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Loss of Heterozygosity and Microsatellite Instability at the Retinoblastoma Locus in Osteosarcomas

[Original Article]

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Abstract

Studies of osteosarcoma cell lines or frozen tissue have detected loss of heterozygosity (LOH) at the retinoblastoma (RB) locus by Southern blot analysis or restriction fragment length polymorphism. Most archived clinical specimens cannot be analyzed by these techniques. We analyzed formalin-fixed, paraffin-embedded samples from 19 cases of osteosarcoma for molecular changes at the RB locus using polymerase chain reaction amplification of polymorphic short tandem repeat sequences (microsatellite repeats). Four repeat sequences, two within and two flanking the RB gene, were analyzed. Fourteen of 18 informative cases (78%) showed molecular changes at the RB locus. LOH was identified in 13 cases (72%). Unexpectedly, microsatellite instability (MI) was found in eight cases (44%). All of the cases of MI involved alterations of more than one repeat unit, and six of eight were associated with LOH. LOH was identified at three unlinked loci in one case and at a single locus in another. Microsatellite analysis of archival tissue yields prevalence rates of LOH comparable to those found by other methods and has the added advantage of showing MI. The ability to use formalin-fixed, paraffin-embedded tissue extends genetic analysis to routinely processed surgical material and may permit molecular confirmation of challenging cases of osteosarcoma.

The retinoblastoma (RB) gene is implicated in the pathogenesis of osteosarcoma, a highly malignant bone tumor primarily affecting adolescents. The RB gene, located on chromosome 13q14, encodes a 105-kd nuclear phosphoprotein (pRB) that binds transcription factors and blocks cell cycle progression from G1 phase to S phase (8,25,26). Restriction fragment length polymorphism analysis of osteosarcoma cell lines and frozen tumor tissue by either Southern hybridization or by polymerase chain reaction (PCR) has demonstrated loss of heterozygosity (LOH) around the RB locus in 63-71% of cases (10,23,24,29). Additional studies aimed at more closely identifying internal alterations of the RB gene have been performed using an RB cDNA probe hybridized to the tumor DNA. These studies have found structural alterations of the RB gene in 35-45% of cases examined (2,19,23) (Table 1). Replacement of a defective RB gene by a normal gene can inhibit growth and suppress tumorigenicity in immunodeficient mice (12,30). These data support the hypothesis that RB plays an integral role in the development of osteosarcoma.

Study	Methods	LOH%	Structural anomalies[a]
Hansen et al. (10)	SR	71% (5/7)	
Toguchida et al. (23)	SC and SR	64% (18/28)	43% (13/30)
Araki et al. (2)	SC		35% (8/23)
Scheffer et al. (19)	SC		45% (5/11)
Yamaguchi et al. (29)	SR	68% (25/37)	
Wadayama et al. (24)	PR and SC	63% (39/62)	
Current study	PM	72% (13/18)	

SR, Southern blot with restriction fragment length polymorphism; SC, Southern blot with cDNA probe; PR, polymerase chain reaction with restriction fragment length polymorphism; PM, polymerase chain reaction–microsatellite.

[a] Structural anomalies refers to internal alterations in the sequence of the RB gene as identified by Southern blot hybridization with normal RB gene-derived cDNA as probe.

TABLE 1. *Molecular changes at the RB locus in osteosarcomas*

The primary objective of this study was to develop a technique that would enable retrospective identification and analysis of molecular changes at the RB locus. Ultimately, we intend to develop a clinical assay that can exploit these molecular alterations prospectively, providing a basis for distinguishing osteosarcomas from their benign histologic mimics. A second objective was to map regions of LOH on a relatively fine scale by choosing four short tandem repeats (STRs) to span the RB locus.

MATERIALS AND METHODS

Specimens

All tissues were fixed in formalin as standard surgical specimens, usually for <=24 h, before embedding in paraffin. Eighteen cases of osteosarcoma were retrieved from the pathology archival files of the M. S. Hershey Medical Center, and one case was obtained from Oakland Children's Hospital, California. Fourteen cases were subtyped as osteoblastic, one as fibrohistiocytic, two as telangiectatic, and one as chondroblastic. One periosteal osteosarcoma arose after radiation and is not further subclassified (16). In five cases (four of the osteoblastic subtype and one telangiectatic), both primary and metastatic tumors were available for analysis. In four cases (all of the osteoblastic subtype), tumor was available from both the initial biopsy and the posttherapy resection specimen. (Chemotherapy is often administered before resection of the primary lesion.) Frozen and formalin-fixed tissue was analyzed in parallel from one case and was consistently heterozygous at all loci. DNA from the Saos-2 osteosarcoma cell line (gift of Dr. N. Chandar), containing a homozygous deletion of the 3' end of the RB gene (21), was used as a control for the demonstration of LOH.

DNA Extraction

A single 15-μm section from each case was cut from formalin-fixed, paraffin-embedded tumor tissue blocks. Whenever possible, nondecalcified tissue was used, with the initial frozen section block being preferentially chosen. Tumor tissue was carefully dissected off the slide with a sterile scalpel to obtain a sample as free of contamination normal tissue as possible. Normal, nontumorous tissue was obtained separately for each patient.

The tissue was placed in 200 µL of lysis buffer consisting of 10 mmol/L Tris at pH 8, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.45% Tween 20, and ddH_2O (18) and digested with 200 µg proteinase K for 24 h at 55° with constant shaking. After the first 24 h a second 200-µg aliquot of proteinase K was added to each test tube, and digestion continued for an additional 24 h. The sample was purified through a Sephadex G50 column. DNA was quantitated by microfluorometry (model TKO 100; Hoefer, San Francisco, CA, U.S.A.) according to the manufacturer's directions before Sephadex purification.

STRs

Primer sequences for four highly informative microsatellite repeats (polymorphic information content >70%) were obtained from the Genome Data Bank (Johns Hopkins University). Genetic loci were chosen to lie within (D13S153 and RB1.20) and adjacent to the gene (D13S118 and D13S119) (Table 2 and Fig. 1A). In 10 cases exhibiting microsatellite instability and/or LOH, two additional chromosomes were analyzed using three highly polymorphic microsatellite DNA markers (D15S119, D15S122, D19S206) (Table 2). All primers, with the exception of RB1.20, were obtained from Research Genetics. RB1.20 was analyzed using either a nested amplification strategy (B57 and B103 followed by reamplification with RBA and RBB) or a heminested strategy (RBA and RBB followed by reamplification with one inner and one outer primer).

Locus	Primer name	Sequence	Annealing temperature
D13S153	AFM058xd6a	AGCATTGTTTCATGTTGGTG	57
	AFM058xd6m	CAGCAGTGAAGGTCTAAGCC	
D13S118	1312TG	CCACAGACATCAGAGTCCTT	54
	1312R	GAAATAGTATTTGGACCTGGG	
D13S119	1310R	AAGACTTTGAATGAAATTCCC	54
	1310L	TTATTGCCTTTGTAGATCATTG	
RB1.20 (inner pair)	RBA	GAGAGACAGGCATTTGGACC	59
	RBB	CTCCAGCCTGGGTAACAGAG	
RB1.20 (outer pair)	B57	TGTATCGGCTAGCCTATCTC	54
	B103	AATTAACAAGGTGTGGTGGT	
D15S119	AFM150xf4m	ACTTTTGTGCCATTTAGAGATT	55
	AFM150xf4a	AACAGAAATCCGTAACATAACATA	
D15S122	AFM200wb4a	GATAATCATGCCCCCCA	55
	AFM200wb4m	CCCAGTATCTGGCACGTAG	
D19S206	kp/1521	TTCATCAAGTCTGTTCCAGCC	55
	kp/1713	AGCCGAAGTCTTTTCACAAGAG	

TABLE 2. *STR oligonucleotide primers*

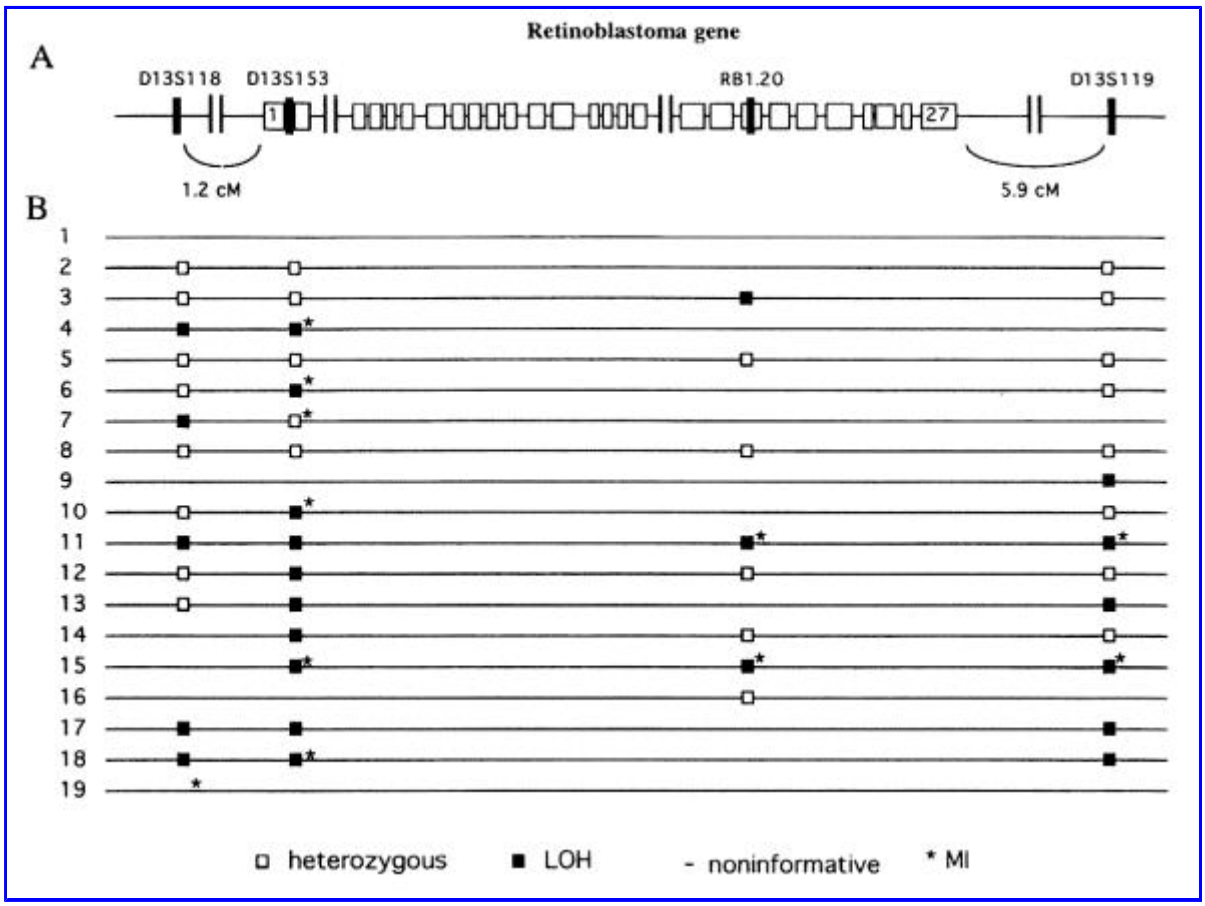

FIG. 1. Loss of heterozygosity and microsatellite instability in osteosarcomas. **A:** Position of STRs with respect to the retinoblastoma locus. Open boxes indicate exons 1-27 of RB, parallel lines denote large introns. Schematic is not drawn to scale. cM, centiMorgan. **B:** Location of LOH and MI at the RB locus in each patient (cases 1-19).

PCR Conditions

PCR was performed on a Perkin-Elmer Thermocycler 480. Each amplification reaction contained 1× magnesium-free *Taq* buffer (Promega, Madison, WI, U.S.A.), 50-100 ng DNA, 1 U *Taq* polymerase (Promega), and 1 µg RNAse A; it also contained 400 µmol/L dNTPs and 1 µmol/L each upstream and downstream primer in a final reaction volume of 10 µl. Optimal $MgCl_2$ concentrations varied, depending on the amplification primers. One primer of each pair was end-labeled with ^{32}P (17). DNA samples were heated to 94° before adding *Taq* polymerase ("hot start"). PCR amplification was carried out using the following parameters: 1 min at 94°, 1 min 24 s annealing, and 2 min at 72° for 30-35 cycles with a final 10-min extension at 72°. Alleles were separated by 4% denaturing polyacrylamide gel electrophoresis at 1,700 V for 2-3 h (17). The gels were dried and exposed to non-preflashed film at either room temperature or -70° for 2-24 h. Autoradiographs were scanned on a Molecular Dynamics Model 100A laser densitometer, and bands from both normal and tumor tissue were quantitated.

Interpretation

In order for a result to be informative, two different alleles (one from each parent) had to be present in the normal tissue, indicating a heterozygous state. Noninformative results were characterized by either homozygosity of the normal tissue or failure of one or more of the samples to amplify. LOH was defined as either complete absence of one allele in the tumor tissue that was present in the normal tissue or a reduction in the ratio of intensities of one allele to the other allele of >50%. Contaminating normal stromal or inflammatory cells account for the faint residual bands. The presence of new bands migrating differently than in the patient's normal tissue represents instability or mutation of the STRs. Two types of microsatellite instability have been

defined: type I shows large (multiple unit) deletions or additions in the sequences, and type II shows small (single unit) changes (1,22).

RESULTS

Informative results were obtained with at least one of the markers in 18 cases. Fourteen of the 18 (78%) showed evidence of either loss of heterozygosity at the RB locus or microsatellite instability (MI) (Fig. 1B). Thirteen (72%) tumors showed LOH with one or more of the markers (Fig. 2). LOH was most frequently identified with D13S153 (10 cases). D13S118 and D13S119 showed approximately equal rates of LOH (five and six cases, respectively). In all but one instance, the lost allele was reduced in intensity >61%. RB1.20 was the least useful marker because of frequent failure to amplify, which was not improved by nested or heminested PCR. Eight specimens (44%) showed MI, all type I, with six of the eight evident at D13S153 (Fig. 3). In six cases, LOH and MI were present at the same locus or loci. Only one instance of MI in the demonstrable absence of LOH was found. The case in which both frozen and formalin-fixed tissue was evaluated failed to show LOH or MI in either preparation.

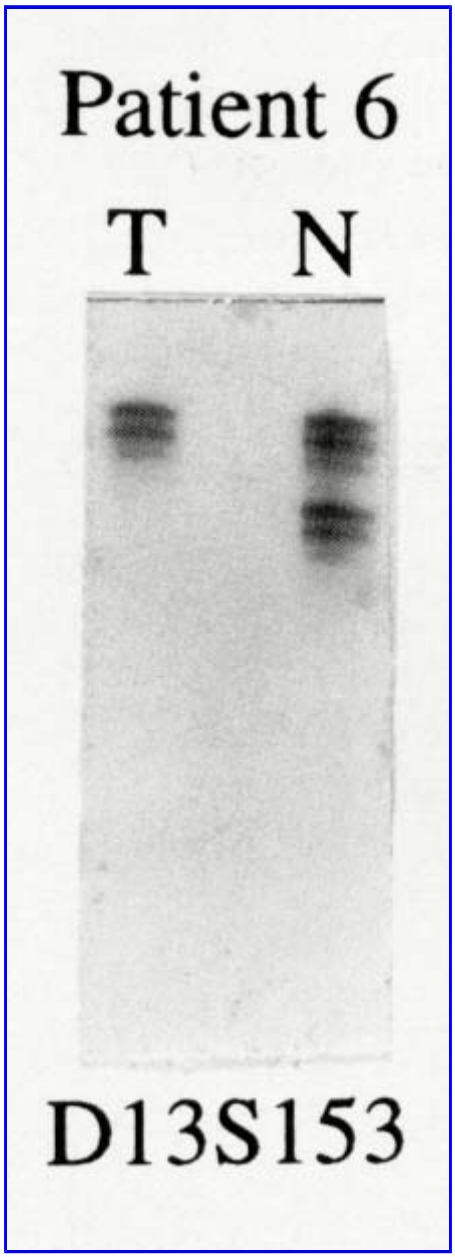

FIG. 2. Loss of heterozygosity in the retinoblastoma gene (patient 6) is demonstrated by loss of one microsatellite allele in comparison with normal.

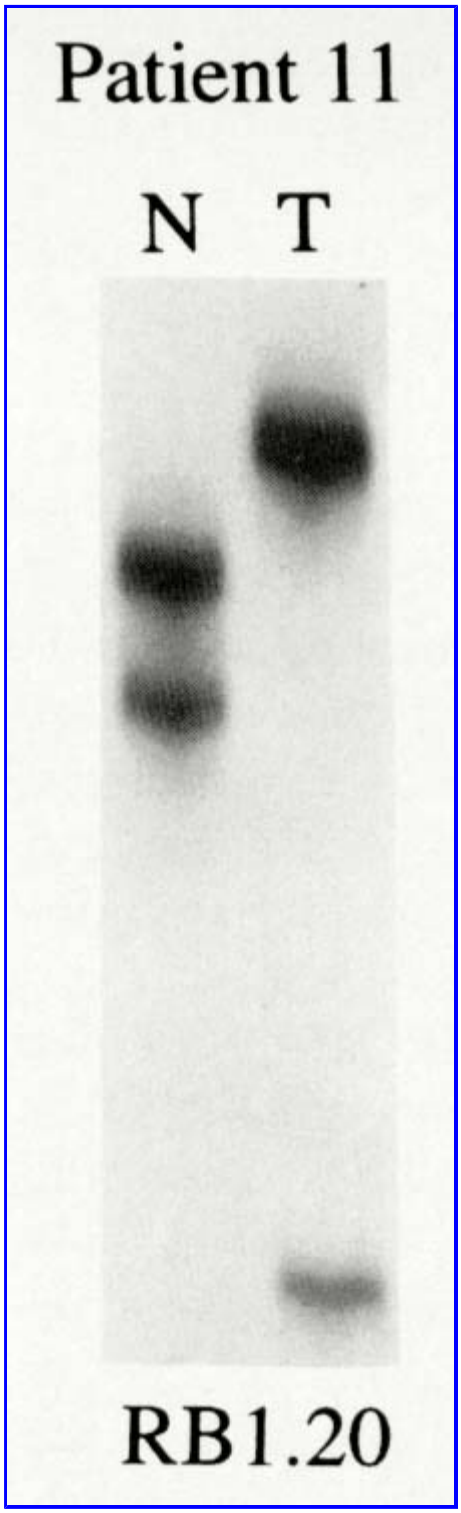

FIG. 3. Microsatellite instability (patient 11) is evidenced by the altered bands in tumor sample as compared with normal. Note also the absence of both normal alleles.

Of the five cases in which both primary and metastatic tumor were available for analysis, two cases (both osteoblastic) showed LOH in the primary tumor and both LOH and MI in the metastatic tumor. Two other cases (both osteoblastic) showed only LOH in both the primary and the metastatic lesion, and one case (telangiectatic) was noninformative. Four cases (all osteoblastic) with both pre- and posttherapy specimens were analyzed. Two demonstrated LOH in both specimens, and the other two showed LOH only in the primary tumor. Histologically, the posttherapy specimens consisted almost entirely of viable-appearing tumor, making contamination with normal tissue unlikely. One posttherapy resection specimen showed MI as well as LOH. Ten of the cases that

showed MI and/or LOH were examined for similar phenomena at other chromosomes (15 and 19). LOH was found at all three loci (D15S119, D15S122, and D19S206) in one case and at a single locus (D15S119) in a second case. MI was not observed at these loci in any case.

DISCUSSION

The RB gene is the prototypical tumor suppressor gene. Alterations of either the RB gene or its expression pattern have been described for a wide variety of soft-tissue tumors and carcinomas (4,27). The role played by RB in these different tumors seems to vary. In some neoplasms the presence of LOH at the RB locus is associated with a worse prognosis or more aggressive behavior (5,15,27). In other tumors, including osteosarcomas, molecular alterations at RB appear to be more closely related to the early stages of tumorigenesis (3,28). Initial work has frequently demonstrated alterations in the RB gene in both cell lines and primary tumors (Table 1).

We have shown that analysis of archival tissue using PCR amplification of polymorphic STRs identifies genetic alterations with the same frequency as techniques requiring frozen tissue. Using formalinfixed, paraffin-embedded tissue, LOH was seen in 72% of tumors and MI in 44%, with a total of 78% of the tumors showing evidence of molecular aberrations at the RB locus. This method does not allow us to distinguish between LOH due to a deleted allele and LOH due to a gene conversion event, but the latter has not been detected in Southern hybridization-based studies (23).

By selecting STRs that span the RB locus, we have also succeeded in mapping, on a relatively fine scale, the location of the abnormalities within the RB locus. For example, in cases 3, 10, and 12, only a single STR showed LOH, whereas flanking regions remained heterozygous. Interestingly, loss of only the 5' (case 14) or 3' (case 3) end of the gene can be observed, suggesting that loss of either is sufficient for tumorigenesis or tumor progression. However, our data show that larger deletions involving most or all of the RB gene and flanking regions are more common, as others have reported (24). The STR in the first intron of the RB gene (D13S153) showed LOH or MI in 73% of informative cases, much higher than other STRs (Fig. 1). Whether this finding is indicative of greater susceptibility of this region to in vivo alteration or is the result of small sample size is uncertain. Analysis of additional STRs within the locus may provide additional information about the gene and its role in tumor development.

Chromosome regions other than the RB locus also show elevated rates of allelic loss in osteosarcomas (9,20,23,29). Two of the 10 tumors analyzed in this study showed LOH on other chromosomes, a proportion lower than that of similar regions (47%) (23). Examination of other chromosomes, particularly around the p53 tumor suppressor gene on chromosome 17, will confirm whether the tumors we have studied are distinct in their genomic instability.

An unexpected finding in our study was the presence of MI. Microsatellite instability, initially described in colorectal carcinomas (22), has since been found in many tumors (6,7). The frequency of this finding varies considerably, with tumors from patients with hereditary nonpolyposis colorectal cancer syndrome (HNPCC) showing mobility shifts in <=77% of carcinomas (13), whereas only 13% of sporadic colorectal carcinomas showed these changes. These changes are thought to represent errors in DNA repair and replication, often involving such DNA repair enzymes as hMLH1 and hMSH2 (14). Different patterns of MI have been noted. The first, exemplified by HNPCC, is characterized by finding MI at multiple loci on different chromosomes, perhaps indicating an inherited genetic instability. The other pattern is characterized by MI apparently restricted to certain loci (22). We did not detect MI at loci other than RB. There was no history of a hereditary cancer syndrome in any of the patients studied. One tumor, a postradiation osteosarcoma, arose as a second malignancy in a child with rhabdomyosarcoma treated with radiation (case 11). Osteosarcoma is the most common secondary malignancy to occur in children with rhabdomyosarcoma (11). This tumor showed extensive

molecular derangement of the RB locus involving all four markers analyzed.

In summary, PCR analysis of STRs for LOH detection is a sensitive and rapid technique that has distinct advantages over other methods. First, it is capable of retrieving tumor DNA sequences from archival specimens, allowing retrospective analysis of rare tumors, such as osteosarcomas. As a result, studies of large, well-characterized groups of patients with long-term follow-up are possible. Second, microdissection of formalin-fixed tumors to homogeneity is simple. Third, relatively large amounts of DNA are obtained, so that numerous STRs may be studied. Fourth, the method obviates the need to grow the tumor in culture or in animals, avoiding the introduction of any in vitro molecular alterations. Fifth, in diagnostically challenging cases, the finding of LOH at the RB locus might provide helpful confirmation of the malignant nature of a tumor. We have obtained preliminary results in 31 benign histologic mimics of osteosarcoma that indicate no loss of heterozygosity (Belchis et al., unpublished observations). These data support the hypothesis that aberrations of the RB gene relate directly to the malignant state of osteosarcomas and that identification of these changes may confirm a malignant diagnosis.

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Key Words: Osteosarcoma; Loss of heterozygosity; Retinoblastoma gene; Polymerase chain reaction; Microsatellite instability

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