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Genetics

Mechanisms of p53 loss in human sarcomas

(tumor suppressor gene/rhabdomyosarcoma/osteosarcoma)

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ABSTRACT **An important role for the p53 gene in neoplastic transformation *in vitro* and *in vivo* has been imputed by functional studies and identification of tumor-acquired gene defects or alterations in its expression. To study the generality and mechanisms of p53 alteration in human cancer, we examined 241 tumors of several types for structural aberrations of the locus. Alterations of the gene or its RNA or protein products consistent with loss of function by either recessive or dominant mechanisms were identified among this set uniquely in rhabdomyosarcomas and osteosarcomas. The alterations of p53 in rhabdomyosarcoma tumors included cases with complete deletion of both p53 alleles, complete deletions of one allele with or without point mutation of the remaining allele, and absence of detectable RNA. Similarly, we detected homozygous deletion and lack of expression of p53 RNA or aberrant expression of p53 protein in osteosarcomas. These observations provide strong support for the inclusion of the p53 locus in the group of loci whose functional inactivation by either dominant or recessive modes plays a significant role in human cancer.**

Evidence for a genetic component in the etiology of human neoplastic disease is accumulating through cytogenetic (1) and molecular genetic (2) identification of tumor-specific genomic changes. In the latter case, these events have been recognized as specific deviations from the germ-line genotype that result in homozygosity (3) for mutant alleles at loci that appear to function normally as suppressors of the tumorigenic phenotype (2). The paradigm case for involvement of this type of locus in human cancer is the embryonal tumor, retinoblastoma (4). Tumor-specific somatic loss of heterozygosity for loci in the chromosome 13q14 region has led to a general model of tumorigenesis requiring loss of function of both alleles at a tumor-suppressor locus (3, 4). A gene with properties consistent with such a role has been isolated (5–7) and its authenticity inferred from genetic behavior in tumors including homozygous or heterozygous structural alterations arising from deletion, rearrangement, or point mutation (5–10) as well as absence or altered size of RNA transcripts (5–9) or proteins (11) encoded by the gene. The recessive nature of retinoblastoma gene mutations is further suggested by complementation of inactivated alleles by expression of an exogenous retinoblastoma (RB) gene *in vitro* (11).

Regions of the genome that show frequent genetic alterations or isodisomy in tumors suggest potential localizations for other tumor suppressor genes (2, 3). One such region is the 17p12–17p13.3 area, which has shown frequent losses of heterozygosity in glioma, colon carcinoma, small cell lung carcinoma, breast carcinoma, and osteosarcoma (12–16). The gene for p53, a protein that has been implicated in tumorigenesis in mice and transformation *in vitro* (reviewed in ref. 17) and that has shown structural alterations in some tumors and tumor cell lines (13, 18–21), has been mapped to this region (22). The p53 gene encodes a 53-kDa nuclear phosphoprotein that is detectable in some transformed cell lines of murine and human origin as well as in developing tissues (reviewed in refs. 17, 23, and 24). The wild-type protein is present at very low levels under normal conditions, regardless of the prevalence of p53 transcript (17, 23, 24). A variety of mutations in the p53 gene can result in a protein with increased stability and may counteract the wild-type product of the normal p53 allele, since elevated levels of mutant p53 protein can lead to cellular transformation (25–28). A similar dominant genetic mechanism for loss of gene function has been inferred in several tumors in which p53 alleles carrying point mutations are detected irrespective of the presence of a wild-type allele (13, 19, 21). Evidence also exists for loss of gene function through recessive genetic mechanisms requiring inactivation of both p53 alleles. For example, genomic rearrangements of the p53 gene have been identified in mouse erythroleukemia cell lines and in human and murine cell lines (17, 18, 29, 30).

To determine the mechanisms of p53 inactivation that occur commonly in human neoplasia, we examined 241 tumors of several types for gross structural alterations of the p53 genomic locus. Only two groups among these, rhabdomyosarcoma and osteosarcoma, showed such aberrations. To assess the generality of p53 mutations and to define the mechanisms by which inactivation may be accomplished, we have analyzed the p53 locus and its products in these tumors.

MATERIALS AND METHODS

DNA Isolation and Analysis. Tumor samples were a random collection acquired without selection through various sources and should be representative for each tumor type. High molecular weight DNA was isolated from tissue and blood samples as described (31, 32). DNA samples were digested with appropriate restriction enzymes, fractionated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized to probes radiolabeled by random oligonucleotide priming (33).

The chromosome 17 loci examined and corresponding DNA probes used were *D17S4* (pTHH59), *D17S78* (p131-A8), *GH1* (C-H800), *D17S71* (pA10.41), p53 (pCDpro53), *MYH2* (p10.5), *D17S1* (pHF12-1), *D17S5* (pYNZ22), *D17S28* (pYNH37.3), *D17S29* (pYNM67), and *D17S31* (pMCT35.1) (refs. 34–42).

RNA Isolation and Analysis. Total RNA was isolated from primary or nude mouse xenografted tissue by the method of Chirgwin *et al.* (43). Thirty micrograms of RNA was electrophoresed through 1% agarose/formaldehyde gels and transferred to Hybond-N membranes (Amersham) using 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

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Northern blots were hybridized to ^{32}P-labeled pCDpro53 (39) or human β-actin probe pHFβA-1 (44) in 50% formamide/0.04 M $NaPO_4$, pH 6.7/5× SSC/0.05% SDS/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin at 42°C for 36 hr.

Protein Isolation and Western Blot Analysis. Crude protein extracts from tumor tissues were prepared in 1% Triton X-100/1% sodium deoxycholate/50 mM Tris·HCl, pH 7.4/0.15 M NaCl/0.1% SDS/2 mM phenylmethylsulfonyl fluoride. Proteins were separated on SDS/10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting at 7 V·cm^{-1} for 12 hr. Western blots were incubated with the human-specific anti-p53 monoclonal antibody, PAb1801, and p53 proteins were visualized with alkaline phosphatase-conjugated anti-mouse IgG (Promega) as described (39).

Amplification and Sequencing of the p53 Locus. The p53 locus was amplified from genomic DNA by the polymerase chain reaction. Five micrograms of genomic DNA was amplified for 30 cycles of 94°C for 1 min, 55°C–60°C for 2 min, and 72°C for 3 min in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/200 μM dNTPs/2.5 units of *Thermus aquaticus* DNA polymerase (*Taq* I DNA polymerase) (Pharmacia) in the presence of 1 μM oligonucleotide primers (13). Polymerase chain reaction amplified sequences were cloned into the *Eco*RI site of pGEM3 (Promega) and sequenced by the Sanger dideoxynucleotide method (45) using Sequenase (United States Biochemical) and alkali-denatured double-stranded templates. Sequence variations were confirmed in a minimum of two clones resulting from independent DNA amplifications.

RESULTS

Constitutional and tumor genotypes at loci on chromosome 17p and the structural integrity of the p53 locus were analyzed for 241 patients with rhabdomyosarcoma (31 cases), osteosarcoma (29 cases), glioma (36 cases), colon carcinoma (11 cases), retinoblastoma (26 cases), Wilms tumor (26 cases), neuroblastoma (14 cases), medullary thyroid carcinoma (12 cases), breast carcinoma (11 cases), renal cell carcinoma (10 cases), Ewing sarcoma (7 cases), and a smaller number of

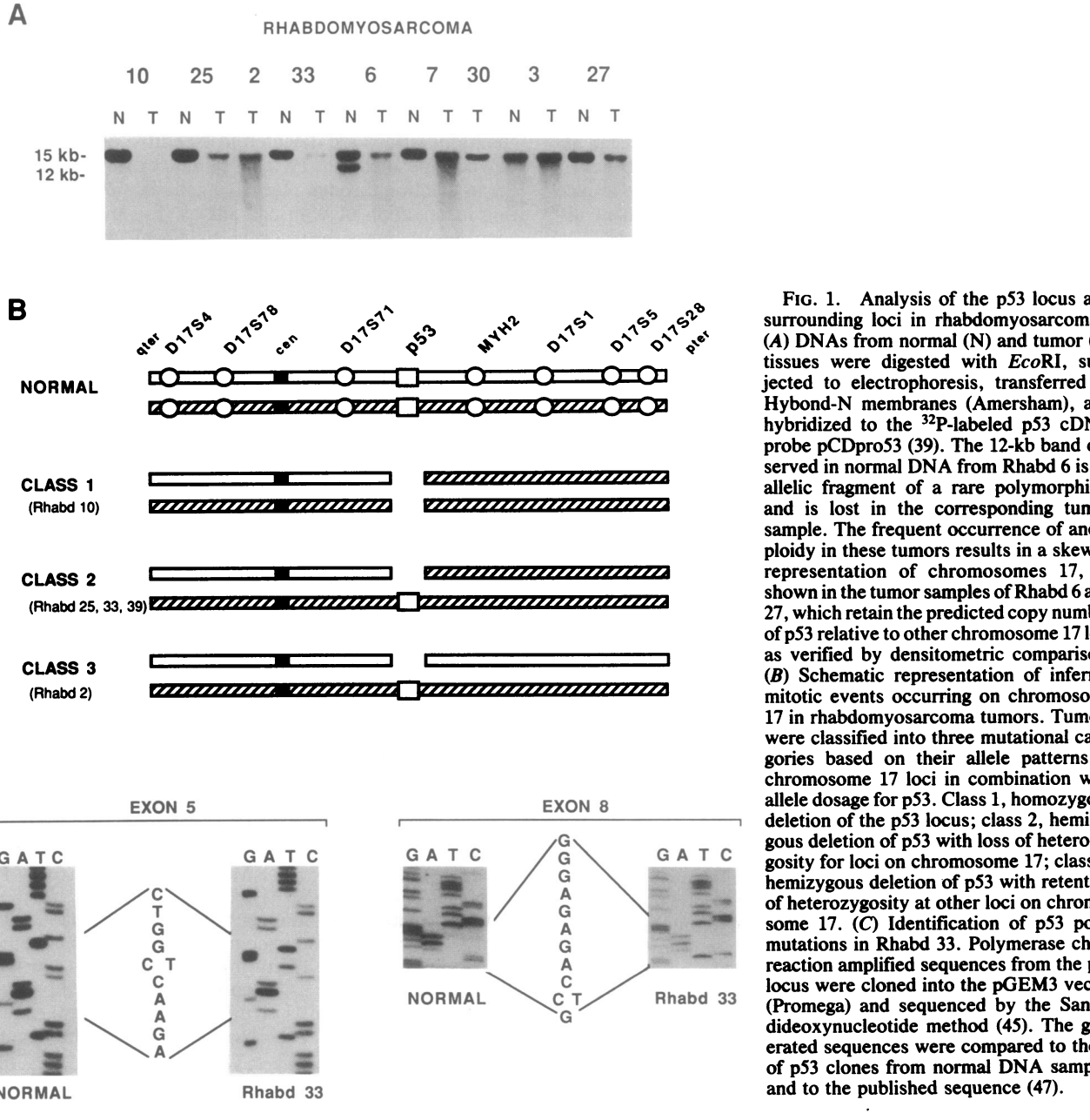

FIG. 1. Analysis of the p53 locus and surrounding loci in rhabdomyosarcomas. (*A*) DNAs from normal (N) and tumor (T) tissues were digested with *Eco*RI, subjected to electrophoresis, transferred to Hybond-N membranes (Amersham), and hybridized to the ^{32}P-labeled p53 cDNA probe pCDpro53 (39). The 12-kb band observed in normal DNA from Rhabd 6 is an allelic fragment of a rare polymorphism and is lost in the corresponding tumor sample. The frequent occurrence of aneuploidy in these tumors results in a skewed representation of chromosomes 17, as shown in the tumor samples of Rhabd 6 and 27, which retain the predicted copy number of p53 relative to other chromosome 17 loci as verified by densitometric comparison. (*B*) Schematic representation of inferred mitotic events occurring on chromosome 17 in rhabdomyosarcoma tumors. Tumors were classified into three mutational categories based on their allele patterns at chromosome 17 loci in combination with allele dosage for p53. Class 1, homozygous deletion of the p53 locus; class 2, hemizygous deletion of p53 with loss of heterozygosity for loci on chromosome 17; class 3, hemizygous deletion of p53 with retention of heterozygosity at other loci on chromosome 17. (*C*) Identification of p53 point mutations in Rhabd 33. Polymerase chain reaction amplified sequences from the p53 locus were cloned into the pGEM3 vector (Promega) and sequenced by the Sanger dideoxynucleotide method (45). The generated sequences were compared to those of p53 clones from normal DNA samples and to the published sequence (47).

cases of hepatoblastoma, fibrosarcoma, pheochromocytoma, papilloma, medulloblastoma, ependymoma, undifferentiated sarcoma, adrenal carcinoma, yolk sac tumor, primitive neuroectodermal tumor, and acute nonlymphocytic leukemia. Our detection of isodisomy for loci on chromosome 17p, without accompanying gross alteration of the p53 locus in colon carcinoma and gliomas, was consistent with previous reports (13, 15) that identified point mutations of p53 in these tumors. However, we detected gross alterations of the p53 gene at high frequency in only a subset of sarcomas, specifically rhabdomyosarcoma and osteosarcoma.

Alterations of p53 in Rhabdomyosarcoma. The first group was the rhabdomyosarcomas, pediatric tumors of striated muscle (31, 46). Upon comparison of tumor and constitutional genotypes at loci on chromosome 17p encompassing the region of the p53 locus, loss of heterozygosity was detected in 7 of 31 tumors examined (Rhabd 6, 7, 10, 21, 25, 33, 39). Structural alterations of the p53 locus were identified in 5 tumors.

The first class of alteration comprised complete deletion of both alleles of the p53 locus as detected in DNA from both primary and xenografted samples of the Rhabd 10 tumor (Fig. 1*A*), indicating a homozygous deletion spanning the coding sequence of the gene, a minimum of 20 kilobases (kb) of genomic DNA (47). Although the end points of this deletion have not yet been precisely mapped, they must lie within the region flanked by the *MYH2* and *D17S71* loci, since these remain intact in the tumor. Dosage of loci on the 17p and 17q arms in the tumor was equivalent (Table 1) and restriction fragment length polymorphism analyses indicated loss of heterozygosity at the *D17S5* and *D17S28* loci. These combined results (Fig. 1*B*) suggest that deletion of the p53 locus on one homologue preceded regional chromosomal loss and duplication, probably through mitotic recombination, and makes two separate intrachromosomal deletions unlikely.

The second class of aberrations is exemplified by Rhabd 25, 33, and 39. These tumors lost heterozygosity at loci on chromosome 17 (Fig. 1*B*) and appeared to have a diminution of copy number of the p53 locus (Fig. 1*A*). Rhabd 2, representing a third class of alterations, demonstrated no loss of heterozygosity on chromosome 17. However, in each of these classes densitometric analysis (Table 1) indicated dosage equivalence of loci on the short and long arms of chromosome 17 and approximately half the p53 locus copy number. The absence of aberrant size fragments in multiple restriction digests indicated that the deletions encompassed the entire p53 gene (Fig. 1*A*).

Table 1. Comparison of the dosage of the p53 locus to other loci on the short and long arms of chromosome 17

Patient	Ratio p53/17q	Ratio 17p/17q
Rhabdomyosarcoma		
Rhabd 10	0.0	0.81
Rhabd 25	0.54	0.87
Rhabd 39	0.51	0.93
Rhabd 2	0.53	0.96
Rhabd 33	0.48	0.88
Osteosarcoma		
Osteo 6	0.0	0.58
Osteo 25	0.11	0.70

DNA samples from normal and tumor tissues were used to prepare Southern blots as described for Fig. 1 and were hybridized sequentially to ^{32}P-labeled probes for loci on chromosome 17p or 17q and the p53 cDNA. The p53/17q ratios are an expression of the relative hybridization intensities at the p53 and *D17S4* loci in tumor compared to normal tissues from the indicated patient or heterologous tissue. The 17p/17q ratio expresses a similar relationship between the chromosome 17p loci, *D17S5* or *D17S28*, and the 17q locus, *D17S4*.

Because these tumors retained single copies of the p53 locus, which were intact by gross structural criteria, we sought to determine whether the remaining alleles contained point mutations or minute alterations undetectable by Southern blot analysis. Using oligonucleotides homologous to sequences that flank the putative mutational hot spots within the p53 gene (21), we amplified and sequenced the remaining p53 allele in tumors from each of these classes (Rhabd 2 and 33) and from Rhabd 7, which was isodisomic for the p53 locus. Whereas no variation from the normal sequence was detected in Rhabd 2 and 7, two point mutations were detected in the retained p53 allele of Rhabd 33. The first was a C to T base-pair substitution within exon 5 at codon 137 (Fig. 1*C*). This would represent a glycine to valine change in the translation product. The second alteration, also a C to T substitution, was in exon 8 at codon 282 (Fig. 1*C*) and would result in an arginine to tryptophan change in the coded protein. Each of these mutations maps to one of the conserved regions subject to frequent point mutations in other tumor types (21).

Since all but one of the tumors examined retained at least one intact p53 allele, we wished to know whether these tumors continued to express the p53 gene product. We analyzed p53 transcript levels in the 18 rhabdomyosarcomas suitable for Northern analysis. As expected, p53 mRNA was not detected in Rhabd 10, the tumor that was homozygously deleted for the p53 locus (Fig. 2). Another tumor, Rhabd 24, had undetectable levels of p53 transcript even though it had two apparently intact alleles (Fig. 2). Each of the other 16 tumors expressed abundant levels of a single 2.8-kb RNA species when compared to a rehybridization of the same Northern filters to the human β-actin probe pHFβA-1 (44).

The abundance of p53 RNA in rhabdomyosarcoma tumors is consistent with its high levels in transformed cell lines and other rapidly dividing cells (reviewed in refs. 17, 23, and 24). Negligible quantities of the p53 protein are present in normal human cells regardless of the prevalence of transcript (48–50). In some transformed cells, dominant mutations of the p53 gene result in the production of mutant protein isoforms with increased stability (25–28). To assess whether alterations in the size or abundance of p53 protein occurred in rhabdomyosarcoma, we performed Western blot analysis, where sufficient tissue was available, using the monoclonal antibody PAb1801, which recognizes a human-specific, denaturation-resistant epitope near the amino terminus of the p53 protein (39). Despite the presence of abundant mRNA, p53 protein was not present at the level of detection possible with this type of analysis in seven of eight rhabdomyosarcomas examined (Table 2), although it was detectable in fetal muscle samples.

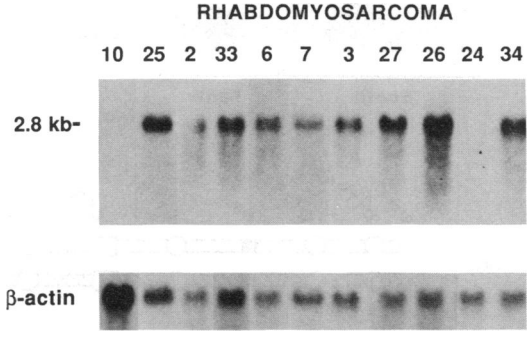

FIG. 2. p53 expression in rhabdomyosarcoma tumors. Northern analysis of p53 RNA. Thirty micrograms of total cellular RNA, isolated by the method of Chirgwin *et al.* (43), was electrophoresed through 1% agarose/formaldehyde gels, transferred to Hybond-N membranes, and hybridized to ^{32}P-labeled pCDpro53. Northern blots were rehybridized with a β-actin probe (44).

Table 2. Summary of p53 alterations

Type of mutation	mRNA	Protein	Tumor
	Rhabdomyosarcoma		
Homozygous deletion	−	−	Rhabd 10
Hemizygous deletion	+	+	Rhabd 2
Hemizygous deletion*	+	NA	Rhabd 33
Hemizygous deletion	+	NA	Rhabd 25, 39
Undetectable mRNA	−	NA	Rhabd 24
Undetectable protein	+	−	Rhabd 3, 18, 19
Loss 17p heterozygosity	+	−	Rhabd 7, 27, 34
Loss 17p heterozygosity	+	NA	Rhabd 6, 21
	Osteosarcoma		
Homozygous deletion	−	−	Osteo 25, 30
Homozygous deletion	−	NA	Osteo 6, 17
Undetectable protein	NA	−	Osteo 7, 42
Abnormal protein size	NA	+	Osteo 5

NA, samples appropriate for Western or Northern blot analysis were not available.
*Point mutated allele retained.

Analysis of p53 in Osteosarcoma. A previous report has suggested that p53 alleles can be rearranged in osteosarcomas (18). We wished to determine whether a variety of mechanisms of p53 inactivation, similar to those described above for rhabdomyosarcomas, also occurred in osteosarcomas. We examined the p53 locus in 29 such tumors and compared these results to the allelic patterns at other chromosome 17 marker loci. Four tumors of the 12 that were constitutionally informative showed loss of heterozygosity at loci flanking the p53 gene (Osteo 4, 9, 23, 32). Homozygous deletions of the p53 gene were detected in 4 primary or xenografted tumors (Osteo 6, 17, 25, 30). In 3 of these tumors (class 1), the deletions were specific to, and inclusive of, the entire p53 gene, as exemplified by Osteo 17 and 25 (Fig. 3*A*). The DNA sample examined in the case of Osteo 25 was extracted from a primary tumor that retained some normal tissue and the signal observed with the p53 probe in this tumor was ≈10% of that in the corresponding normal tissue (Fig. 3*A*). In Osteo 6 (class 2), homozygous deletion for both the p53 and *D17S71* loci was detected (Fig. 3*A*), encompassing a minimum genetic distance of 15 centimorgans (51). Constitutional homozygosity at all chromosome 17p loci tested did not allow us to determine whether this represented a single deletion followed by mitotic recombination or two separate deletions of the p53 alleles. In tumors of each class, loci distal to p53 and on the long arm of chromosome 17 were not deleted (Table 1). As expected, no p53 transcripts were detected in RNA from any of the 4 osteosarcomas with homozygous deletions of the p53 gene. Western immunoblotting using antibody PAb1801 showed no detectable p53 protein in the 2 of these tumors examined (Osteo 6 and 17) or in 2 other osteosarcomas that showed no gross structural alterations of the p53 locus (Osteo 7 and 42). Two additional tumors (Osteo 5 and 16) with intact p53 genes expressed detectable levels of immunoreactive protein, although in the case of Osteo 5 this was of an aberrant size (57 kDa).

DISCUSSION

Our data show frequent inactivation of p53 in rhabdomyosarcomas and osteosarcomas. In summary, 14 of the 31 rhabdomyosarcomas examined showed defects in the structure or expression of the p53 gene, loss of heterozygosity for loci in the vicinity of p53, or failed to express p53 transcript or protein detectable by Western blot analysis (Table 2). Likewise, 12 of the 29 osteosarcomas examined showed homozygous deletions of the p53 locus, loss of heterozygosity at loci flanking the p53 gene, or failed to express or expressed p53 proteins of aberrant size as detected by Western blot analysis. The specificity of these events is suggested by extensive analyses of loci on other chromosomes in these tumors. We detected no additional aberrations other than those previously reported on chromosome 13q in osteosarcomas (52) and chromosome 11p in a subset of rhabdomyosarcomas (31), suggesting that alterations of p53 did not reflect general chromosome instability in these tumors. Alteration of p53 was detected irrespective of events on chromosome 11p in rhabdomyosarcoma or at the RB1 locus in osteosarcoma.

The identification of homozygous deletions of the p53 locus, as well as the failure of several tumors to express its mRNA, implies that truly recessive mechanisms of p53

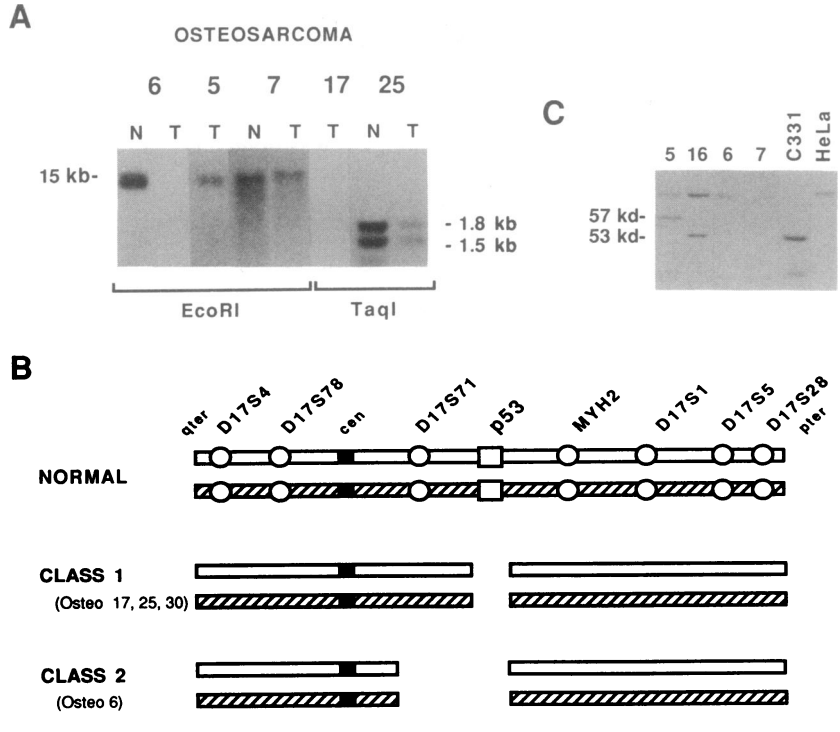

FIG. 3. Analysis of the p53 locus and surrounding loci in osteosarcoma. (*A*) DNAs from normal and tumor tissues were digested with *Eco*RI or *Taq* I and analyzed as described for Fig. 1. The DNA sample examined in the case of Osteo 25 was extracted from a primary tumor that retained some normal tissue and the signal observed with the p53 probe in this tumor was ≈10% of that in the corresponding normal tissue (Table 1). (*B*) Schematic representation of inferred mitotic events occurring on chromosome 17 in osteosarcoma. Tumors were classified into categories based on their patterns of allele dosage for p53 and the surrounding loci. Class 1, homozygous deletion of the p53 locus; class 2, homozygous deletion of the p53 and *D17S71* loci. Since constitutional tissue was not available for some tumors it was not possible to assess loss of heterozygosity for chromosome 17, and consequently isodisomy was not used as a criterion to classify osteosarcomas. (*C*) Immunoblot analysis of p53 expression. Protein extracts from tumor tissues were separated by SDS/PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), incubated with the human-specific anti-p53 monoclonal antibody PAb1801 (39), and p53 protein was visualized by incubation with alkaline phosphatase-conjugated anti-mouse IgG (Promega). The C331 line expresses levels of the p53 protein detectable by immunoblotting while HeLa expresses no p53 transcript or protein (39); they are used here, respectively, as positive and negative controls.

inactivation, analogous to those reported for the retinoblastoma gene (5–8), can contribute to neoplastic growth *in vivo*. These data are consistent with reports implicating loss of function mutations for p53 in pathogenesis of human malignancy based on alterations of p53 in human tumor cell lines (18–20). However, the retention of a point-mutated p53 allele in Rhabd 33 and the expression of an aberrant protein in Osteo 5 provides further support for previous analyses in other tumor types, which suggest genetically dominant loss of function through point mutation (13, 21), resulting in an oncoprotein capable of sequestering or incapacitating wild-type p53. Together, these data indicate that it is the attenuation of normal p53 function and not the genetic mechanism by which this is accomplished that is important for its role in human neoplasia.

The alteration of p53 in tumors for which other, primary mutations have been defined implies that p53 plays a progressional role in tumorigenesis. Particularly interesting in this regard is the absence of gross genomic aberration of the p53 gene in 26 retinoblastoma tumors, despite the frequency with which we detected such alterations in the clinically associated tumor, osteosarcoma (52). This may suggest that the inactivation of the p53 gene in the latter represents a progressional event analogous to the occurrence of an isochromosome 6p in retinoblastoma (53). Divergence of these secondary events is consistent with a model in which the two tumors have common requirements for an initiating mutation, but each tumor accumulates different progressional mutations in its evolution.

Thus, these data provide evidence for the importance of functional loss of p53 in human tumorigenesis. Furthermore, they describe alternative and complementary, recessive and dominant mechanisms whereby alterations at this locus can contribute to human neoplasia, and they provide important clues for delineating the multistep process of these diseases.

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