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EXT-Mutation Analysis and Loss of Heterozygosity in Sporadic and Hereditary Osteochondromas and Secondary Chondrosarcomas

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Summary

Osteochondromas occur as sporadic solitary lesions or as multiple lesions, characterizing the hereditary multiple exostoses syndrome (EXT). Approximately 15% of all chondrosarcomas arise within the cartilaginous cap of an osteochondroma. EXT is genetically heterogeneous, and two genes, *EXT1* and *EXT2*, located on 8q24 and 11p11-p12, respectively, have been cloned. It is still unclear whether osteochondroma is a developmental disorder or a true neoplasm. Furthermore, it is unclear whether inactivation of both alleles of an *EXT* gene, according to the tumor-suppressor model, is required for osteochondroma development, or whether a single *EXT* germline mutation acts in a dominant negative way. We therefore studied loss of heterozygosity and DNA ploidy in eight sporadic and six hereditary osteochondromas. *EXT1*- and *EXT2*-mutation analysis was performed in a total of 34 sporadic and hereditary osteochondromas and secondary peripheral chondrosarcomas. We demonstrated osteochondroma to be a true neoplasm, since aneuploidy was found in 4 of 10 osteochondromas. Furthermore, LOH was almost exclusively found at the *EXT1* locus in 5 of 14 osteochondromas. Four novel constitutional cDNA alterations were detected in exon 1 of *EXT1*. Two patients with multiple osteochondromas demonstrated a germline mutation combined with loss of the remaining wild-type allele in three osteochondromas, indicating that, in cartilaginous cells of the growth plate, inactivation of both copies of the *EXT1* gene is required for osteochondroma formation in hereditary cases. In contrast, no somatic *EXT1* cDNA alterations were found in sporadic osteochondromas. No mutations were found in the *EXT2* gene.

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Introduction

Osteochondromas (or osteocartilaginous exostoses; MIM 133700), defined as cartilage-capped bony protuberances developing from the juxta-epiphyseal regions of the long bones, may occur as sporadic solitary lesions or multiple lesions, characterizing the hereditary multiple exostoses syndrome (EXT). EXT is an autosomal dominant skeletal disorder that, apart from the development of multiple osteochondromas, is characterized by deformities of the forearm and disproportionate short stature. The histogenesis of osteochondroma is still a matter of debate. Many consider it a perversion in the direction of bone growth, resulting from aberrant epiphyseal development, instead of a true neoplasm (Huvos 1991; Dorfman and Czerniak 1998). However, cytogenetic aberrations have been detected (Mertens et al. 1994; Bridge et al. 1998), suggesting a clonal neoplastic growth.

The most serious complication of EXT is sarcomatous degeneration, which is estimated to occur in <5% of cases (Schmale et al. 1994; Wicklund et al. 1995). In 90% of cases, malignant transformation occurs within the cartilaginous cap, leading to a secondary peripheral chondrosarcoma (Willms et al. 1997). Approximately 15% of all chondrosarcomas are documented to arise peripherally and secondary to an osteochondroma, whereas 75% arise centrally in bone, usually without a benign precursor (Mulder et al. 1993; Springfield et al. 1996).

EXT is genetically heterogeneous, and, at present, two genes, *EXT1* and *EXT2*, located on 8q24 and 11p11-p12, respectively, have been cloned (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). Furthermore, linkage to chromosome 19p has been found, suggesting the existence of an *EXT3* gene (Le Merrer et al. 1994). A tumor-suppressor function has been suggested (Hecht et al. 1995; Raskind et al. 1995), on the basis of loss of heterozygosity (LOH) in chondrosarcomas. Recently, the *EXT1* and *EXT2* gene products were shown to be glycosyltransferases required for the biosynthesis of he-

paran sulfate (Lind et al. 1998; McCormick et al. 1998). Furthermore, an *EXT1* homologue in *Drosophila* (*toutvelu*) was demonstrated to be required for diffusion of the morphogen Hedgehog (Bellaiche et al. 1998).

Knudson (1971) postulated the two-hit model for tumor-suppressor–gene inactivation. Both copies of the gene must be inactivated to abolish the normal tumor-suppressor activity. In hereditary cancer, the first hit usually consists of a germline mutation, which has been extensively studied in EXT (Ahn et al. 1995; Stickens et al. 1996; Hecht et al. 1997; Philippe et al. 1997; Wuyts et al. 1998). The second hit is the inactivation of the remaining wild-type copy, which occurs somatically, often either by deletion of part or all of the chromosomal arm that harbors the gene or by mitotic recombination. This results in LOH, detectable with polymorphic markers located in this chromosomal region. Analogously, in sporadic tumors, LOH and mutational inactivation of the retained allele are observed.

LOH at the *EXT* loci has been studied and detected in chondrosarcomas only (Hecht et al. 1995; Raskind et al. 1995). It is therefore still unclear whether the complete inactivation of the *EXT* gene—and, thus, a second somatic hit—is required for osteochondroma development, or whetehr a single *EXT* germline mutation acts in a dominant-negative way causing multiple benign osteochondromas. In the latter case, inactivation of the remaining allele is a prerequisite for malignant transformation. To address this, we performed a study of LOH in eight sporadic and six hereditary osteochondromas in combination with *EXT1*- and *EXT2*-mutation analysis in a total of 34 sporadic and hereditary osteochondromas and secondary chondrosarcomas.

Material and Methods

Material

Tumor tissues were retrieved from the files of the Leiden University Medical Center, a bone-tumor referral center. Fresh-frozen cartilaginous tumor tissue was available for five cases of multiple osteochondromas of three patients and for eight sporadic osteochondromas. Clinical data regarding multiplicity of skeletal lesions and family history were obtained by review of clinical charts. If data were inconclusive, patients were asked to complete a questionnaire, for which the response rate was 83%. An unequivocal family history was not always available. The mean ages at operation were 20.5 years (range 12.4–48.8 years) for sporadic cases (five males and three females) and 24.8 years (range 20.7–27.0 years) for hereditary cases (three males). Of the three patients with multiple osteochondromas, two had a negative family history. Blood of their relatives was not available. Additionally, fresh-frozen tumor tissue of 23 chondrosarcomas secondary to osteochondroma was used for mutation analysis. Both data from patients with peripheral chondrosarcoma and LOH data have been described previously by Bovée et al. (in press). Of 20 patients, 6 demonstrated multiple osteochondromas, 3 of whom had an unequivocal positive family history for EXT. For three other patients, multiplicity of osteochondromas could not be determined.

DNA Isolation

DNA isolation of fresh-frozen tissue was performed with proteinase-K digestion and phenol-chloroform extraction, as described by Devilee et al. (1989), with some modifications: before proteinase-K digestion, cartilaginous tumor tissue was preincubated in a 3 M NaAc buffer (pH 5.6), saturated with hyaluronidase, for 2 h at 37°C. Then, the pH was adjusted by addition of $\frac{1}{8}$ vol 2 M NaOH. DNA from one osteochondroma of patient 428 was derived from cultured cells. For four osteochondromas, the tumor-cell fraction was purified for LOH analysis by microdissection. Haematoxylin- and eosin-stained cryostat sections were visually examined, and fields derived from the cartilaginous cap of the osteochondroma were scraped from 20 adjacent, stained, uncovered 10-μm cryostat sections, with use of a scalpel or syringe. Sections were fixed in methanol 10 min before staining. DNA from this microdissected tissue was isolated as described by Isola et al. (1994). DNA from freshly collected blood samples obtained, with informed consent, from the patients and 96 normal healthy individuals was isolated with a salting-out procedure (Miller and Polesky 1988).

EXT-Mutation Analysis

Mutation analysis was performed on tumor DNA from 11 patients with osteochondromas (range 40%–90% for tumor percentages) and from 23 patients with chondrosarcomas (range 50%–100%). Only one osteochondroma from each patient with multiple osteochondromas was included.

PCR

For exon 1 of the *EXT1* gene, five overlapping fragments were amplified, whereas the other exons were covered with single PCR fragments as described by Wells et al. (1997). Thermal cycling was performed in a programmable heat block (Perkin-Elmer-Cetus) with a "touchdown" PCR program of 47 cycles consisting of 2 cycles with annealing at 65°C, 2 cycles at 64°C, 2 cycles at 63°C, 2 cycles at 62°C, 2 cycles at 61°C, 2 cycles at 60°C, 4 cycles at 59°C, 6 cycles at 58°C, and 25 cycles at 57°C. Some modifications were made, as

Table 1

Constitutional *EXT1* cDNA Alterations Found in Four Patients

Parameter	Patient 282	Patient 428	Patient 427	Patient 17
Diagnosis	Chondrosarcoma	Osteochondromas	Osteochondromas	Chondrosarcoma
Multiple osteochondromas?	No	Yes	Yes	Inconclusive
Family history	Negative	Positive	Negative	Inconclusive
cDNA change	A117G	G490C	702-717delCCCCTCTTTTCTAAG	A947G
Protein change	E39E	D164H	PLFSK235-239del	N316S
Type of mutation	Silent	Missense	In-frame deletion	Missense
Method of confirmation	Restriction *Bsi*HKAI	Hybridization	4% Agarose gel	Hybridization
Frequency in normal individuals	0/92	0/89	0/96	0/92
LOH?	Yes	Yes	Yes[a]	No

[a] LOH detected in one of two osteochondromas.

follows: primers Apr111 and 211 were replaced by primers CAGGCAGGACACATGCAG and CTGGAATCCTCGTTTTCCAA, generating a product of 242 bp, with thermal cycling consisting of the touchdown PCR program decreasing to 59°C instead of 57°C. Primers Apr 109 and 209 were replaced by GCTCCAGGTTCTACACCTCG and TACTGATGCTGGCTTTGGC, and 40 cycles with a constant annealing temperature of 63°C were applied, generating a product of 249 bp. PCR was performed in a total vol of 25 μl containing 15 pmol of each primer, 0.2 mM dNTP (Pharmacia), 50 mM KCl, 10 mM Tris (pH 8.3), 0.2 mg BSA/ml, 2 mM $MgCl_2$, 1 U Ampli*Taq* or Ampli*Taq* GOLD (Perkin-Elmer), and 1 μCi $\alpha[^{32}P]$-dCTP (Amersham).

Amplification of the *EXT2* exons was performed with the primers and PCR conditions described elsewhere (Wuyts et al. 1998). Both primers were ^{32}P labeled with T4 polynucleotide kinase (Pharmacia), and ^{32}P-labeled dCTP was incorporated during the amplification process.

SSCP Analysis

SSCP analysis of the *EXT1* gene was performed on a gel with the GeneAmp Detection Gel kit (Perkin-Elmer), according to the manufacturer's recommendations, for 4 h at 12 W at room temperature. Additionally, samples were mixed with an equal volume of formamide-loading dye (90% formamide, 10 mM EDTA [pH 8.0], 0.3% bromphenol blue, and 0.3% xylene-cyanol), denatured for 5 min at 95°C, and loaded onto a gel consisting of 10% glycerol, 6% polyacrylamide, and 1 × Tris-borate EDTA, for 6 h at 25 W at room temperature. Finally, a 6.5% polyacrylamide gel containing 7 M urea was run under denaturing conditions, to study small insertions or deletions. SSCP analysis of the *EXT2* gene was performed on an MDE polyacrylamide gel (FMC) with 10% glycerol for 16 h (500 V) at room temperature. Gels were dried and exposed to x-ray films. PCR and SSCP analysis were repeated on samples showing aberrant bands, and constitutional DNA from the same patient was also analyzed.

Sequencing Analysis

PCR was repeated, without $\alpha[^{32}P]$-dCTP, on samples showing aberrant bands on SSCP analysis, and PCR products were purified with MicroSpin G-50 columns (Pharmacia Biotech). Subsequent sequencing reactions were performed, with the same primers and use of the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's recommendations (Perkin-Elmer Applied Biosystems). Both strands were sequenced. Samples were run on the ABI 377 semiautomated sequencer (Perkin-Elmer). Sequences were analyzed with Factura 2.0.1 software (Perkin-Elmer) and were aligned with each other, and the normal cDNA sequence (Ahn et al. 1995) electronically by ClustalW multiple-sequence alignment (EMBL Outstation European Bioinformatics Institute). Nucleotide numbering was done on the basis of the sequence provided by Ahn et al. (1995), and mutation nomenclature was according to the Nomenclature Working Group.

Testing for Sequence Variations

To confirm cDNA alterations and to exclude that these alterations are polymorphisms occurring in the normal population, PCR-fragment analysis was performed on normal and tumor DNA of the patient and on peripheral-blood DNA from 89–96 normal healthy blood donors, by use of either oligonucleotides designed to contain the mismatch, restriction-enzyme analysis, or gel electrophoresis (table 1). PCR products of *EXT1* fragments containing the mutated sequence (the third and fifth fragment of exon 1) were blotted on nylon membranes, as described by Devilee et al. (1991*b*). Oligonucleotides TTTAGACAGACACCAGTTGT (G490C) and GACAGAGACAGCACCGAGTA (A947G) were hybridized on the blots at 50°C and 60°C, respectively. Restriction-enzyme analysis was performed to study

A117G by use of 2 μl PCR product (the first fragment of exon 1) that was incubated with 2 U *Bsi*HKAI (New England BioLabs), and fragments were separated onto 1.5% agarose gels. The enzyme could only digest when the cDNA alteration was present.

To study 702-717delCCCCTCTTTTCTAAG, PCR products (the fourth fragment of exon 1) were electrophoresed on an agarose gel containing 3% NuSieve GTG (FMC BioProducts) combined with 1% Seakem GTG (FMC BioProducts).

LOH Study

Analysis of microsatellite markers was performed by PCR on 100 ng DNA from each of 14 osteochondromas from 11 patients, as described by Weber and May (1989), with 1 μCi α[^{32}P]-dCTP in a total vol of 12 μl. Thermal cycling was performed in a programmable heat block (MJ research) consisting of 27 cycles with an annealing temperature of 55°C. For PCR on microdissected DNA, 33 cycles were applied. Apart from loci harboring the *EXT* and *EXT*-like regions, primers were chosen in regions described to be involved in chondrosarcoma, including the p53 and Rb regions (Yamaguchi et al. 1996) at 9p21 (Jagasia et al. 1996) and at chromosome 10 (Raskind et al. 1996) (table 2). Polymorphic markers are described in the Genome Database. After electrophoresis on a 6.5% polyacrylamide gel containing 7 M urea, gels were dried and exposed to x-ray films. Signal intensities were measured by Phosphor Imaging (Molecular Dynamics; see fig. 1). LOH was scored when the quotient of the ratios of both alleles of normal and tumor samples was ⩾1.7 (Gruis et al. 1993). Ratios of 1.3–1.7 were regarded as inconclusive and were excluded from the analysis (Devilee et al. 1991*a*). LOH had to be reproducible to be included.

Flow Cytometry

DNA-flow cytometry was performed on 12 osteochondromas from 11 patients. Single-cell suspensions from fresh-frozen tissue for single-parameter nuclear-DNA flow cytometry (FCM) were prepared by use of the method of Vindelov et al. (1983*a*). A minimum of 10,000 cells/sample was measured on an FACSCalibur flow cytometer (Becton Dickinson). Trout red blood cells served as an internal standard for determination of the $G_{0,1}$-cell DNA content (Vindelov et al. 1983*b*). ModFitLT V2.0 software was used for data acquisition. DNA histograms were evaluated according to accepted criteria (Hiddemann et al. 1984).

Results

EXT Mutation Analysis

cDNA alterations were detected exclusively in exon 1 of the *EXT1* gene in 4 (12%) of 34 samples (table 1 and fig. 1). Two (22%) of nine patients demonstrating multiple osteochondromas showed a constitutional *EXT1* mutation. In addition, one patient with sporadic chondrosarcoma and one patient with chondrosarcoma for whom EXT status was unclear demonstrated constitutional *EXT1* cDNA alterations. No somatic alterations were found.

The aberrant bands, displayed in figure 1, were detected by both nondenaturing gel conditions. Denaturing gel electrophoresis demonstrated an additional band in patient 427 (fig. 1).

A DNA sequence variant (GAA→GAG in codon 39) that did not result in an amino acid change (Glu→Glu) was found in a sporadic peripheral chondrosarcoma-tumor sample from patient 282 and was also heterozygously present in DNA extracted from peripheral blood from the same patient. This variant, which can be detected by *Bsi*HKAI digestion of the first fragment of exon 1, proved to be rare, since it was not found in the 33 other tumor samples or in 92 normal individuals. However, the GAG codon is more frequent in human than is the GAA codon (DNA Information and Stock Center), thus excluding the possibility that this alteration makes transcription of the DNA sequence more difficult (Conrad et al. 1983). Combined with the lack of an EXT phenotype, this finding implies that this silent alteration is not disease causing.

A heterozygous constitutional missense mutation, GAC→CAC (Asp→His), was found in codon 164 of patient 428, demonstrating EXT with a positive family history. A heterozygous constitutional 15-bp deletion of codons 235–239, deleting Pro-Leu-Phe-Ser-Lys from the protein, was detected in patient 427, demonstrating multiple osteochondromas with a negative family history. Finally, in codon 316, a heterozygous missense alteration, AAC→AGC (Asn→Ser), was found in constitutional DNA from patient 17, with peripheral chondrosarcoma. This patient did not demonstrate an obvious EXT phenotype and had a family history of "knee operations." None of these alterations was detected in DNA from 89–96 normal individuals.

LOH Analysis

LOH was detected by microsatellite analysis in six osteochondromas, consisting of four sporadic and two hereditary cases (table 2 and fig. 2). Five (36%) of 14 osteochondromas showed LOH restricted to chromosome 8q24, where the *EXT1* gene is located. In two sporadic cases LOH was found only for marker D8S522,

Table 2

Microsatellite Markers with Chromosomal Localization, and Percentage of Heterozygosity

	Marker Information		Frequency of Osteochondromas with LOH [a]	
Gene and Marker	Location	Heterozygosity (%)	Sporadic (n = 8)	Hereditary (n = 6)
EXT1:				
D8S85	8q23.3	.74	0/5	1/6
D8S547	8q24.11	.66	1/5	2/4
D8S522	8q24.12-13	.71	3/6	2/6
D8S198	8q24.13	.83	1/6	1/3
EXT2:				
D11S905	11p13-p12	.74	0/7	0/1
D11S903	11p13-q13	.74	0/4	0/4
D11S554	11p11.2-12	.91	0/7	0/4
EXT3:				
D19S216	19pter-qter	.75	0/4	0/4
D19S413	19pter-qter	.76	0/7	0/2
D19S221	19p13.2	.86	0/7	0/4
EXTL1:				
D1S436	1p36	.75	0/6	0/4
D1S470	1p36	.76	0/7	0/5
EXTL2:				
D1S206	1p11-12	.82	0/4	0/5
D1S248	1p11-12	.82	1/7	0/3
EXTL3:				
D8S1130	8p12-p22	.93	0/7	0/3
GATA119C06	8p12-p21		0/5	0/4
D8S283	8p12-p21	.78	0/6	0/4
D8S1820	8p	.73	0/6	0/6
p16/p15:				
D9S43	9p21	.83	0/4	0/2
D9S171	9p21	.80	0/5	0/2
10:				
D10S89	10pter-p11.2	.80	0/7	0/6
D10S604	10pter-qter	.66	0/6	0/4
D10S538	10pter-qter	.73	0/4	0/3
D10S109	10q11.2-qter	.71	0/8	0/6
D10S110	10q11.2-qter	.58	0/4	0/0
Rb:				
D13S153	13q14.1-14.3	.82	0/7	0/4
D13S155	13q14.3-q21.2	.83	0/7	0/6
p53:				
TP53	17p13.1	.69	0/7	0/6
D17S513	17p13.3	.89	0/4	0/3

[a] Frequencies of LOH for osteochondromas are given as the no. of tumors showing LOH/the no. of informative cases.

with retention of adjacent markers, whereas in one hereditary and one sporadic case the whole chromosome arm was involved (table 3). One additional osteochondroma demonstrated LOH at D1S248, a marker flanking the *EXTL2* gene. Four sporadic and four hereditary osteochondromas showed retention of heterozygosity at all loci tested. Apart from the loci at 8q and 1p, none of the other loci demonstrated LOH in osteochondroma. In total, three (38%) of eight sporadic and two (33%) of six hereditary osteochondromas demonstrated LOH at 8q24.

In the two EXT patients demonstrating germline cDNA alterations, loss of the wild-type allele was demonstrated in three of four osteochondromas. In patient 428, sequence analysis demonstrated the predominance of the mutated allele—and, thus, loss of the wild-type allele—in both osteochondroma samples, compared with normal DNA (fig. 1). LOH at D8S547 and D8S522 was found in one of them (fig. 2). In patient 427, one of two osteochondromas demonstrated loss of the wild-type allele (fig. 1), on the denaturing gel, and LOH, on microsatellite analysis. The chondrosarcoma of patient

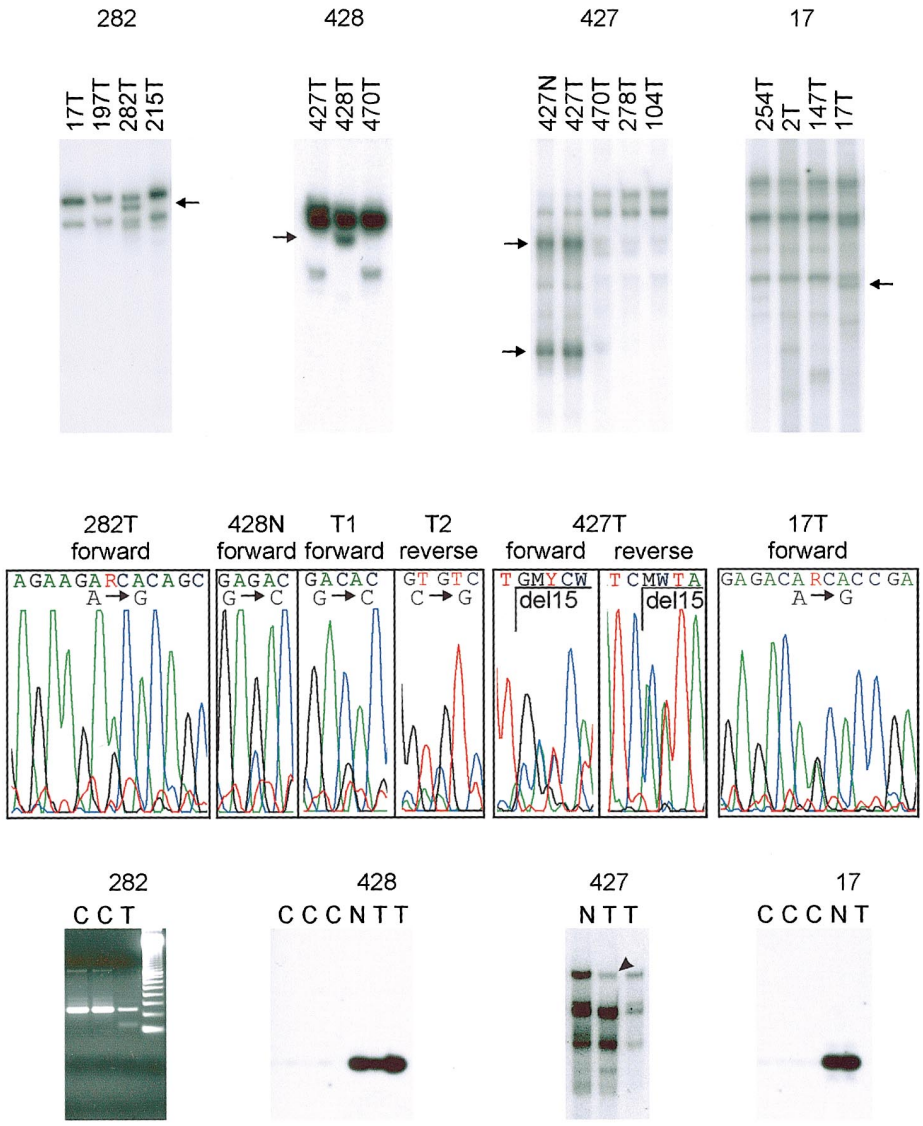

Figure 1 *Upper panel,* aberrant bands (indicated by arrows) detected by SSCP analysis in both normal (only shown for patient 427) and tumor DNA of four patients. *Middle panel,* corresponding sequence data. In patient 428, both normal and tumor DNA are shown, which illustrates the predominance of the mutated allele and thus loss of the wild-type allele in both osteochondromas. *Lower panel,* confirmation of cDNA alterations and screening of normal individuals. The A117G variant of patient 282 generated a *Bsi*HKAI restriction site. Normal DNA of this patient could also be digested (not shown). The mutations G490C (patient 428) and A947G (patient 17) were confirmed by oligo hybridization. For patient 427, the PCR fragment containing the mutation was run on a denaturing gel, illustrating loss of the wild-type allele (arrow) in one of two osteochondromas. Abbreviations: N = normal, T = tumor DNA, and C = control DNA from normal individual.

282 demonstrated LOH on microsatellite analysis, whereas the chondrosarcoma of patient 17 did not show LOH on either microsatellite analysis or sequence analysis.

DNA Flow Cytometry

Four of 10 osteochondromas showed DNA aneuploidy. DNA indices are listed in table 3. For two osteochondromas, adequate DNA histograms could not be obtained because of low cellularity. Six osteochondromas showed a diploid DNA content. Three osteochondromas showed an aneuploid DNA content, with DNA indices of 1.14, 1.17, and 1.2. One osteochondroma demonstrated a hypodiploid (DNA index 0.88) as well as a hyperdiploid (DNA index 1.13) clone.

Discussion

Until now, it has been unclear whether an osteochondroma is a developmental disorder or a true neoplasm.

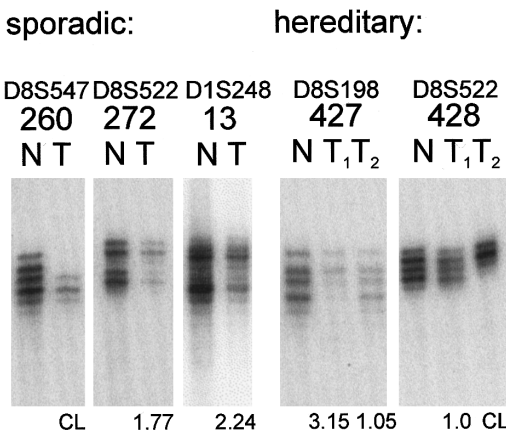

Figure 2 Examples of autoradiograms on LOH analysis. Microsatellite markers are indicated above each panel. Allelic-imbalance ratios, as measured by phosphor imaging, are indicated below each panel. For patient 260, tumor DNA was microdissected, and for patient 428, T_2 tumor DNA was isolated from cultured cartilaginous tumor cells. In both hereditary cases LOH was found in one of two osteochondromas, by microsatellite analysis. Abbreviations: N = normal DNA, T = tumor DNA, and CL = complete loss.

Furthermore, it is unknown whether the complete inactivation of an *EXT* gene, according to the tumor-suppressor model, is required for osteochondroma development, or whether a single *EXT* germline mutation acts in a dominant-negative way causing multiple benign osteochondromas. In the latter case, inactivation of the remaining allele would be a prerequisite for malignant transformation. The current study provides substantial proof that osteochondroma is a true neoplasm and that *EXT1* is compatible with the tumor-suppressor–gene model in osteochondroma. We studied LOH and DNA ploidy in eight cases of sporadic and in six cases of multiple osteochondromas. Furthermore, the *EXT1* and *EXT2* genes, which were reported to show linkage in ~70% of the EXT families (Legeai-Mallet et al. 1997*a*; Raskind et al. 1998), were screened for mutations in a total of 34 sporadic and hereditary osteochondromas and secondary peripheral chondrosarcomas.

Osteochondroma is generally considered a perversion in the direction of bone growth, resulting from aberrant epiphyseal development with displacement of physeal cartilage subsequently growing at right angles to the bone, instead of as a true neoplasm. This is because the lesion is formed by enchondral ossification and because the bone substance produced is in every way normal (Huvos 1991; Dorfman and Czerniak 1998). In the present study, however, we show osteochondroma to be a true neoplasia, since the presence of LOH in 6 of 14 and of aneuploidy in 4 of 10 osteochondromas indicates a clonal origin for the cartilaginous tissue of osteochondroma. Also, in the literature, clonal karyotypic abnormalities have been described (Mertens et al. 1994; Bridge et al. 1998). It remains unclear, however, whether cells forming the stalk of osteochondroma are also neoplastic or are reactive.

EXT1-mutation analysis was performed by running PCR fragments, of size 203–271 bp, on two nondenaturing gels with different SSCP conditions, to improve the detection rate (Sheffield et al. 1993). However, all band shifts were detected under both conditions. Therefore, the *EXT2* fragments were analyzed under only one condition. Four constitutional cDNA alterations of the *EXT1* gene were found. Of nine patients demonstrating multiple osteochondromas, only two (22%) demonstrated a constitutional *EXT1* mutation, whereas no *EXT2* mutations were found. This is lower than what would be expected on the basis of linkage (Legeai-Mallet et al. 1997*a*). However, an adequate family history was not always available, and in only five of nine cases with multiple osteochondromas was an unequivocal positive family history reported, although EXT is considered to have a high, although incomplete, penetrance (Wicklund et al. 1995; Legeai-Mallet et al. 1997*b*). Therefore, the remaining four patients may also have multiple osteochondromas in a setting other than familial EXT, such as Langer Gideon syndrome. In addition, the low mutation incidence may be explained in part by the 70%–90% sensitivity of SSCP mutation analysis. It has also been reported that analysis of the open reading frame of *EXT1* does not always result in the identification of a mutation in families demonstrating linkage to 8q24 (Hecht et al. 1997; Wells et al. 1997).

The cDNA alterations found in the present study have not been described previously (Wuyts et al. 1998 review all mutations). Remarkably, all alterations were located in exon 1, whereas only 40% of described mutations occur in exon 1 (Wuyts et al. 1998). Furthermore, in contrast to most *EXT1* alterations described—80% of which result in a truncated protein and 20% of which are missense—no frameshifts leading to truncation were found in the present study. This may be due to the population of both sporadic and hereditary osteochondromas and chondrosarcomas, which is different from the populations comprising exclusively patients with EXT that have been analyzed so far.

Evidence that both copies of the *EXT1* gene need to be inactivated to allow the development of an osteochondroma is provided by the two patients with EXT in the present study, demonstrating, in three of four osteochondromas, *EXT1* germline mutations combined with loss of the wild-type allele. One osteochondroma failed to show LOH, indicating that a small somatic mutation could have inactivated the wild-type allele. The amount of DNA from fresh-frozen tumor tissue available from this osteochondroma was not enough to allow us to detect this possible mutation.

LOH found exclusively at 8q24 in 5 of 14 osteo-

Table 3

Results of *EXT1*-Mutation Analysis, LOH Analysis, and DNA-Flow Cytometry for 11 Patients with Osteochondroma

Patient	Multiple Osteochondromas	*EXT*1 Mutation	LOH at Marker[a]				LOH[b] (%)	DNA Index
			D8S85	D8S547	D8S522	D8S198		
272	No	No	R	R	L	R	8	…
260	No	No	NI	L	L	L	8	1.0
165	No	No	R	R	NI	R	0	1.20
425	No	No	R	NI	NI	R	0	1.14
13	No	No	NI	NI	R	NI	8	1.0
396	No	No	R	R	R	R	0	1.0
481	No	No	R	R	L	R	8	1.0
484	No	No	NI	…	R	NI	0	.88+1.13
277 (n = 2)	Yes	No	R	NI	R	NI	0	1.0
428 (n = 2)[c]	Yes	Yes	R	L	L	…	8	…
427 (n = 2)[c]	Yes	Yes	L	L	L	L	8	1.17

Note.—LOH and ploidy data for peripheral chondrosarcomas were described previously by Bovée et al. (in press). Abbreviations: L = LOH, R = retention of heterozygosity, and NI = homozygous for the marker investigated.

[a] For loci 8q24.

[b] For all loci. Percentage of LOH was calculated, per tumor, as the percentage of loci showing LOH.

[c] Only the osteochondroma showing LOH on microsatellite analysis is given.

chondromas points to an important role for the *EXT1* gene in the development of osteochondromas. Cytogenetic abnormalities involved 8q22-24.1 in 10 of 30 sporadic and in 1 of 13 hereditary osteochondromas (Mertens et al. 1994; Bridge et al. 1998). In contrast, the *EXTL2* locus was the only other locus demonstrating LOH in one sporadic osteochondroma, whereas in the literature a deletion of 11p11-13 was observed in one sporadic osteochondroma (Bridge et al. 1998). This suggests that, in contrast to hereditary cases—in which all three *EXT* loci may be involved with a slight predominance of *EXT1* over *EXT2* and *EXT3* (Legeai-Mallet et al. 1997*a*; Raskind et al. 1998)—in sporadic cases almost exclusively *EXT1* is involved.

If the tumor-suppressor function of the *EXT1* gene is eliminated in an epiphyseal cartilage cell, then probably normal regulation of chondrocyte proliferation is disturbed, giving rise to a clonal proliferation with normal differentiation. Both the role of the resulting altered heparan-sulfate expression on the cell surface (Lind et al. 1998; McCormick et al. 1998) and the abnormal diffusion of the Hedgehog proteins (Bellaiche et al. 1998) still need to be investigated. Indian Hedgehog is normally expressed in the growth zone and prehypertrophic chondrocytes of cartilage (Hammerschmidt et al. 1997).

However, in sporadic osteochondromas no mutations were detected. So far, only one somatic mutation in the *EXT1* gene has been described in a sporadic chondrosarcoma (Hecht et al. 1997). Future studies on a larger panel of tumors should reveal whether the situation is similar to the *BRCA1* gene, for which only germline mutations in hereditary breast cancer have been described. In sporadic breast cancer, no somatic mutations in *BRCA1* are found, whereas LOH at 17q is a frequent phenomenon (Futreal et al. 1994). It is also possible that either mutations in regulatory sequences or large deletions of *EXT1* may occur or that alternative mechanisms of inactivation may act, for instance, as transcriptional down-regulation. Investigation of transcriptional inactivation will entail northern analysis or immunohistochemistry. However, so far, EXT1 antibodies are not available. Alternatively, mutations in *EXT1* may not be critical to the development of sporadic osteochondroma, and, instead, another locus at 8q may be involved.

In a previous study, we investigated LOH and DNA ploidy in 20 peripheral chondrosarcomas. In contrast to chondrosarcomas not secondary to osteochondroma, a high percentage of LOH was found at all loci studied in peripheral chondrosarcoma, and DNA indices had a range of 0.56–2.01 (Bovee et al., in press). Combining those results and data from the present study, we want to propose a genetic-progression model for peripheral cartilaginous tumorigenesis. First, inactivation of both copies of the *EXT1* gene in cartilaginous cells of the growth plate is required for osteochondroma formation, as was shown by loss of the remaining wild-type allele in hereditary osteochondromas in the present study. Whether complete inactivation occurs in sporadic cases remains to be investigated. One or more additional genetic alterations are then required for peripheral chondrosarcomas to arise within the benign precursor. The process of malignant transformation is genetically represented by chromosomal instability and may be caused by defective cell-cycle checkpoints. We showed mild aneuploidy to be present in the benign precursor, with DNA indices showing a range of 0.88–1.2, whereas in the peripheral chondrosarcomas more-severe aneuploidy

is seen, with DNA indices of 0.56–2.01. Additionally, in osteochondromas LOH is restricted to 8q24, whereas in its malignant counterparts a high percentage of LOH is found at all loci tested.

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Electronic-Database Information

The accession numbers and URLs for data in this article are as follows:

DNA Information and Stock Center, http://www.dna.affrc.go.jp/ (for codons used)

EMBL Outstation European Bioinformatics Institute, http://www2.ebi.ac.uk/clustalw/ (for ClustalW)

Genome Database, http://gdbwww.gdb.org (for polymorphic markers)

Nomenclature Working Group, http://ariel.ucs.unimelb.edu.au:80/~cotton/nomenclature.htm (for mutation nomenclature)

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim (for osteochondromas [MIM 133700])

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