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Evidence for a Novel Osteosarcoma Tumor-Suppressor Gene in the Chromosome 18 Region Genetically Linked with Paget Disease of Bone

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Summary

Paget disease of bone, or "osteitis deformans," is a bone disorder characterized by rapid bone remodeling resulting in abnormal bone formation. It is the second most common metabolic bone disease after osteoporosis, affecting 3%–5% of subjects aged >40 years. Recent evidence suggests that predisposition to Paget disease may have a genetic component. Genetic linkage analysis of families with multigenerational Paget disease shows linkage to a region of chromosome 18q near the polymorphic locus D18S42. Approximately 1% of Paget patients develop osteosarcoma, which represents an increase in risk that is several thousandfold over that of the general population. Osteosarcoma in Paget patients is the underlying basis for a significant fraction of osteosarcomas occurring after age 60 years. Our analysis of tumor-specific loss of constitutional heterozygosity (LOH) in 96 sporadic osteosarcomas has identified a putative tumor-suppressor locus that maps to chromosome 18q. We have localized this tumor-suppressor locus between D18S60 and D18S42, a region tightly linked to familial Paget disease. Analysis of osteosarcomas from patients with Paget disease revealed that these tumors also undergo LOH in this region. These findings suggest that the association between Paget disease and osteosarcoma is the result of a single gene or two tightly linked genes on chromosome 18.

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Introduction

Paget disease (MIM 167250) is a condition in which activation of osteoclastic bone resorption and the resulting stimulation of osteoblastic repair occur over a period of years. This results in excessive new bone formation in a chaotic manner, resulting in an overall decrease in bone strength and an increase in susceptibility to fractures. The most frequently affected bones include the pelvis, lumbar spine, sacrum, skull, femur, and tibia. Although Pagetic lesions may occur in multiple sites (polyostotic Paget disease), the disease does not spread from bone to bone.

Paget disease is most common in Caucasians of European descent, but it also occurs in African Americans. It is rare in those of Asian descent. Paget disease is seldom diagnosed in people aged <40 years, but has an estimated incidence of up to 5% in individuals aged >55 years (Ooi and Fraser 1997). It affects both sexes, but is slightly more common in men than women.

At present, the etiology of Paget disease is uncertain. The role of viruses in the pathogenesis of the disease has been investigated extensively; however, no causative link has been demonstrated between any infective agents and Paget disease (Thomas and Shepherd 1994). Reports of familial aggregation of Paget disease, including occurrence in successive generations, are common (Jacobs et al. 1979; Brenton et al. 1980; Nassar and Gravanis 1981; Sofaer et al. 1983; Wu et al. 1991), with as many as 40% of Paget disease patients having an affected first-degree relative (Morales-Piga et al. 1995). The risk for first-degree relatives of a Pagetic patient to develop Paget disease was seven times greater than for an individual with no affected relatives (Siris 1994), suggesting that there may be a genetic component to predisposition for the disease.

Familial expansile osteolysis (FEO) is a disorder similar to Paget disease. FEO has been described in a single extended family as an autosomal dominant bone dysplasia with some histological similarity to Paget disease (Osterberg et al. 1988; Wallace et al. 1989). Unlike con-

ventional Paget disease, the onset of the skeletal lesions in FEO patients occurs in late adolescence to early adulthood. It has been proposed that Paget disease and FEO are allelic variants of the same gene (Hughes and Barr 1996). Precedence for this is seen with Duchenne and Becker muscular dystrophies, in which different mutations in the same gene lead to different phenotypic presentations (Koenig et al. 1989).

Hughes and coworkers demonstrated linkage between FEO and a region of chromosome 18q21.1–q22 flanked by polymorphic loci D18S51 and D18S64 (Hughes et al. 1994; Hughes and Barr 1996). We have performed linkage studies on a large kindred with Paget disease (Cody et al. 1997). A total of 12 short tandem-repeat polymorphic (STRP) loci from the region of human chromosome 18 surrounding the FEO locus were used to screen DNA samples from the kindred. No recombinants were observed between the Paget disease locus and D18S42, giving a maximum LOD score of 3.40 (Cody et al. 1997) (this same marker showed no recombination with FEO [Hughes and Barr 1996]). Thus, the evidence strongly indicates that a locus for Paget disease resides on 18q, which is either the same locus as FEO or is tightly linked to the FEO locus.

One of the most serious complications of Paget disease is osteosarcoma. By 1889, Paget had observed sarcomas arising in 5 of his 23 patients with osteitis deformans (Paget 1889). Various reports have placed the incidence of osteosarcoma in Paget disease at 0.7%–5% (Freydinger et al. 1963; Wick et al. 1981; Greditzer et al. 1983; Hadjipavlou et al. 1992), with the lower frequency reported in studies where both asymptomatic and symptomatic cases of Paget disease were followed. Although the incidence of osteosarcoma in Paget disease is relatively low, it contributes significantly to the mortality and morbidity of Pagetic patients (Klein and Norman 1995). Osteosarcoma related to Paget disease represents 20% of the patients with osteosarcoma who are over the age of 40 years (Wick et al. 1981) and as many as 50% of the patients with osteosarcoma over the age of 60 years (Huvos 1986), making this complication a significant geriatric health risk.

Evidence that the osteosarcoma is secondary to Paget disease comes from several areas. Osteosarcoma associated with Paget disease occurs at an age when osteosarcoma is otherwise rare in the population (Sweetman 1982). In the majority of patients, there is evidence of symptomatic Paget disease before the identification of the tumor. Furthermore, the osteosarcoma always develops in the bones that are affected by Paget disease (Moore et al. 1991). The osteosarcoma that develops in the Pagetic bone is characterized by the presence of a large number of osteoclastic giant cells and atypical osteoblasts that resemble an exaggerated form of the remodeling process seen commonly in Paget disease. The majority of Paget disease patients who develop osteosarcoma have polyostotic Paget disease (Huvos et al. 1983; Schajowicz et al. 1983; Seret et al. 1987; Wu et al. 1991), which occurs more commonly in patients with a genetic predisposition for Paget disease (Wu et al. 1991), suggesting that there may be a link between genetic predisposition to Paget disease and the occurrence of osteosarcoma in those patients.

Consistent or frequent LOH of all or part of a chromosomal arm in a tumor is a good predictor of the presence of a tumor-suppressor gene (Hansen and Cavenee 1988). Yamaguchi and coworkers used LOH analysis to examine osteosarcomas for LOH on each chromosomal arm (Yamaguchi et al. 1992). They found that in their sampling of osteosarcomas, as in other tumors, LOH for any single chromosome occurred in a detectable fraction of the tumors, but when they compared the relative frequency of LOH for each individual arm of the chromosomes separately they found that LOH occurred more frequently on some chromosomal arms than on others. The chromosomal arms involved most frequently were 3q, 13q, 17p, and 18q, suggesting that these chromosomal arms may harbor tumor-suppressor genes important in osteosarcoma tumorigenesis. In two cases, the tumor-suppressor loci are already known: the retinoblastoma susceptibility gene RB1 on 13q (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987) and the p53 gene on chromosome 17p (Masuda et al. 1987), suggesting that this approach is a valid one for identifying regions that contain tumor-suppressor genes.

Our hypothesis was that there was a gene on chromosome 18q that predisposed to both osteosarcoma and familial Paget disease. To test this, we mapped the minimum region of LOH for sporadic and Pagetic osteosarcomas to determine whether the minimum region of overlap included the region containing the chromosome 18q familial Paget disease locus.

Material and Methods

Patient Samples

Osteosarcoma samples were obtained from the primary or metastatic tumor sites in 96 cases. Matched normal samples were obtained from these osteosarcoma patients either as peripheral blood or as adjacent normal tissue from surgical specimens. Some of these samples were used for a previous study to map a chromosome 3q tumor-suppressor gene (Kruzelock et al. 1997). All of the sporadic osteosarcoma patients were under 35 years of age, and none had been diagnosed with Paget disease. Samples of osteosarcomas from patients with Paget disease were obtained as formalin-fixed postsurgical tissue samples of Pagetic bone and tumor. This research was done under the approval of the Institutional

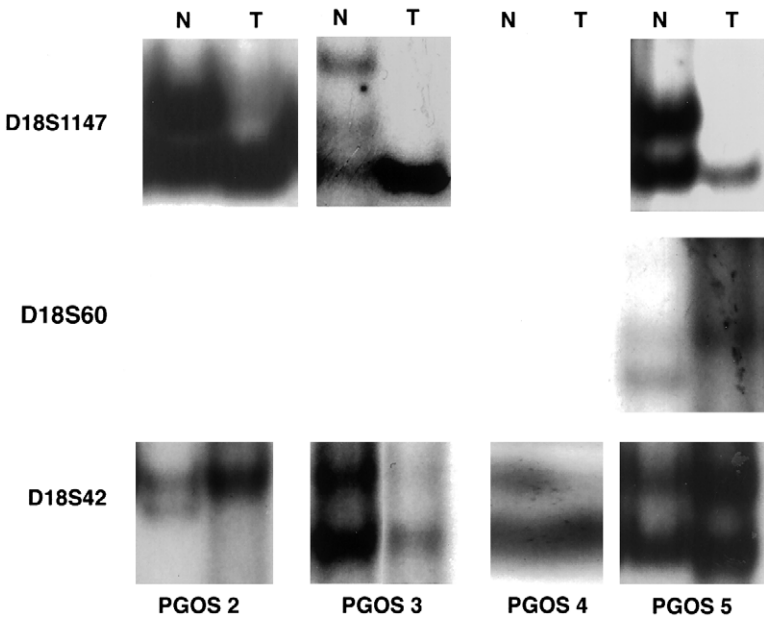

Figure 1 Representative LOH analysis of chromosome 18q loci in osteosarcoma tumors from patients with Paget disease, using STRPs. Matched normal (N) and tumor (T) samples were amplified by PCR by means of primers that amplify each specific polymorphic locus. Shown are results from D18S1147, D18S60, and D18S42.

Review Board, and informed consent was obtained from patients or their legal guardians in all cases prior to sample acquisition.

DNA Isolation

Osteosarcoma tumors were snap frozen at −80°C. DNA was isolated from frozen tumor samples and needle biopsies by a slight modification of a procedure reported elsewhere (Hansen et al. 1985). Care was taken during the tumor DNA isolation to minimize the amount of contaminating normal tissue. Instead of an initial hypotonic lysis, the frozen tumor sample was powdered in a liquid nitrogen–cooled mortar. The frozen powder was then mixed with a cell lysis buffer and incubated for 1 h at 42°C, followed by phenol/chloroform extraction and sodium acetate/ethanol precipitations. Matched normal DNA was extracted either from adjacent normal tissue as described above or from peripheral blood samples as described elsewhere (Miller et al. 1988). In either case, the final DNA pellet was suspended in TE^{-4} (10 mM Tris, pH 7.5, 0.1 mM EDTA).

The specimens from the Paget disease patients were obtained as formalin-fixed samples. In these cases, DNA was isolated as described elsewhere for paraffin-embedded tumor samples (Schubert et al. 1993).

Loss of Heterozygosity Analysis

Ten polymorphic loci were used for the LOH analysis. The simple sequence–length polymorphisms (dinucleotide, trinucleotide, and tetranucleotide repeats) were D18S487, D18S64, D18S499, D18S1148, D18S1147, D18S60, D18S42, D18S51, D18S55, and BCL2 (listed in proximal-to-distal order). The forward primer was end-labeled with polynucleotide kinase and $\gamma[^{32}P]$-ATP. The radiolabeled primer was then used in a standard PCR reaction to amplify matched normal and tumor DNA samples. The radiolabeled PCR amplification products were then electrophoresed on nondenaturing polyacrylamide gels (0.5 × TBE [0.045M Tris borate, 0.001M EDTA], 8% acrylamide) in a 38 × 50 cm sequencing gel apparatus. The gels were then dried and exposed to X-ray film for 6–24 h at −70°C with or without an intensifying screen.

In some cases, we used nonradioactive analysis in our LOH screening of tetranucleotide repeats. In these cases, the polymorphic loci were amplified as before, except that no radiation was added. The samples were run on a 1.5 mm × 20 cm × 20 cm nondenaturing polyacrylamide gel (1 × TBE, 8% acrylamide) in a vertical gel apparatus at 50V for 12 h and then stained with ethidium bromide and photographed under UV light.

YAC Contig Construction

In order to isolate yeast artificial chromosomes (YACs) spanning the crucial region, we first queried the CEPH/Généthon QUICKMAP database at the "level 7" confidence index. Level 7 includes *Alu* PCR hybridization and YAC fingerprinting data as well as STS-content of

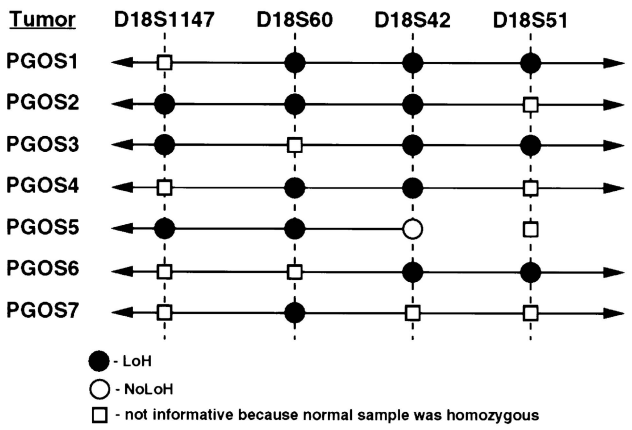

Figure 2 Summary of the data from the LOH analysis of osteosarcomas from Paget disease patients. Solid lines and arrows depict the region and direction of LOH for each tumor. Note that PGOS5 shows LOH only for the region proximal to D18S42.

YACs for polymorphic markers described by Weissenbach et al. (1992). The STS markers used to identify LOH were used to identify YACs in this region. The chromosome 18q YACs were individually arrayed and screened with all available chromosome 18 STSs (sequence information was obtained from the Genome Database) by PCR amplification. The PCR data were converted to a STS × YAC content data table and analyzed with the SEGMAP computer program. SEGMAP is a software package that analyzes biological physical mapping data and then creates contig maps from these data (Green and Green 1991). SEGMAP generates maps based on a set of clones and the site content information for that set. Clone size information is used to estimate site spacings. Each site is tested against a set of clones, the best order of sites is determined, and a map is constructed that agrees with this order. In the case of data inconsistencies, the inferred data errors in site content data are determined. Once a YAC contig map had been constructed, Généthon's genetic maps enabled us to orient the contig with respect to the centromere and telomere.

Results

LOH Analysis of Chromosome 18 in Osteosarcoma Samples

DNA samples from seven osteosarcomas from patients with Paget disease as well as matching Pagetic bone tissue were analyzed for LOH with use of the polymorphic loci D18S1147, D18S60, D18S42, and D18S51. Six of the seven osteosarcomas from patients with Paget disease showed LOH for the complete region analyzed. The seventh Pagetic osteosarcoma showed LOH for the region proximal to D18S42. Examples of this analysis are shown in figure 1 and the data are summarized in figure 2.

We also analyzed DNA from matched normal and tumor tissues from 96 sporadic osteosarcoma cases with up to 10 polymorphic loci. Of the 96 osteosarcoma tumors examined, 61 (64%) showed LOH for all or part of the region examined, while 35 (36%) showed no LOH for the region examined.

Identification of Minimal Region of LOH

Eight tumor samples underwent mitotic recombination with breakpoints in or near the region of chro-

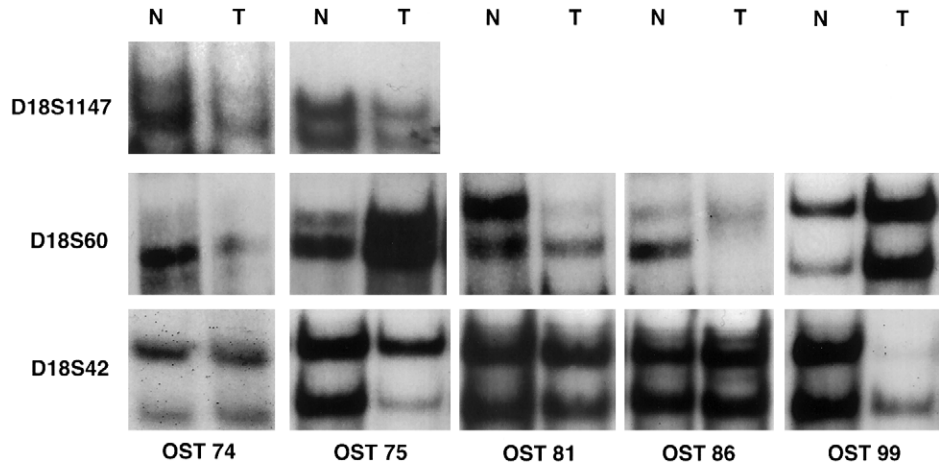

Figure 3 Representative LOH analysis of chromosome 18q loci in sporadic osteosarcoma tumors, using STRPs. Matched normal (N) and tumor (T) samples were amplified by PCR by means of primers that amplify each specific polymorphic locus. Shown are results from D18S1147, D18S60, and D18S42 that define the minimal region of LOH.

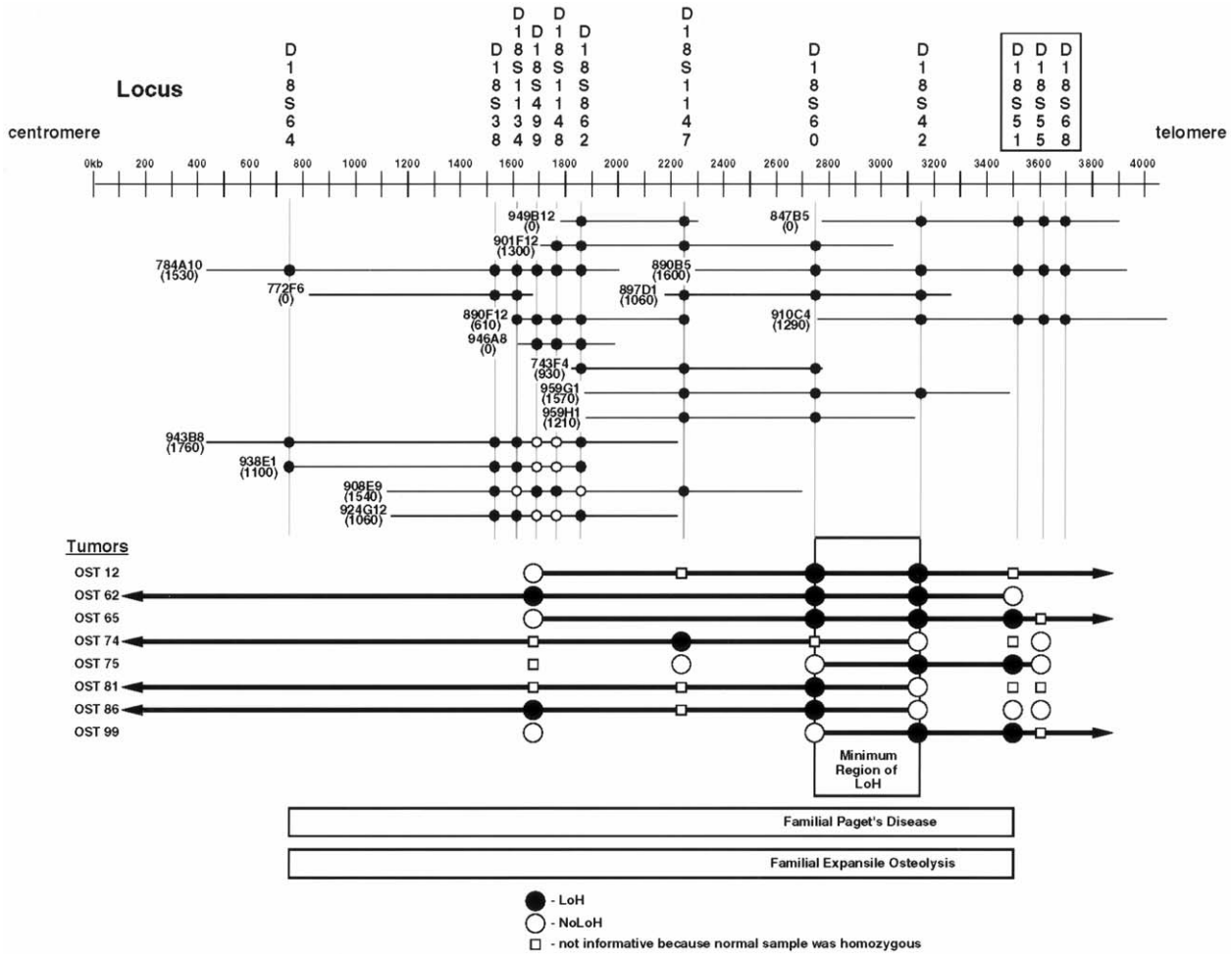

Figure 4 Map of the minimal region of LOH on chromosome 18q in osteosarcoma tumors. The polymorphic loci and the physical distances between them in kilobases, as defined by the YAC contig, are shown at top. The names of the YACs and their known sizes (in parentheses) are shown to the left of each YAC. Blackened circles represent positive PCR amplification with primers for a locus, unblackened circles represent negative PCR amplification with primers for a locus. Note that D18S51, D81S55, and D18S68 cannot be ordered in the contig and are shown within a box, to represent this point. Below the YAC contig data, the solid lines and arrows depict the region and direction of LOH for each tumor. The minimal region of LOH in the sporadic osteosarcomas as well as the region containing the familial Paget loci and the region containing the FEO locus are shown as boxes below the arrows.

mosome 18q that contained the Paget disease/FEO loci. Examples of the LOH analysis of this region from some of these tumors are shown in figure 3. The regions of LOH in these tumors were mapped to determine the location of the minimal region of LOH common to all nine tumor samples. Five tumor samples revealed breakpoints that defined this minimal region of LOH (fig. 4). OST 74, OST 81, and OST 86 underwent mitotic recombination such that the region proximal to D18S42 underwent LOH. OST 75 and OST 99 underwent mitotic recombination such that the region distal to D18S60 underwent LOH. From these we were able to conclude that the minimal region of LOH was between D18S60 and D18S42 (fig. 4).

Discussion

To test the hypothesis that osteosarcoma, familial Paget disease, and FEO involve either the same locus or closely associated loci on chromosome 18q, we examined osteosarcomas from Paget disease patients for LOH in the same chromosomal region as the familial Paget disease and FEO loci. The discovery that the osteosarcomas from Paget disease patients show LOH for the same region strongly suggests that there is a genetic link between the development of osteosarcoma and the gene that predisposes to Paget disease and FEO. LOH has been strongly associated with the presence of a tumor-suppressor gene within the region of LOH (Cavenee et

al. 1983; Hansen and Cavenee 1988; Yamaguchi et al. 1992). Interestingly, LOH for this region also occurs in sporadic osteosarcomas, suggesting that this region may contain a tumor-suppressor gene that is involved in both sporadic and Pagetic osteosarcoma. Using the sporadic osteosarcomas, we began to localize the region of LOH to determine whether the minimal region of LOH in the sporadic osteosarcomas coincided with the region linked to both Paget disease and FEO. To identify the region in which the tumor-suppressor gene was located, we used a technique called mitotic mapping (Scrable et al. 1987), which relies on the tendency of LOH to occur by mitotic recombination rather than by mitotic disjunction with loss of the entire chromosome, to sublocalize the region of LOH on the chromosomal arm. By examining a large number of tumors for LOH by mitotic recombination, it is possible to localize the smallest region of LOH, which should contain the tumor-suppressor locus, to an area that can be physically isolated and analyzed directly for the gene. By this analysis we were able to define a minimal region of LOH between genetic markers D18S60 and D18S42 on chromosome 18q. This region does not include the 18q tumor-suppressor genes DCC (Fearon et al. 1990) or DPC4 (Hahn et al. 1996), which have been implicated in the development of colon cancer and pancreatic cancer, respectively. However, it does overlap the region in which the predisposition to both familial Paget disease and FEO have previously been mapped (Hughes et al. 1994; Hughes and Barr 1996; Cody et al. 1997; Haslam et al. 1998) (fig. 4), suggesting that there may be a genetic association between Paget disease and osteosarcoma.

Similar associations between growth disorders or developmental disorders and cancer have also been found to result from mutations in other tumor-suppressor genes. Mutations in the WT1 gene have been implicated in both Denys-Drash syndrome and Wilms tumor (Pelletier et al. 1991); mutations in the NF1 gene have been linked to both von Recklinghausen neurofibromatosis and development of neurofibrosarcomas (Hartley et al. 1988, 1990); and mutations in the RET gene have been linked to both Hirschsprung disease and multiple endocrine neoplasia type 2 (Ponder 1994); whereas patients with Beckwith-Wiedemann syndrome (a hemihypertrophy growth syndrome) are at increased risk of rhabdomyosarcoma, hepatoblastoma, adrenocortical carcinoma, and Wilms tumor (Sotelo-Avila et al. 1980; Schneid et al. 1997). Inherited mutations in the familial adenomatous polyposis (FAP) locus have been shown to result in hyperproliferation of the normal colonic tissue (Vogelstein et al. 1988). Vogelstein and coworkers demonstrated that within these hyperproliferating cells a number of somatic events occurred that eventually gave rise to carcinoma. These additional genetic events included the homozygous inactivation of the FAP locus (Vogelstein et al. 1988).

An alternative model to explain the association between Paget disease and osteosarcoma would assert that, in those patients with both Paget disease and osteosarcoma, the association is the result of a deletion that inactivates two adjacent genes. This would be similar to the molecular events that give rise to the association between aniridia and Wilms tumor. In this case, there are two genes located within the WAGR contiguous gene–syndrome locus on chromosome 11p (Baird et al. 1992), in which multigenic deletions inactivate both the WT1 gene and the PAX6 gene and predispose to both disorders. If this model is true in Paget disease, then the mapping of the osteosarcoma tumor-suppressor gene and the subsequent analysis of osteosarcomas from Paget patients would reveal deletions that extend beyond the osteosarcoma gene and into an adjacent gene, providing us with a tool to identify the Paget disease locus.

Evidence exists that at least one other familial Paget disease locus exists in the genome. In at least one Paget disease family, the complete chromosome 18 candidate region has been excluded for harboring the disease-causing mutation (Haslam et al. 1998). Sporadic osteosarcomas have been shown to undergo LOH for other chromosomes as well (Yamaguchi et al. 1992), suggesting that there may be further associations between Paget disease predisposition and osteosarcoma tumorigenesis that could be identified through LOH analysis.

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Electronic-Database Information

Accession number and url for data in this article are as follows:

Genome Database, http://www.gdb.org (for sequence information used in YAC contig construction)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Paget disease [MIM 167250])

CEPH/Généthon QUICKMAP Database, http://www.ceph.fr/quickmap.html (for YAC mapping information)

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