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Loss of Heterozygosity in Chondrosarcomas for Markers Linked to Hereditary Multiple Exostoses Loci on Chromosomes 8 and 11

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Summary

Hereditary multiple exostoses (EXT; MIM 133700) is an autosomal dominant condition characterized by growth of multiple benign cartilage-capped tumors. EXT greatly increases the relative risk to develop chondrosarcoma, although most chondrosarcomas are sporadic. This observation suggests that, like the genes responsible for retinoblastoma and other dominantly inherited cancer susceptibility disorders, the genes that cause EXT may have tumor-suppressor function and may play a role in the pathogenesis of the related sporadic tumors. To investigate this hypothesis, we evaluated chondrosarcomas for loss of constitutional heterozygosity (LOH) at polymorphic loci linked to three recently identified genomic regions containing genes involved in EXT. LOH for markers linked to EXT1 on chromosome 8 was detected in a chondrosarcoma that arose in a man with EXT. Four of 17 sporadic tumors showed LOH for markers linked to EXT1, and 7 showed LOH for markers linked to EXT2 on chromosome 11. In all, LOH was observed for markers linked to EXT1 or EXT2 in 44% of the 18 tumors, whereas heterozygosity was retained for markers on 19p linked to EXT3. These findings support the hypothesis that genes on 8q and the pericentromeric region of 11 have tumor-suppressor function and play a role in the development of chondrosarcomas.

Introduction

After multiple myeloma, chondrosarcoma is the second most common primary malignant neoplasm of bone in adults, increasing in frequency with age, from ~0.3/100,000 at age 50 years to ~0.7/100,000 by age 75 years (Dorfman and Czerniak 1995). The majority of chondrosarcomas are sporadic, but the presence of hereditary multiple exostoses (EXT; MIM 133700) greatly increases the risk of developing this tumor (Gitelis et al. 1981; Garrison et al. 1982; Voutsinas and Wynne-Davies 1983). EXT is an autosomal dominant disorder with a prevalence of $\geq$1/50,000 (Schmale et al. 1994) and is characterized by growth of multiple exostoses. Exostoses are cartilaginous capped exophytic osseous lesions that result from the exuberant growth of bone and/or cartilage at either the epiphyseal plate or the adjacent periosteal surface (Peterson 1989; Huvos 1991). Although such tumors may develop in any bone that has a cartilaginous origin, they typically appear in the metaphyseal regions of the proximal tibia, distal femur, and proximal humerus or in the pelvis or scapula. Malignancy, usually chondrosarcoma and rarely osteosarcoma (Dahlin and Unni 1986; Tsuchiya et al. 1990) or spindle-cell sarcoma (Matsuno et al. 1988), can develop as a secondary change. There is wide variation in the reported rates of malignant degeneration in EXT; the more recent series conclude that the risk is 1%–6% (Solomon 1974; Gordon et al. 1981; Voutsinas and Wynne-Davies 1983). However, the relative risk for malignancy in EXT has been estimated as ~2,500-fold (Schmale et al. 1994). EXT is genetically heterogeneous; EXT genes have been assigned to 8q24 (EXT1; Cook et al. 1993), the pericentromeric region of 11 (EXT2; Wu et al. 1994), and 19p11-p13 (EXT3; Le Merrer et al. 1994). It is not known whether genes at all three loci confer the same risk of chondrosarcoma.

Familial cancer syndromes, although rare, may provide clues to understanding the complex multistep pathogenesis of cancer, by identifying the gene(s) involved in the more common sporadic forms of the same cancer. Retinoblastoma (Benedict et al. 1990) and the Li-Fraumeni syndrome (Srivastava et al. 1990) are prototypes of autosomal dominant cancer-predisposition syndromes. Knudson (1971) postulated that a pattern of autosomal dominant inheritance of cancer reflects an autosomal recessive loss-of-function mutation at the cellular level. For the oncogenic effect to occur, it is neces-

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sary that both copies of the gene that normally functions to suppress tumorigenesis be eliminated. In familial cancer syndromes, the first mutation is inherited and the second is somatically acquired; in sporadic cases, both mutations occur somatically. A common mechanism for inactivation of the tumor-suppressor locus is point mutation of one allele, with loss of the remaining normal allele by a chromosomal event such as nondisjunction, interstitial deletion, or gene conversion (Cavenee et al. 1983; Orkin et al. 1984). The effect is to render the cell homozygous or hemizygous for an inactivated tumor-suppressor gene. Because the process that eliminates the normal allele often involves large stretches of DNA, it can be detected by comparing the genotypes of tumor tissue and normal tissue at polymorphic loci in the vicinity of the tumor-suppressor gene.

There are now many examples of such a pathogenesis occurring in human tumors, including retinoblastoma (Cavenee et al. 1983; Benedict et al. 1990), Wilms tumor (Orkin et al. 1984; Koufos et al. 1985), and familial adenomatous polyposis (Kinzler et al. 1991). The study of these inherited disorders that predispose to malignancy, most notably retinoblastoma, has led to discoveries regarding molecular changes involved in carcinogenesis in general. In an analogous manner, somatic mutations in the EXT genes may be involved in development of sporadic chondrosarcomas. We initiated this study to determine if chondrosarcomas show loss of heterozygosity (LOH) at any of the loci related to EXT.

Chondrosarcomas are slow-growing tumors that often have a diploid DNA content and a low-grade histological appearance. The degree of cellular differentiation does not always correlate with biological behavior (Kreibergs et al. 1982), and prognostic counseling is made more difficult by the lack of tumor markers. Development and progression of cancer appears to be a multistep process in which genetic alterations accumulate in the same cell. It has been reported that 25% of high-grade chondrosarcomas contain a mutated p53 sequence (Wadayama et al. 1993). p53 is a tumor-suppressor gene located on chromosome 17p13 and is inactivated with varying frequency in tumors of many types (Hollstein et al. 1991). Because it is possible that patterns of inactivation of the p53 and EXT genes may be of prognostic significance, we evaluated chondrosarcomas for LOH at these loci.

Material and Methods

Sample Acquisition

Eighteen patients with chondrosarcoma evaluated at the University of Washington Medical Center were included in this study. One patient, D0178, had EXT; this diagnosis was excluded in the remaining 17 patients by physical examinations and bone scans performed as part of the routine preoperative evaluation. Portions of the chondrosarcomas were obtained at surgery, and aliquots were frozen at −70°C in optimum-cutting-temperature (i.e., O.C.T.) compound or in RPMI medium containing 8% bovine calf serum and 8% dimethylsulfoxide. The sample was taken from a region of each tumor that visibly appeared to be composed of active tumor tissue, as determined by the orthopaedic surgeon (E.U.C.). Blood samples were drawn into EDTA-containing Vacutainer tubes under protocols approved by the Human Subjects Committee of the University of Washington.

Histological Evaluation

Fresh tumor samples were decalcified and formalin fixed. Sections were stained with hematoxylin and eosin and were evaluated by a hospital staff pathologist. Grades 1–3 were assigned on the basis of the Evans criteria (Evans et al. 1977).

Detection of Allelic Losses

White blood cells were recovered from a buffy-coat preparation or from a discontinuous Ficoll-Histopaque gradient (specific gravities 1.077 and 1.119). DNA was extracted from cryopreserved chondrosarcomas or leukocytes by a modified salting-out procedure (Miller et al. 1988), and 50–300 ng of target DNA in 25 μl of reaction mixture was PCR amplified at the following loci: D8S200, D8S85, D8S547, D8S527, D8S522, D11S905, D11S1355, D11S903, D11S1313, D11S1319, D11S554, D19S221, D19S226, D19S216, D18S71, and p53 (Gyapay et al. 1994; Wu et al. 1994). For the latter locus both a pentanucleotide *Alu* sequence repeat in the first intron and an intragenic dinucleotide repeat were evaluated. Primers for the p53 pentanucleotide-repeat polymorphism were synthesized in the Molecular Pharmacology Facility at the University of Washington; primers for the remaining loci were obtained from Research Genetics. One primer for each locus was end-labeled with [γ^{32}]P by a T4 kinase reaction (Sambrook et al. 1989). Glycerol (10%) was added to the reaction mixtures. For amplification, samples were initially denatured for 1 min at 92°C, followed by 35 cycles of 30 s at 92°C, 30 s at the appropriate annealing temperature (55°C–58°C), and 90 s at 75°C. Final extension was performed for 5 min at 72°C. Amplified products were electrophoresed through 6% polyacrylamide gels containing 7 M urea and 30% formamide. Dried gels were autoradiographed, and the band patterns of normal and tumor cells were quantitated by a Hoefer GS300 scanning densitometer with GS370 software (Hoefer Scientific Instruments). Because bands corresponding to the two alleles in normal tissue were often not equal in peak area, numeric results were normalized

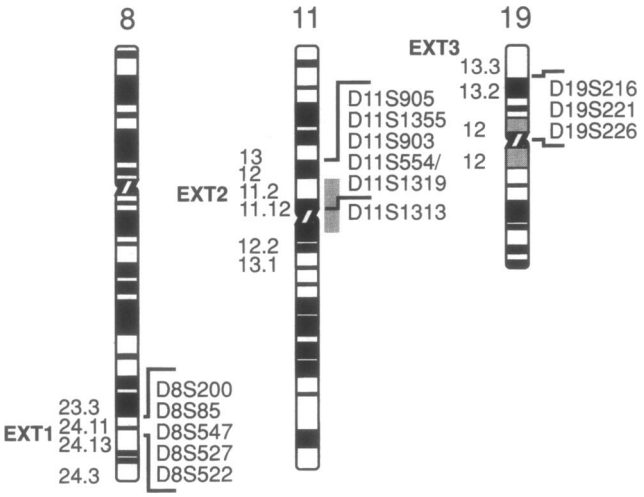

Figure 1 Ideograms of chromosomes 8, 11, and 19, showing approximate locations of the EXT genes and the polymorphic microsatellite markers used to evaluate LOH. The relative order of markers is according to the CEPH linkage maps (Gyapay et al. 1994; James et al. 1994; Matise et al. 1994), with the exception of the non-Généthon marker D11S554 (Wu et al. 1994). The position of D11S1313 relative to the centromere is not known (*shaded area*).

by dividing the ratio of alleles in the tumor tissue by the ratio of alleles in the normal tissue control $[(t_a/t_b)/(n_a/n_b)]$, as described by Cawkwell et al. (1993). If this calculated value was >1, the reciprocal was used. A change 50% in the normalized value was accepted as evidence of LOH.

Results

LOH at the EXT Loci

To detect somatic loss of chromosomes 8, 11, and 19 sequences, DNA from normal cells and from tumors was typed at polymorphic loci linked to the EXT genes. Figure 1 shows the approximate locations of highly polymorphic microsatellite markers linked to EXT genes on chromosomes 8 (EXT1), 11 (EXT2), and 19 (EXT3) (Cook et al. 1993; Le Merrer et al. 1994; Wu et al. 1994). Combined information from several maps suggests that marker pairs D8S200 and D8S522, D11S905 and D11S1319, and D19S216 and D19S226 define regions of 5.4 cM, 9 cM, and 25 cM, respectively (Gyapay et al. 1994; James et al. 1994; Matise et al. 1994).

A chondrosarcoma that developed in a patient with EXT (D0178) exhibited LOH for all five chromosome 8 markers tested, whereas heterozygosity was retained for chromosomes 11 and 19 markers (fig. 2). Small family size precluded mapping; therefore, it is not known which locus is involved in EXT in this case.

Seventeen sporadic chondrosarcomas were then evaluated. Seven of these showed LOH for markers in one or more chromosomal regions associated with EXT. The alteration in band intensity patterns for markers linked to EXT2 seen in an eighth tumor, D0359, could have resulted from amplification rather than from loss of one allele. Genotype results are shown in table 1, and representative autoradiograms are shown in figure 3.

In all, LOH at chromosome 8q24 was detected in 28% (5/18) of the chondrosarcomas, one of which had developed in a man with hereditary multiple exostoses. Seven (39%) of 18 tumors exhibited LOH for chromosome 11 markers linked to EXT2, whereas none of 18 showed LOH for chromosome 19 markers linked to EXT3. One tumor showed LOH for chromosome 8 markers only, three tumors showed LOH solely for chromosome 11 markers, and four tumors showed LOH for markers on both chromosomes. In most cases, concordant results were obtained for all markers evaluated in a region. For instance, for patient D0178, genotypes at the five chromosome 8 markers all showed LOH in the tumor (normalized ratios of .18–.34), and heterozygosity was retained for all four informative chromosome 11 markers tested (normalized ratios of .76–.95) (Cawkwell et al. 1993). In contrast, LOH was not detected for marker D11S554 in tumors D0547 and D0621, although these tumors exhibited allelic loss for other chromosome 11 markers. To evaluate whether widespread and nonspecific LOH occurred in the chondrosarcomas, a locus on chromosome 18 (D18S71) was genotyped as a control. Only 1 of 12 tumors showed LOH at this locus; the remaining six patients were not informative.

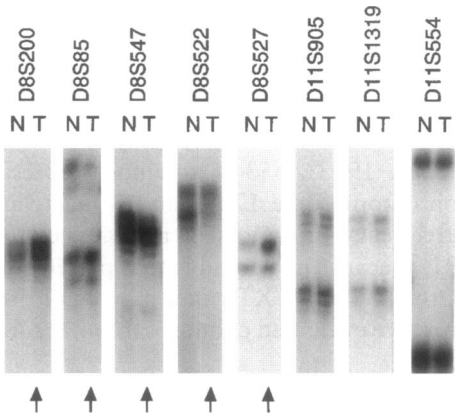

Figure 2 Evaluation of an EXT-associated chondrosarcoma from patient D0178. The left lane (N) of each pair contains DNA from corresponding normal tissue; and the right lane (T) contains DNA from tumor. LOH was detected for all five chromosome 8 markers (*arrows*) but for none of four chromosome 11 markers tested (D11S1313 is not shown). LOH was not detected for two chromosome 19 markers (autoradiograms not shown).

Table 1

Summary of Evaluation of 18 Chondrosarcomas for LOH

Patient (Grade)	D8S200	D8S85	D8S547	D8S527	D8S522	D11S905	D11S1355	D11S903	D11S1319	D11S554	D11S1313	D19S216	D19S221	D19S226	D18S71	TP53[a]
D0196 (2)	(−) .88	(−) .70	NI	NI	ND	(+) .37	ND	(+) .39	(+) .28	(+) .4	ND	(−) .82	(−) .55	(−) .87	(−) .61	(−) .81–NI
D0178[b] (1)	(+) .18	(+) .22	(+) .31	(+) .34	(+) .26	(−) .76	NI	ND	(−) .89	(−) .93	(−) .95	(−) .76	(−) .65	NI	(−) .96	(−) .56–.63
D0237 (2)	(+) 0	(+) .02	(+) 0	(+) 0	(+) 0	(+) 0	NI	(+) 0	NI	(+) .15	(+) 0	(−) .78	(−) .57	NI	NI	(+) 0–NI
D0239[c,d] (2)	(+) .20	NI	(+) .26	(+) .28	NI	(+) 0	NI	(+) .13	NI	(+) .29	(+) .27	(−) .68	(−) .81	(−) .80	(−) .98	(+) .38–.49
D0359[e] (2)	(−) .98	(−) .98	(−) .87	NI	(−) .67	(+) .32	(+) .32	NI	(+) .36	(+) .49	(+) .46	NI	(−) .98	(−) .92	(−) .55	(−) .84–.92
D0418 (1)	(−) .90	NI	(−) .59	ND	(−) .83	(−) .66	NI	ND	NI	(−) .96	(−) .74	(−) .97	(−) .88	NI	(−) .54	(−) NI–.92
D0547 (2)	(−) .95	NI	(−) .81	NI	(−) .71	NI	NI	(+) .29	(+) .37	(−) .56	(+) .37	(−) .57	NI	NI	(−) .91	NI
D0550[c] (2)	(−) .80	NI	(−) .78	NI	NI	(−) .94	NI	(−) .70	(−) .69	(−) .89	(−) .99	NI	(−) .86	(−) .99	NI	(+) .22–NI
D0562[c,d] (3)	NI	NI	NI	NI	(−) .91	NI	(−) .84	(−) .80	(−) .77	(−) 1.0	(−) .99	(−) .72	(−) .94	NI	NI	NI
D0575 (2)	(−) .91	(−) .92	NI	NI	(−) .94	NI	NI	(+) .11	(+) 0	(+) .14	(+) .26	(−) .72	NI	NI	NI	(+) .46–NI
D0587[f] (2)	(−) .80	(−) .78	(−) .75	ND	NI	NI	(−) .89	NI	(−) .69	(−) .99	(−) .55	(−) .62	NI	(−) .94	NI	NI
D0598 (1)	(−) .90	NI	(−) .77	NI	NI	NI	NI	(−) .88	(−) .63	(−) .90	(−) .98	NI	NI	(−) .89	(−) .98	(−) .80–.92
D0612 (1)	(−) .94	(−) .71	(−) .96	ND	ND	ND	ND	ND	NI	(−) .91	ND	(−) .74	(−) .83	ND	(+) .45	(−) NI–.76
D0613[g] (1)	(−) .89	NI	NI	NI	NI	NI	(−) .93	(−) .89	NI	(−) .89	ND	(−) .98	(−) .62	NI	NI	(−) .89–.95
D0614 (2)	(+) .28	(+) .11	(+) .26	NI	ND	NI	(+) 0	(+) .17	NI	(+) .13	(+) 0	(−) .95	NI	NI	(−) .54	(−) NI–.81
D0621 (1)	(+) .37	(+) .30	NI	NI	(+) .38	(+) .44	(+) .47	ND	NI	(−) .67	NI	NI	NI	(−) .87	(−) .92	(−) NI–.95
D0622 (1)	NI	(−) .65	(−) .92	NI	ND	(−) .56	NI	NI	NI	NI	NI	(−) .91	(−) .94	(−) .76	(−) .82	(−) .89–NI
D0623 (1)	NI	(−) .95	NI	NI	(−) .85	(−) .53	NI	(−) 1.0	(−) .93	(−) .82	(−) .74	(−) .99	(−) .85	(−) .84	(−) .96	NI

NOTE.—(+) = LOH detected; (−) = LOH not detected; NI = not informative; ND = not determined (untested or not evaluable); normalized densitometry ratios were calculated as described in Material and Methods.

[a] Two intragenic polymorphisms were evaluated—densitometry results are given as VNTR − STRP.

[b] Patient with EXT. Although the histology was low grade, this tumor metastasized widely and led to the death of the patient.

[c] Tumor developed into a cell line during culture.

[d] Dedifferentiated chondrosarcoma.

[e] The pattern of band-intensity alteration in this tumor might have resulted from amplification of one allele, rather than from LOH.

[f] Mesenchymal chondrosarcoma.

[g] Myxoid chondrosarcoma.

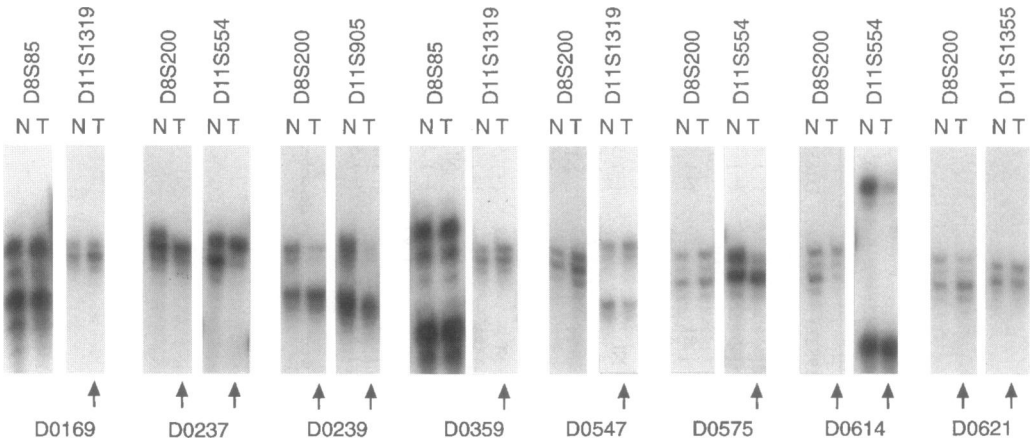

Figure 3 Autoradiograms showing genotypes at representative markers on chromosomes 8 and 11 of eight sporadic tumors that exhibit LOH or other change in band pattern for one or both of these chromosomes. For each marker, DNA from normal tissue is in the left lane (N); and DNA from tumor is in the right lane (T). Patient numbers are shown below each pair of lanes. Arrows indicate tumor DNA with altered band intensities. It is possible that the alteration in band intensities at locus D11S1319 in tumor tissue from D0359 result from amplification of the larger allele rather than from loss of the smaller allele; this tumor showed similar patterns of band alterations for the other chromosome 11 markers tested.

Evaluation of p53 for LOH

Loss of one p53 allele was detected in 4 (29%) of the 14 tumors that were informative for at least one of two intragenic polymorphisms in the p53 locus tested (table 1). Figure 4 shows an autoradiograph containing results for three of these tumors, D0239, D0550, and D0575. A similar analysis of tumor DNA from D0237 revealed complete loss of one band (autoradiograph not shown).

Lack of Alteration in Size of Microsatellite Alleles

Some tumor types, notably hereditary nonpolyposis colon cancer, exhibit microsatellite-length instability manifested by altered band mobilities in simple repeated sequences such as the polymorphisms evaluated in the present study (Fishel et al. 1993; Leach et al. 1994; Bonner et al. 1994; Papadopoulos et al. 1994). In addition to the markers shown in table 1, tumors were also genotyped for markers on several other chromosomes. At least 10 markers were evaluated per case. The instability phenotype was not observed in any of the chondrosarcoma samples tested.

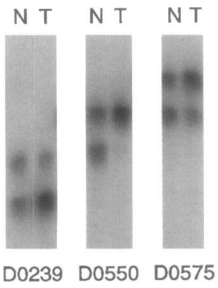

Figure 4 LOH detected at the p53 locus (i.e., VNTR) in chondrosarcoma cells from three patients. N = normal tissue; and T = tumor tissue. LOH was also detected in the chondrosarcoma from patient D0237 (data not shown).

Discussion

Tumor-suppressor genes have been shown to be involved in regulating the orderly process of cell proliferation. Loss of function of these genes leads to increased (or unregulated) proliferation and may result in tumor initiation and/or progression (Sager 1989; Marshall 1991). Although germ-line mutations in tumor-suppressor genes may be involved in the pathogenesis of disease that is transmitted in a dominant fashion, a characteristic of a tumor-suppressor gene is its recessive behavior at the cellular level. For expression of the phenotype, both normal copies of the gene must be inactivated. Segmental DNA deletions detected near tumor-suppressor genes in inherited tumors led to their implication in the pathogenesis of the more common isolated cases of the same tumor types (Koufos et al. 1985; Solomon et al. 1987). It is postulated that other tumor-suppressor genes are contained in regions of the genome where reductions to hemizygosity or homozygosity are frequently found in sporadic tumors of a given type (Sager 1989).

The observations that chondrosarcoma, like retinoblastoma and Wilms tumor, exists in both hereditary and nonhereditary forms and that EXT confers an increased relative risk to develop chondrosarcoma suggest that some or all of the genes that cause EXT may have

tumor-suppressor function. The finding that a chondrosarcoma in a man with hereditary multiple exostoses showed LOH for the region on the long arm of chromosome 8 that contains the EXT1 gene is consistent with this hypothesis. Furthermore, the observation that 41% of 17 sporadic chondrosarcomas had LOH for at least one region associated with EXT genes suggests that, as with retinoblastoma or p53, somatic mutations in the EXT genes may play a role in the formation of tumors that occur in the absence of an inherited predisposition. It must be mentioned, however, that the borders of LOH were not defined in our study, and it is possible that genes distinct from the EXT loci are the critical ones. Tumors D0547 and D0621 showed LOH for three and two chromosome 11 markers, respectively, but not for D11S554. The position of this marker relative to the others is not known. The band-intensity changes in all five chromosome 11 markers tested in tumor D0359 exhibited a pattern that might reflect amplification rather than loss of one allele. The biological significance of this observation is not clear and merits further investigation.

In all, 44% (8/18) of the chondrosarcomas had LOH or other band-pattern alteration for markers linked to EXT1 or EXT2. This is a conservative estimate, because we required a 50% relative change in band area in order to distinguish LOH. Others have used a criterion of 30% (Hampton et al. 1994). It might not be expected that all chondrosarcomas would show abnormalities at EXT1, EXT2, or EXT3, because additional EXT loci may exist. There are several reasons why LOH may not be seen even if the locus does contain a relevant tumor-suppressor gene. The length of contiguous-DNA loss varies and can be very short (Jones et al. 1994). LOH would not be detected if the markers tested all lie outside the deleted area. A patient may not be heterozygous at the crucial marker. Somatic point mutations can also inactivate the normal allele; this change would not result in LOH. Because tumor cells were not dissected out of the tissue samples that we tested, it is possible that, in some of the negative cases, LOH was masked by the presence of normal cells. Arguing against the possibility that this explains all of the negative cases is the demonstration of LOH for p53 without LOH for EXT markers in tumor D0550.

The chondrosarcomas represent a diverse group of tumors, with respect to histology, cytogenetics, and biological behavior. For instance, translocations involving the long arms of chromosomes 9 and 22 are associated with myxoid chondrosarcomas. It is possible that mutations in the EXT genes contribute only to the pathogenesis of specific subtypes of chondrosarcomas. LOH for the EXT genes was not detected in either the myxoid tumor, D0613, or the mesenchymal chondrosarcoma, D0587. More tumors will need to be evaluated before a relationship between pattern of LOH and histological subtype can be determined.

In our study, all four tumors exhibiting LOH for p53 were biologically aggressive, and two of them developed into long-lived lines during culture. The tumor that gave rise to the third cell line was homozygous for both p53 markers tested. In contrast, LOH for chromosomes 8 and 11 was seen in a low-grade, less aggressive tumor (patient D0621). This observation suggests that mutations in the EXT genes may be earlier events and that mutations in p53 are associated with progression of disease. Study of additional tumors will be necessary to evaluate this hypothesis.

Recently, mutations in the human homologues of genes involved in mismatch repair in yeast have been found to be associated with microsatellite-locus instability in several types of human cancer (Fishel et al. 1993; Leach et al. 1994; Bonner et al. 1994; Papadopoulos et al. 1994). No alterations in band size were observed at any of the loci tested in any of the 18 chondrosarcomas that we evaluated. Therefore, we find no evidence that chondrosarcomas exhibit microsatellite-length instability. In addition, the low frequency of LOH for markers on chromosomes 19 (0/18 tumors) and 18 (1/12 tumors) argues against widespread, nonspecific, genomic alterations.

In summary, the finding of LOH at two of the three known EXT loci in sporadic chondrosarcomas and in a chondrosarcoma that developed in a man with hereditary multiple exostoses suggests that the genes at EXT1 and EXT2 may have tumor-suppressor functions whose loss is related to the development of a subset of chondrosarcomas. In tumors displaying LOH, the region of syntenic allele loss extends a variable distance along the chromosome (Fujiwara et al. 1993; Hampton et al. 1994). By evaluation of shared regions of LOH in many tumors, a smallest region of overlap can be delineated. This strategy can contribute to fine-structure mapping of an area containing a tumor-suppressor gene and can complement the information obtained by linkage analysis in pedigrees. Although in this study the tumors showed concordant loss of alleles at most informative markers tested in a region, evaluation of additional markers and additional tumors may provide valuable information regarding the likely location of the EXT genes and facilitate their positional cloning.

Acknowledgments

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