. Hansen, unpublished data). Raskind et al. (1996) reported that 67% of isolated chondrosarcomas underwent LOH for at least one chromosome 10q locus. The RET proto-oncogene maps within the region of LOH, on chromosome 10q, that is common to all tested chondrosarcomas. Mutations in the RET proto-oncogene have been identified in multiple endocrine neoplasia types 2A and 2B, as well as in sporadic medullary thyroid carcinoma and Hirschsprung disease (Mulligan et al. 1993; Angrist et al. 1995; Attie et al. 1995; Bolino et al. 1995; Eng et al. 1995). LOH for chromosome 3q has been reported in 75% of sporadic osteosarcomas (Yamaguchi et al. 1992; R. P. Kruzelock, E. C. Murphy, L. C. Strong, and M. F. Hansen, unpublished data). The discovery of LOH for both 3q and 10q in an EXT-related chondrosarcoma suggests that loci within these regions may play a role in EXT tumor development.

One chondrosarcoma demonstrated LOH for the chromosome 19 region containing the EXT3 locus, as well as LOH for the chromosome 8q region containing the EXT1 locus. Raskind et al. (1995, 1996) also reported LOH for chromosome 19 loci in 2 of 20 chondrosarcomas. These results, together with linkage data from previous studies (LeMerrer et al. 1994), provide additional support for the involvement of chromosome 19

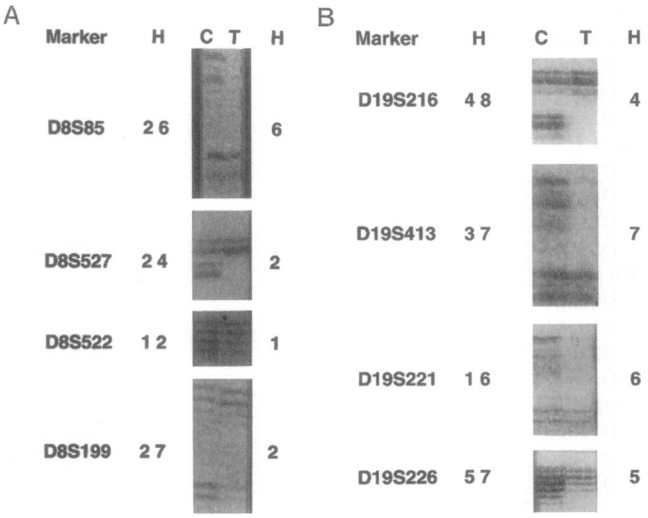

Figure 5 Constitutional (lane C) and chondrosarcoma (lane T) DNA pair demonstrating LOH for *(A)* chromosome 8 polymorphic markers D8S85, D8S527, and D8S199 and *(B)* chromosome 19 polymorphic markers D19S216, D19S413, D19S221, and D19S226. H = haplotype.

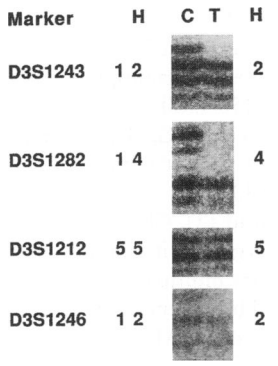

Figure 6 Constitutional (lane C) and chondrosarcoma (lane T) DNA pair demonstrating LOH for chromosome 3 polymorphic markers D3S1243, D3S1282, and D3S1246. D3S1212 was not polymorphic. H = haplotype.

in EXT. The discovery of a somatic mutation in the EXT1 gene, as well as LOH for chromosome 19, suggests a model in which multiple genetic events may be necessary for tumor development.

Finally, a mutation in exon 6 of the EXT1 gene was found in the constitutional DNA of an individual with EXT; however, the mutation was not found in the tumor DNA, which had undergone LOH for the chromosome 8q region containing the EXT1 gene. Analysis of other polymorphic loci confirmed that the constitutional and tumor DNA were derived from the same individual. The retention of the normal allele was an unexpected finding, on the basis of other models of tumorigenesis, such as retinoblastoma, in which the mutant allele is retained. However, if multiple genetic events are required for tumorigenesis, and since we do not know the order in which these multiple events must occur, it is possible to propose a model in which a predisposing mutation is lost subsequent to other tumorigenic events. Consistent with this model is the observation that the tumor also had undergone LOH for chromosome 3q, which suggests that multiple genetic events had occurred during tumorigenesis.

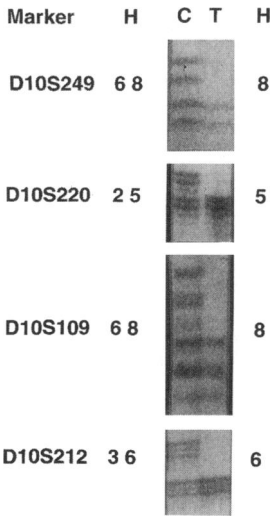

Figure 7 Constitutional (lane C) and chondrosarcoma (lane T) DNA pair demonstrating LOH for chromosome 10 polymorphic markers D10S249, D10S220, D10S109, and D10S212. H = haplotype.

In summary, exostoses may arise either on a familial background or sporadically. Our data suggest that the development of exostoses is dependent on mutations that inactivate both alleles at an EXT locus. Our model for the development of exostoses predicts that each exostosis arises from a cell in which the second mutation has occurred in the other allele at the predisposing EXT locus. This would allow for initiation of inappropriate growth. Thus, in inherited cases, exostoses would arise early because the first mutation is inherited. Differential timing of acquisition of a second mutation would account for the asymmetry that is observed when bony involvement is compared, even though almost every bone may show involvement in inherited cases. A sporadic exostosis would arise only after two mutations had occurred in the same cell. This would account for the later occurrence and sparsity of exostoses in sporadic cases. Evidence to support this hypothesis has been provided in an isolated exostosis in which both copies of chromosome 8 were rearranged (Ogle et al. 1991). One copy was deleted at 8q22–8qter, and the other chromosome was disrupted by a translocation involving 8q24.1.

It follows that the development of malignancy in EXT would depend on the inactivation of one or more additional EXT loci, loss of other chromosomal regions containing tumor-suppressor genes, or mutations in protooncogenes. The mechanism by which these events affect the growing bones and lead to malignancy will provide fruitful areas of future research.

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