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Somatic loss of an *EXT2* gene mutation during malignant progression in a patient with hereditary multiple osteochondromas

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Multiple osteochondromas (MO) is an autosomal-dominant skeletal disorder caused by mutations in the exostosin-1 (*EXT1*) or exostosin-2 (*EXT2*) genes. In this study, we report the analysis of the mutational status of the *EXT2* gene in tumor samples derived from a patient affected by hereditary MO, documenting the somatic loss of the germline mutation in a giant chondrosarcoma and in a rapidly growing osteochondroma. The sequencing of all exons and exon–intron junctions of the *EXT1* and *EXT2* genes from blood DNA of the proband did not reveal any mutation in the *EXT1* gene but did demonstrate the presence of the transition point mutation c.67C > T in the *EXT2* gene, determining the introduction of a stop codon in the coding sequence (p.Arg23*). A mutational analysis of other members of the family and the presence of osteochondromas in the metaphysis of long bones confirmed the diagnosis of hereditary multiple osteochondromas. Direct sequencing from DNA extracted from different sites of two tumor samples (a small rapidly growing osteochondroma and a giant peripheral secondary chondrosarcoma, each located at different chondrocostal junctions) revealed the loss of the germline *EXT2* mutation. Analysis of microsatellite polymorphic markers in the 11p region harboring the *EXT2* gene did not reveal any loss of heterozygosity. This observation supports a recent model of sarcomagenesis in which osteochondroma cells bear *EXT* homozygous inactivation, whereas chondrosarcoma-initiating cells are EXT-expressing cells.

Keywords Hereditary multiple osteochondromas, tumor suppressor gene, chondrosarcoma, hereditary cancer

Multiple osteochondromas (MO) is an autosomal-dominant skeletal disorder characterized by the formation of multiple cartilage-capped bony protuberances or osteochondromas. MO is caused by mutations in either of two genes: exostosin-1 (*EXT1*), which is located on chromosome 8q24.11–q24.13, and exostosin-2 (*EXT2*), which is located on chromosome 11p12-p11 (1–3). However, in 5–30% of patients, no *EXT1* or *EXT2* mutation can be detected (4,5). Most germline mutations found in the *EXT* genes are nonsense, frameshift, or splice-site mutations leading to a premature stop codon inactivating the gene.

According to the two-hit model (6) for classic tumor suppressor genes, both alleles of *EXT* would need to be inactivated for osteochondroma formation, with one allele constitutionally inactivated and the other somatically mutated. The second somatic hit is the inactivation of the remaining wild-type copy, which occurs often either by deletion of the chromosomal region that harbors the gene or

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by mitotic recombination. This results in a loss of heterozygosity (LOH), which is detectable with polymorphic markers located in this chromosomal region. Indeed, LOH of *EXT1* or *EXT2* and loss of the wild-type allele were shown in hereditary osteochondromas, supporting the two-hit model (7–11). However, the absence of LOH or of biallelic inactivation of the *EXT* genes in a substantial proportion of osteochondromas (12–16) resulted in a longstanding controversy as to whether osteochondromas could arise through haploinsufficiency (i.e., the single functional copy of the gene does not produce enough gene product to bring about the wild-type condition, leading to osteochondromas).

Mouse experimental genetic models have provided strong evidence in favor of the role played by biallelic inactivation of *EXT* genes in chondromagenesis in accordance with the classic two-hit model for tumor suppressor genes (17,18). Indeed, somatic biallelic inactivation of *EXT1* in a fraction of chondrocytes generates osteochondromas of the long bones in these mouse models (17,18). Moreover, the discovery that chondrocytes constituting such experimental osteochondromas are mixtures of mutant and wild-type cells may explain the difficulties in detecting homozygous inactivation of the *EXT* genes in several human studies.

The most important complication of osteochondroma is the malignant transformation into a secondary peripheral chondrosarcoma, which is estimated to occur in 1–5% of patients (19). The presence of genetic aberrations not related to *EXT* has been shown during chondrosarcoma progression (12,20–22), and it is assumed that additional genetic alterations (extra-hits) are necessary to progress into malignancy, although different hypotheses have been suggested regarding the mutational status of the *EXT* genes in the secondary chondrosarcoma–initiating cells (16,23,24).

In this study, we report the analysis of the mutational status of the *EXT2* gene in two tumor samples (an osteochondroma and a peripheral secondary chondrosarcoma, each located at different chondrocostal junctions) taken from a patient affected by hereditary MO. The main point of this communication is the surprising observation that the germline-mutated *EXT2* allele is somatically lost in both examined tumor samples, a result with interesting implications for the mechanism of such alteration and for the possible role of functional *EXT* genes in chondrosarcoma progression (25).

Materials and methods

Research participants

The patient affected by multiple osteochondromas and other participants belonging to the same genealogic tree were recruited for this study. Every participant provided a written informed consent for participating in the study.

DNA extraction, PCR amplification, and sequence analysis

Genomic DNA (gDNA) was extracted from blood cells using the QIAamp DNA blood mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The concentration and the quality of the DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Approximately 200 ng of DNA from each individual was used in the PCR reactions. The QIAamp DNA formalin-fixed, paraffin-embedded (FFPE) tissue kit (Qiagen) was used according to the manufacturer's instructions to extract gDNA from FFPE tissue.

The PCR was performed with oligonucleotide primers specific for the coding exons of the *EXT1* and *EXT2* genes (24,26). Exon 1 of *EXT1* was split into three overlapping fragments. DNA was added to a mix containing 25 pmol of each primer (forward and reverse), PCR buffer 10 ×, 10 μM dNTP, 50 mM magnesium chloride, and 5 units of Taq polymerase, to give a total reaction volume of 50 μL. Thermal conditions required for the reaction were a preheating step at 95°C for 30 seconds, followed by the addition of Invitrogen Platinum Taq DNA polymerase (Thermo Fisher Scientific) and 35 cycles at 95°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. All PCR-product sizes were confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The PCR products were purified and 10 ng of each PCR product was sequenced on an ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific) using the Applied Biosystems BigDye terminator cycle sequencing ready reaction kit (Thermo Fisher Scientific) as previously described (27).

Pyrosequencing method

Human genomic DNA (10 ng), extracted as previously described from blood or FFPE tissues, was PCR amplified using conditions optimized for pyrosequencing analysis (PyroMark PCR Kit, catalogue number [cat. no.] 978703; Qiagen). PCR was performed with oligonucleotide primers specific for pyrosequencing (PyroMark custom assay, cat. no. 978776; Qiagen). DNA was added to a mix containing 0.2 μM of each primer (forward and reverse) in the PyroMark PCR MasterMix (Qiagen), up to a total reaction volume of 25 μL. Thermal conditions required for the reaction were a preheating step at 95°C for 15 minutes; 45 cycles at 95°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. PCR-product sizes were confirmed by 1.2% agarose gel electrophoresis. The Qiagen protocol was used for immobilization to Sepharose beads: PyroMark Q24 advanced reagent, cat. no. 970902 (Qiagen) and Streptavidin Sepharose High Performance (cat. no. 17-5113-01, GE Healthcare, Chalfont St. Giles, UK), using 10 μL of PCR product. After immobilization, samples were incubated with PyroMark annealing buffer (cat. no. 979009; Qiagen) and sequencing primer and run on the PyroMark Q24.

11p LOH analysis by microsatellite analysis

Polymorphic microsatellites located in the *EXT2* locus (D11S903) or surrounding it in the 11p chromosome (D11S1355, D11S1313, D11S1319) were analyzed by PCR amplification and measurement of their sizes on the ABI Prism 310 Genetic Analyzer.

Results

Clinical case description

In May 2006, patient II-1, a 22-year-old man, was examined by one of the authors (F.P.C.) because of a slow-growing, painless mass on the anterior chest wall (Figure 1A). Results of a CT scan and MRI showed a well-circumscribed, 18 × 14 × 15-cm, expansile mass with amorphous calcifications inside. Intralesional radiodensities consisted of calcified cartilage that manifested as popcorn-like speckled foci. The tumor arose from the right costochondral junction of the seventh rib, with involvement of the fifth, sixth, and eighth ribs as well as partial diaphragmatic and sternal involvement. The mass extrinsically compressed the liver and had minimal contact with the right lung (Figure 1B). A preoperative biopsy was performed, and the diagnosis of low grade chondrosarcoma was made. The patient presented with multiple osteochondromas, providing proof of the multicentricity of the disease (an X-ray image of osteochondromas of the left arm is shown in Figure 1C). The presence of a small osteochondroma (8 mm), originating from the inner side of the anterior arc of the left third rib, was also detected. In July 2006, the patient underwent a surgical resection of the giant thoracic chondrosarcoma, and the postoperative histopathological analysis confirmed a completely resected low grade chondrosarcoma (Figure 1D, sample T1). The tumor's weight was 5,400 g.

The patient was discharged with a strict follow-up program, including a scheduled CT scan every 6 months, because of the presence of other osteochondromas. In August 2007, a CT scan of the chest revealed that the painless 8 mm osteochondroma, which originated from the inner side of the anterior arc of the left third rib, had increased in size to 1.5 cm (Figure 1E). In September 2007, the patient underwent rib resection. Histopathology confirmed a completely resected osteochondroma (sample T2). No local recurrences have been observed at 6 years post–thoracoabdominal excision and 5 years after left rib resection. Other osteochondromas are stable to date.

Mutational analysis of the *EXT* genes in blood and tumor DNA

The sequencing of all exons and exon–intron junctions of the *EXT1* and *EXT2* genes from blood DNA of patient II-1 did not reveal any mutation in the *EXT1* gene but did demonstrate the presence of the transition point mutation c.67C > T (counting from start site in NM_207122.1) in the *EXT2* gene, determining the introduction of a stop codon in the coding sequence (p.Arg23*) (Figure 2A). An analysis of DNA from other family members revealed the presence of the same mutation in the father (I-1), in the paternal uncle (I-2), and in one first cousin (II-2) of the proband (Figure 2B). None of the affected relatives developed chondrosarcomas, although the presence of multiple osteochondromas was documented radiologically.

Direct sequencing of the *EXT2* gene by the classic chain-termination method did not reveal the presence of the mutation p.Arg23* in DNA extracted from sample T1 (secondary peripheral chondrosarcoma) nor T2 (osteochondroma) obtained from patient II-1 (Figure 2A). The analyses were repeated in two different sites for each tumor sample.

We repeated the sequencing reactions with a more sensitive and quantitative method (pyrosequencing). With this method, we could observe the presence of the mutation c.67C > T in T1 and T2 tumor samples. However, the normal

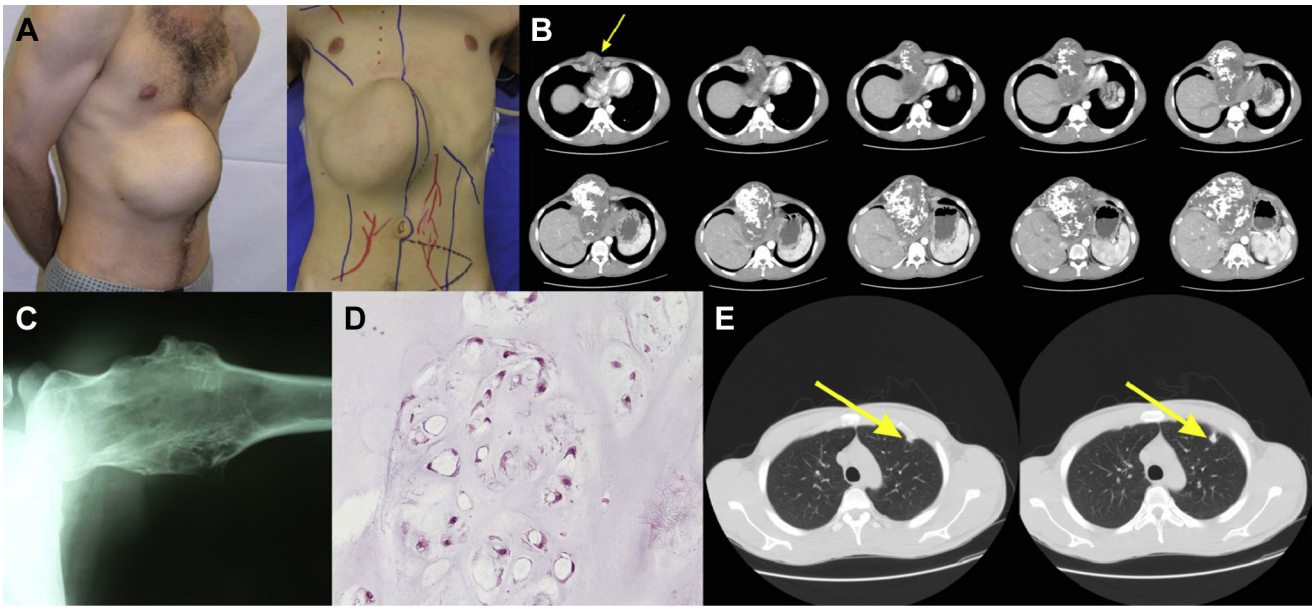

Figure 1 (A) Lateral and frontal views of the mass on the right lower anterior chest wall. (B) Results of a CT scan of the chondrosarcoma shows a large inhomogeneous mass containing scattered calcified areas. Yellow arrows show evidence of a radiological stalk. (C) X-ray images show multiple osteochondromas leading to a deformity of the left arm. (D) A histopathological image of chondrosarcoma of the chest wall after the surgical resection of the mass (T1 sample). (E) Results of a CT scan show exostoses of the inner side of the anterior arc of the left third rib (T2 sample). (Color versions of these illustrations are available on the journal's website at www.cancergeneticsjournal.org.)

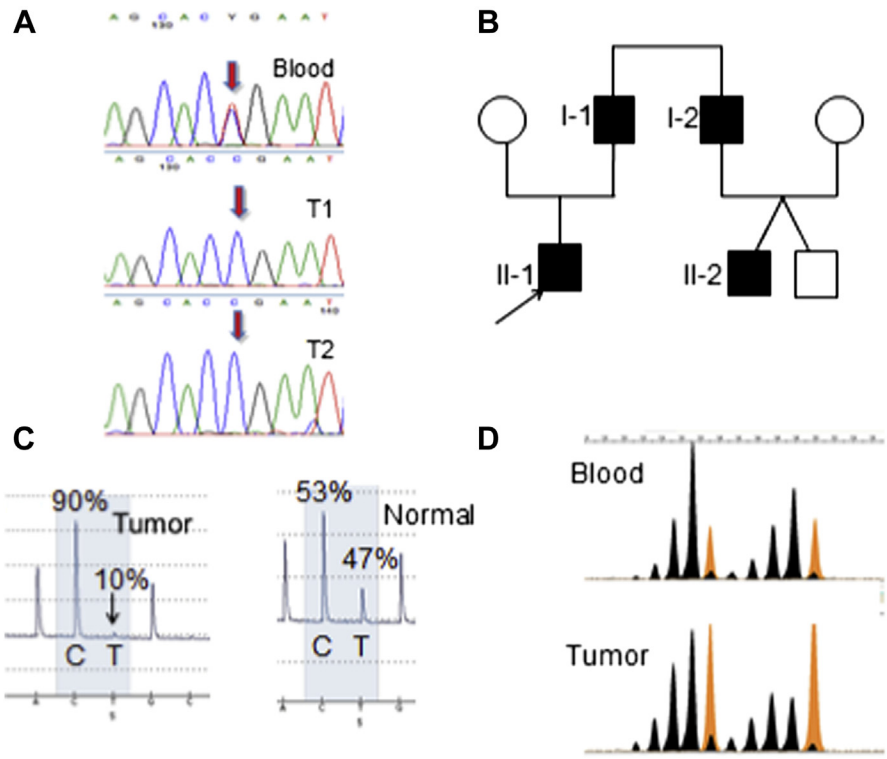

Figure 2 (A) Sequencing electropherograms show the presence of the mutation c.67 C > T in the *EXT2* gene in blood DNA from patient II-1 and the absence of the mutation in tumors T1 and T2 from the same patient. (B) The family tree of patient II-1. Black symbols indicate the presence of the mutation. (C) Pyrograms show the mutation c.67 C > T in sample T2 (Tumor) and in normal chondrocostal tissue present in the same paraffin-embedded block (Normal). The percentage above the corresponding peak indicates the allele frequency. (D) An electropherogram shows PCR amplification of microsatellite D11S903 at the *EXT2* locus, in blood and tumor T1 (black peaks). (Size standards are depicted as orange peaks). Similar results have been obtained in tumor T2. (Color versions of these illustrations are available on the journal's website at www.cancergeneticsjournal.org.)

C allele was present in the vast majority of the DNA molecules (see Figure 2C for sample T2 with 90% C and 10% mutated T alleles). DNA extracted from tissue slices of the same paraffin block that contained normal chondrocostal tissue showed the mutation with an allele frequency of 53% for C and 47% for T, as expected for a germline heterozygous mutation. Analysis by pyrosequencing of DNA extracted from blood of II-1 and I-1 confirmed the presence of the mutation c.67C > T with the expected allele frequency for the heterozygous mutation.

11p Loss of heterozygosity (LOH) analysis

Analysis of polymorphic microsatellite markers (see Materials and methods) surrounding the *EXT2* gene, located at 11p12-p11, excluded the presence of an LOH region in this chromosomal region. Representative results for the microsatellite D11S903, contained in the *EXT2* locus, are shown in Figure 2D.

Discussion

In this report, we document that the germline mutation of the *EXT2* gene, causally linked to hereditary MO (5,26,28), is somatically lost during tumorigenesis in two different tumoral samples derived from the same member of an affected family.

In the classic two-hit model (6) of hereditary tumorigenesis, the second somatic event is usually responsible for the loss of the wild-type allele, whereas in the case here reported, we observed the loss of the mutated allele and the retention of the wild-type one. Such a condition could be referred to as an "isoallelic two-hit model" to highlight that the same allele is affected twice, as opposed to the classic "alloallelic two-hit hypothesis" that predicts the different alleles are affected by the two hits (Figure 3).

Interestingly, a similar observation was reported in 1997 for the *EXT1* gene, the other gene responsible for MO. Hecht et al. (29) analyzed a chondrosarcoma derived from a patient bearing a constitutional heterozygous *EXT1* mutation (a single-base insertion resulting in a frameshift and premature stop codon) and found that the tumor underwent LOH for chromosome 8q24.1, retaining the wild-type *EXT1* allele and losing the mutated one. Indeed, a partial or complete loss of the mutation-bearing chromosome can be considered a likely mechanism for the loss of the mutated allele. It has been repeatedly reported (7,9,12,21) that, in chondrosarcomas, the majority of the autosomes, including chromosome 11, frequently displayed LOH as a result of chromosome loss (deletion-LOH) and, in some cases, subsequent duplication of the remaining homologue gives rise to acquired uniparental disomy or copy-neutral LOH (CN-LOH) (22). Segmental CN-LOH can also take place in a single step: for instance, through a mitotic recombination. In such case, a biallelic wild-

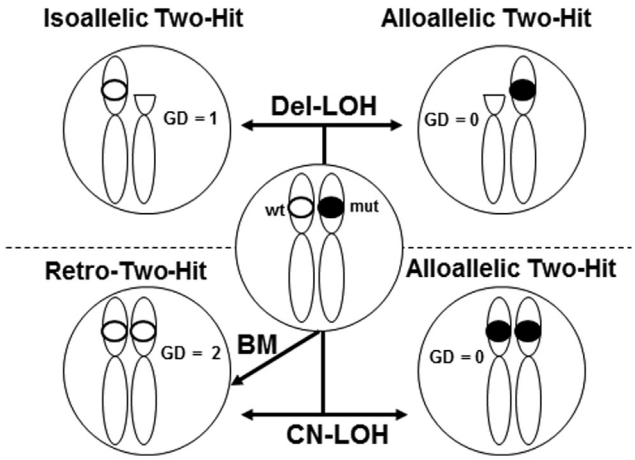

Figure 3 A schematic representation of a cell bearing a heterozygous inactivating mutation (mutated (mut) allele and wild-type (wt) allele) undergoing a second somatic hit in the wt allele (alloallelic two-hit or classic two-hit model) or in the mut allele (isoallelic two-hit or retro-two-hit). *Abbreviations*: Del-LOH: LOH due to deletion of a chromosomal region or an entire chromosome; BM: back-mutation; GD: gene dosage.

type condition +/+ would appear in a tumoral lineage in the context of a heterozygous organism +/-. Such somatic reversion of an inherited mutation in the context of a tumor can be referred to as a "retro two-hit model" (Figure 3). However, in the present case, the analysis of polymorphic microsatellite markers did not reveal any type of LOH in the chromosomal region harboring the *EXT2* gene. Other mechanisms may be responsible for the observed somatic loss of the inherited mutation, such as a small focal deletion larger than the sequenced region but not involving the polymorphic markers, or a true back mutation. Indeed, examples of somatic back mutations and revertant somatic mosaicism have been reported in genetic diseases (30,31). Whatever the mechanism of the loss of the mutated allele (focal CN-LOH, focal deletion, or true back-mutation) the fact that most cells in the tumor samples show a normal C allele suggests that the predominating cell type in chondrosarcoma (T1) or in rapidly growing osteochondromas (T2) did not originate from cells bearing a biallelically inactivated *EXT2* gene.

The importance of our observation mainly relies on its impact on some aspects of the present conception of hereditary tumorigenesis. Indeed, inactivating mutations of the *EXT2* gene are causally linked to the hereditary susceptibility to osteochondromas development and, in this sense, the *EXT2* mutations can be considered driver mutations for osteochondromas. As reported in the introduction, either haploinsufficency or a two-hit mechanism can be invoked to explain the role of the *EXT2* gene in the genesis of osteochondromas (11). However, the molecular cytogenetic characterization of human tumors and recent genetic mouse models support a two-hit mechanism, leading to biallelic inactivation of the *EXT* genes, as a necessary step for chondromagenesis (17,18,32). Moreover, in such experimental osteochondromas, wild-type cells with functional *EXT* genes were being integrated into the mutated cartilaginous tumor tissue, thus producing a genetic mosaic. On the basis of such a hypothesis, osteochondromas of patient II-1 should contain a mixture of cells with different *EXT2* genotypes: a population of cells +/- bearing a monoallelic *EXT2* inactivation because of the first hereditary hit, and a population of cells -/- bearing a biallelic inactivation of the *EXT2* gene generated by the second somatic hit. Therefore, the cellular and molecular events that lead to the transformation of osteochondromas in secondary chondrosarcoma should take place in the context of this genetic mosaic. The current multistep genetic model for secondary peripheral chondrosarcoma formation assumes that osteochondroma cells with homozygous inactivation of *EXT1* or *EXT2* are the origin of chondrosarcoma-initiating cells that acquire one or more additional genetic alterations to progress into malignancy. However, it has been suggested (25) that it is unlikely that such cells (-/-) can generate a cell population bearing the *EXT2* wild-type allele. Indeed, the presence of a vast cell population bearing only the wild-type *EXT2* allele in a peripheral secondary chondrosarcoma sample (as observed in the T1 sample of this study) suggests that chondrosarcoma-initiating cells might be the constitutional +/- cells, and not the double-hit -/- ones. Moreover, in this study we detected similar findings in a rapidly growing osteochondroma (T2 sample), which probably represents an early stage of progression toward a peripheral secondary chondrosarcoma.

Our results are in agreement with recent data by de Andrea et al. (25) that challenged the notion that osteochondroma cells with homozygous inactivation of *EXT1* or *EXT2* are the cellular origin of secondary peripheral chondrosarcomas. By immunohistochemical analysis, these researchers confirmed the presence of cells with dysfunctional *EXT1* in sporadic and hereditary osteochondromas and showed cells with functional *EXT1* in secondary peripheral chondrosarcomas. Homozygous inactivation of the *EXT1* locus in sporadic secondary peripheral chondrosarcoma (2/17 cases, 12%) is much less frequently detected than expected based on the assumption that it originates in a sporadic osteochondroma, in which homozygous inactivation of *EXT1* is found in 80% of cases (11,32). These results point to a model of sarcomagenesis in which the osteochondroma cells with homozygous inactivation of *EXT* create a niche (a permissive microenvironment), which enables cells with functional *EXT* to acquire other genetic changes that give rise to secondary peripheral chondrosarcoma (25).

The results of this study, considered in light of other recent investigations (23,25), support the idea that osteochondromas and their associated secondary peripheral chondrosarcomas have different initiating cells. However, the conclusive claim that chondrosarcoma arises predominantly from cells with a normal *EXT* gene will require the accurate molecular characterization of a large collection of tumor samples deriving from well-characterized families affected by this rare genetic disease.

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