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Rearrangement of the p53 gene in human osteogenic sarcomas

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ABSTRACT **p53 is a 53-kDa nuclear protein that is associated with malignant transformation in several tumor model systems. In a survey of 134 human carcinomas, sarcomas, leukemias, and lymphomas obtained at surgery or from peripheral blood, we found rearrangements of the p53 gene only in osteogenic sarcomas (3 of 6 osteogenic sarcomas examined). Normal tissue from one of these patients had an unrearranged gene, indicating that the genetic abnormality in the tumor was acquired. Two of the sarcomas with rearranged genes expressed levels of p53 protein that were elevated relative to other tumors. Rearranged p53 genes were also found in human osteogenic sarcoma cell lines.**

A 53-kDa cellular phosphoprotein known as p53 has been associated with malignant transformation in several model systems (1–3). Elevated levels of p53 are detected in transformed primate and rodent cell lines. The protein is detectable in many actively proliferating, nontransformed cells but is undetectable or present at low levels in resting cells (4). The synthesis of p53 increases in resting cells after stimulation with mitogen (5), and stimulated cells can be blocked from entering S phase by microinjections of p53-specific antibody (6). Changes in levels and synthesis of p53 are often concomitant with malignant transformation (1, 7, 8). Protein encoded by the p53 gene can complement activated *ras* genes in *in vitro* transformation of primary rodent cell cultures (9, 10) and can prolong the lifespan of such cells (11). These observations suggest a direct role for p53 in oncogenesis of at least some cell types and are further supported by observations on the Abelson virus-transformed murine cell line L12, which does not normally express p53 because its gene has been inactivated by insertion of a retrovirus-like sequence within its first intron. L12 cells do not induce progressively growing tumors in syngeneic mice, but, when a functional p53 gene is introduced into these cells, transformed L12 cells do induce tumors (12).

Because of these associations of the p53 gene with neoplasia in model systems, we undertook a systematic investigation of this gene in fresh human tumors. We found that alterations of this gene were present in three of six human osteogenic sarcomas *in vivo* but in no other tumor studied. Two of the fresh tumors expressed an immunoreactive protein of 53 kDa. Alterations of the p53 gene were also found in three human osteogenic sarcoma cell lines.

MATERIALS AND METHODS

Solid tumors and accompanying normal tissues were obtained at surgery, and leukemic cells were obtained from peripheral blood. Samples were obtained before chemotherapy or radiotherapy and stored at −70°C. Eighty-five percent of adenocarcinomas and squamous carcinomas were primary tumors, and 15% were metastatic. All other malignancies were primary tumors or leukemias.

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Genomic DNA was extracted by described methods (13) using cesium chloride density gradient ultracentrifugation and was digested with appropriate restriction endonucleases according to the directions of the supplier. The resulting fragments were separated by electrophoresis on 0.8% agarose gels, transferred to coated nylon filters, and hybridized to nick-translated ^{32}P-labeled probes as described (13). Autoradiographs of the hybridized filters were made with Kodak intensifying screens. Probes included (*i*) pR4-2, a cDNA probe encompassing exons 2–10 and parts of exons 1 and 11 (14), generously provided by E. Harlow (Cold Spring Harbor Laboratory); (*ii*) pBT53, a fragment encompassing part of the first exon of p53, prepared by *Xba* I and *Bam*HI digestion of clone pBT53 (15); and (*iii*) p53-2-5, a fragment encompassing exons 2–5 prepared by *Nco* I digestion. The p53 gene, the probes used in analysis, and the various anticipated restriction fragments are shown in Fig. 1.

Osteogenic sarcoma cell lines were obtained from the American Type Culture Collection and maintained under conditions recommended by the supplier.

RESULTS

Hybridization Analysis. We analyzed DNAs from 2 benign tumors and 132 malignant tumors including 25 breast carcinomas, 25 colon and rectum carcinomas, 11 lung carcinomas, 7 ovary carcinomas, 5 stomach carcinomas, 3 kidney carcinomas, and 2 head and neck region carcinomas, 8 neuroblastomas, 6 other childhood tumors, 4 germ-cell tumors, 22 leukemias and lymphomas, 3 nerve tumors, 5 soft tissue sarcomas, and 6 osteogenic sarcomas. DNAs were digested with *Eco*RI and hybridized with pR4-2, a p53 probe that covers the known 11 exons of the p53 gene (Fig. 1). Abnormalities of the p53 gene restriction pattern were detected in 3 osteogenic sarcomas (Fig. 2) but in no other tumor DNA. Analyses with the additional restriction enzymes *Bam*HI, *Hin*dIII, and *Bgl* II and additional p53 probes limited to the 5′ portion and the midportion of the gene were used to define the likely sites of the rearrangements in these tumors.

Using the pR4-2 probe, osteogenic sarcoma 1 (OS1) had normal 15- and 3.7-kb *Eco*RI bands as well as a larger-than-normal *Eco*RI band (Fig. 2*A*, lane c). OS1 DNA had the predominant 7.8-kb *Bam*HI fragment (Fig. 2*B*, lane c) and a normal *Hin*dIII restriction pattern (Fig. 2*C*, lane c), but had a *Bgl* II fragment smaller than the normal 12-kb size (Fig. 2*D*, lane c). Additional tests with the 5′ probe (pBT53) and *Eco*RI digestion generated the normal 3.5-kb fragment suggesting that the region encompassing the first exon was intact. This analysis indicated that the p53 gene in this tumor was rearranged within the first intron and that the restriction fragments containing the coding sequences were apparently intact. The rearrangement was homozygous since no normal *Bgl* II 12-kb band was detected. Normal tissue from this patient contained a p53 gene that had the germ-line configuration (data not shown), indicating that the alteration of this gene in the sarcoma was an acquired abnormality and not a polymorphism.

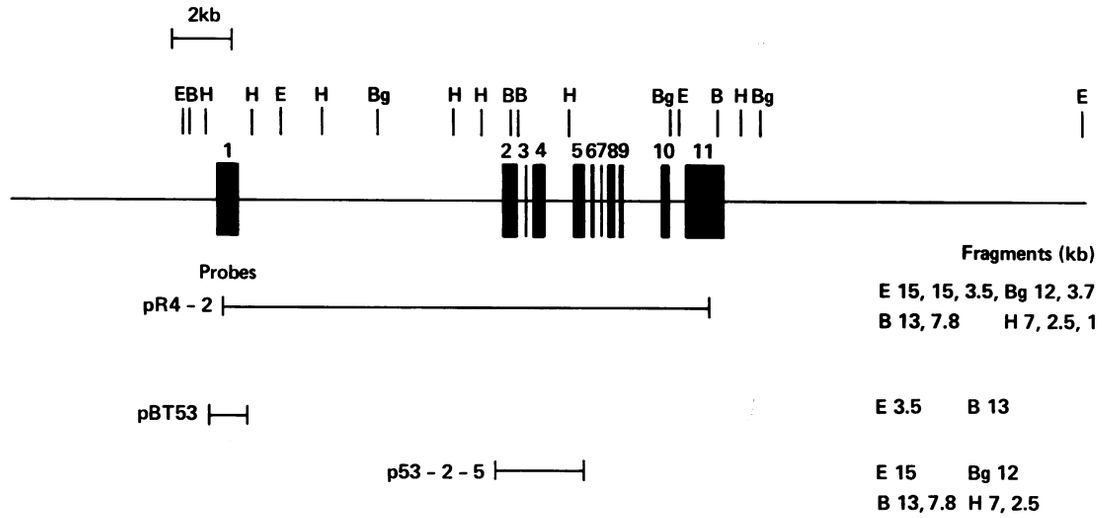

FIG. 1. Map of the human p53 gene (after Lamb and Crawford, ref. 15) showing probes and anticipated restriction fragments used in analysis of p53 rearrangements in osteogenic sarcomas. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl* II.

Osteogenic sarcoma 2 (OS2), when analyzed with the exon 11 pR4-2 probe, had two *Eco*RI bands [one was larger than normal (Fig. 2*A*, lane d)] and the normal major hybridizing bands generated with *Bam*HI, *Hin*dIII, and *Bgl* II (Fig. 2 *B*, *C*, and *D*, lanes d). When a *Bam*HI digest of DNA from this tumor was analyzed with the exon I probe (pBT53), only a band of ≈5 kb was observed (Fig. 3). This is smaller than the normal 13-kb *Bam*HI fragment that spans the first exon and first intron (Fig. 1). However, the first intron *Eco*RI fragment of the p53 gene in this tumor was normal with the pBT53 probe. We conclude that the p53 gene was rearranged in this tumor in the first intron on the 5′ side of the *Bgl* II site and on the 3′ side of the *Eco*RI site.

Osteogenic sarcoma 3 (OS3) had a 13-kb *Eco*RI fragment in addition to the normal 15-kb fragment when analyzed with probe pR4-2 (Fig. 2*A*, lane e). The first exon p53 probe (pBT53) revealed a normal 3.5-kb *Eco*RI fragment, but the p53-2-5 probe revealed only an *Eco*RI fragment of ≈13 kb (i.e., smaller than the normal 15-kb 5′ *Eco*RI fragment). The *Bam*HI fragments of 7.8 kb (Fig. 2*B*, lane e) were normal, as were the major *Hin*dIII and *Bgl* II fragments (Fig. 2 *C* and *D*, lanes e). These data indicate that the likely site of the p53 gene

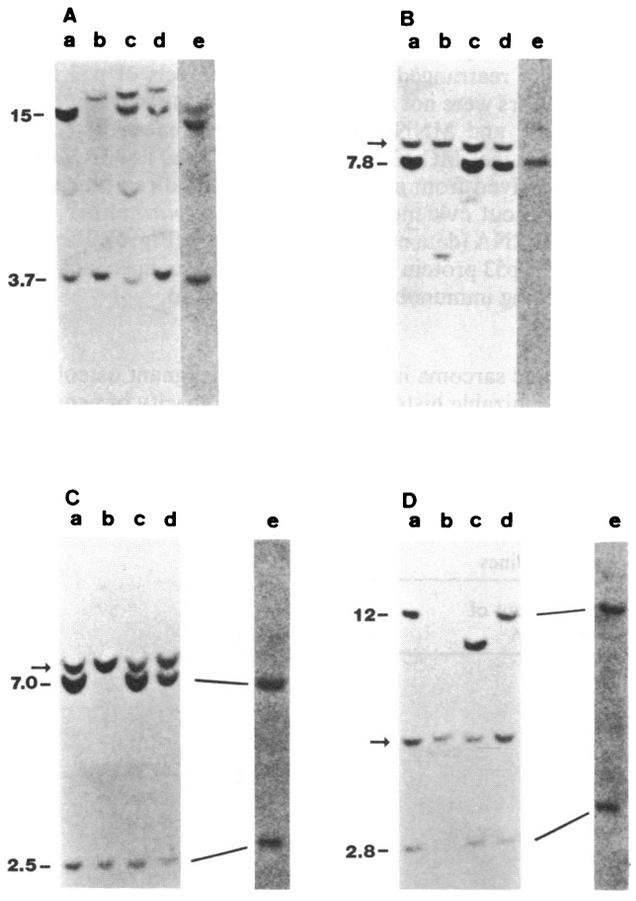

FIG. 2. DNA was isolated from the following cells. Lanes: a, B-cell line BV173 with an unrearranged p53 gene; b, cell line COLO 667 with a rearranged p53 gene; c, OS1; d, OS2; e, OS3. Cells in lanes c–e are osteogenic sarcomas. The DNA was digested with various enzymes and hybridized with a p53 cDNA probe (pR4-2, ref. 14) or with a human β-globin probe. β-Globin bands are indicated by arrows, and sizes are indicated in kilobases (kb). (*A*) *Eco*RI. (*B*) *Bam*HI. (*C*) *Hin*dIII. (*D*) *Bgl* II.

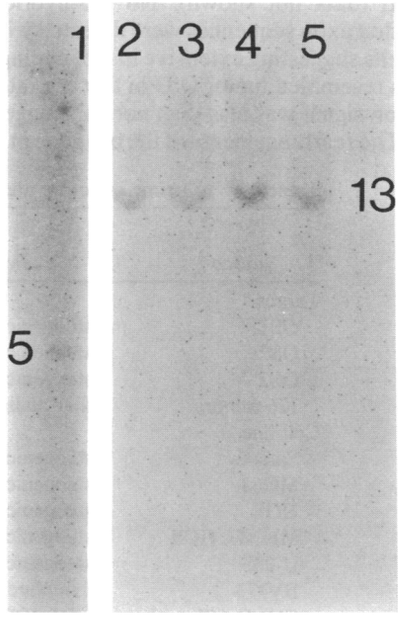

FIG. 3. DNA from OS2 and controls was digested with *Bam*HI and hybridized with pBT53, a p53 first exon probe. Lanes: 1, 0S2; 2–5, control DNAs without rearranged p53 genes. Sizes of bands in kb are shown.

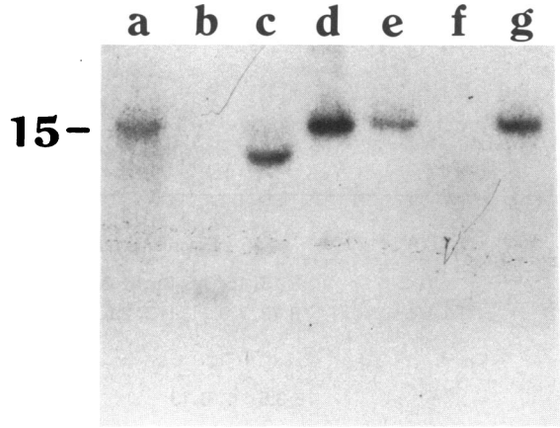

FIG. 4. DNA from cell lines was digested with *Eco*RI and hybridized to a p53 probe. Lanes: a, HEL cells (unrearranged p53 gene); b, HL-60 cells (deleted p53); c, MGG3; d, MNNG/HOS; e, U-20S; f, Saos-2; g, HOS. Cells in lanes c–g are osteogenic sarcoma cell lines.

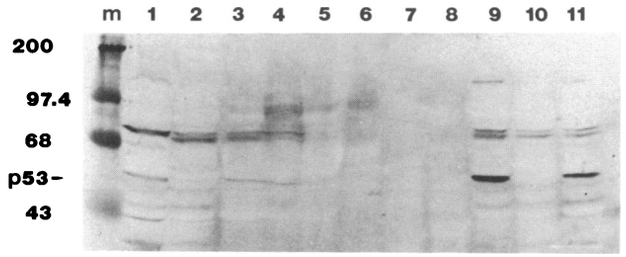

FIG. 5. Immunoblot of extracts of tumors and cell lines utilizing monoclonal antibody pAb122 to p53 protein (13) and immunoperoxidase technique. m, Molecule weight markers. Lanes: 1, BV173 cells (positive control); 2, COLO 667 cells (negative control); 3, osteosarcoma OS1; 4, osteosarcoma OS2; 5 and 6, osteosarcomas without rearranged p53; 7 and 8, colon cancers without rearranged p53; 9, MNNG/HOS cells (an osteosarcoma line); 10, MG63 (an osteosarcoma line with rearranged p53); 11, HOS (an osteosarcoma line).

rearrangement is in the first intron on the 5′ side of the *Bgl* II site and on the 3′ side of the *Eco*RI site. OS3 probably had a single copy of the p53 gene as determined by the data with the exon 2–5 probe and quantitative densitometry of the hybridization signal. DNA from tumors OS1 and OS2 were examined with hybridization probes for 13 and 7 protooncogenes, respectively, in addition to p53 as described (13); none of these was detectably rearranged in analyses with one or two appropriate restriction enzymes. OS1 had a deletion of one allele of *MYB*, a common finding in solid tumors heterozygous at the *MYB* locus (13).

Because of the unusual frequency of rearrangements of the p53 gene in osteogenic sarcomas, we analyzed five human cell lines derived from four osteogenic sarcomas. Three lines from various tumors had abnormalities of the p53 gene. MG63 was homozygous for a p53 gene with an alteration between the first and second exon as determined by hybridization with pR4-2 and digestion with *Eco*RI (Fig. 4), *Bam*HI, and *Hin*dIII (data not shown). No p53-hybridizing bands other than first exon sequences were detected with this probe in Saos-2 cells suggesting extensive loss of coding sequences. U-20S cells resembled tumor OS3 in that the intensity of the hybridization signal was less than normal, suggesting loss of one allele. The rearrangements of the p53 gene in the cell lines were confirmed by analysis with the cDNA probe encompassing only the first exon pBT53.

Expression of p53. Fresh tumor tissues were not available for mRNA analyses; however, sufficient tissue was available from tumors OS1 and OS2 for immunologic analysis of p53 levels using the monoclonal antibody PAb122 (16). As seen in Fig. 5, OS1 and OS2 had distinct p53 bands that were as intense as bands from the actively proliferating B-lymphocyte cell line BV173 and that were more intense than bands from two osteogenic sarcomas and two colon cancers without detectably rearranged p53 genes. The levels of p53 in the fresh tumors were not as great as levels in the two related cell lines HOS and MNNG/HOS derived from an osteogenic sarcoma. Lines MG-63 (osteogenic sarcoma) and COLO 667 (a line derived from a Hodgkin tumor) had rearranged p53 genes without evidence of expression *in vitro* either at the level of mRNA (data not shown) or protein (Fig. 5). We could not detect p53 protein in the osteogenic sarcomas Saos-2 and U-20S using immunoblots (data not shown).

DISCUSSION

Osteogenic sarcoma is a neoplasm of malignant osteoblasts. It is recognizable histologically by the capacity of neoplastic cells to synthesize osteoid. Like most other sarcomas it is thought to originate in cells arising from the embryonic mesoderm. It is the most common primary tumor of bone and occurs both in sporadic and familial forms. Clinically the

Table 1. p53 gene structure and expression in tumors and cell lines

Source	Type	Rearrangement of p53 DNA	p53 mRNA	p53 Protein
Tumor				
OS1	Osteogenic sarcoma	Intron 1	ND	+
OS2	Osteogenic sarcoma	Intron 1	ND	+
OS3	Osteogenic sarcoma	Intron 1	ND	ND
126 tumors	Other malignancies	nd	ND	(−)*
Cell line				
Saos-2	Osteogenic sarcoma	Deletion coding region	(−)	(−)
MG63	Osteogenic sarcoma	Intron 1	(−)	(−)
HOS	Osteogenic sarcoma	nd	+++	+++
MNNG/HOS	Osteogenic sarcoma	nd	(−)	(−)
U-20S	Osteogenic sarcoma	Single gene deletion	(−)	(−)
BV173	B lymphocyte	nd	+	+
COLO 667	Hodgkin disease	3′ deletion	(−)	(−)

ND, not done; nd, none detected; (−), undetectable by RNA gel blotting (mRNA) or immunoblotting (protein); +, intensity of signal observed in proliferating cell line BV173; +++, a signal of >2-fold relative intensity.

*Undetectable in two osteogenic carcinomas and two colon cancers without rearranged p53.

tumor is characterized by occurrence in young people, aggressive growth, and frequent metastases to lung.

The human genome contains a single p53 gene containing 11 exons and spanning ≈20 kb (14). The alterations of the p53 gene observed in osteogenic sarcoma tumors *in vivo* and in cell lines *in vitro* are summarized in Table 1. Our analysis of osteogenic sarcoma tumors and cell lines indicates that rearrangements of the p53 gene occurred in the first intron in three tumors and one cell line. One cell line had lost all coding sequences other than those in the first exon.

The human osteogenic sarcoma cell lines have extensive chromosomal rearrangements rendering complex the interpretation of the rearrangements of the p53 gene on chromosome 17 (17). U-20S varies from hypodiploid to near tetraploid with abnormalities including dicentrics, breaks, rings, and minute markers. Saos-2 varies from hyperdiploid to hypopentaploid with chromosome breaks, secondary constrictions, and double minutes. Karyotype analysis of the fresh tumors was not possible. The significance of frequent rearrangements of the p53 gene in osteogenic sarcomas *in vivo* (3 of 6 osteogenic sarcomas examined) contrasted with its rarity in other fresh human tumors (0 of 126 tumors) is uncertain. Two of the sarcomas with rearrangements in the first intron synthesized immunologically detectable p53. This protein was not detected in four other tumors lacking p53 rearrangements.

p53 levels are elevated in many murine tumors and tumor cell lines. The elevated levels may result both from increased mRNA synthesis and from stabilization of a protein with a very short half-life (18–20). Interaction with the large tumor antigen of simian virus 40 or the adenovirus E1b 58-kDa protein can also stabilize p53 and increase the concentration from a few hundred molecules to ≈10,000 molecules per cell (21, 22). It is not certain, therefore, whether the relatively high levels of p53 protein observed in two osteogenic sarcomas (OS1 and OS2) *in vivo* reflected rearrangements of the p53 gene or post-transcriptional modifications. The elevation of p53 levels in these tumors probably did not simply reflect higher levels of cell proliferation since histologic analysis did not reveal an unusually high frequency of mitoses when compared to the osteogenic sarcomas without detectable p53 rearrangement. The possibility that a rearrangement involving the first exon of p53 in osteogenic sarcomas alters transcriptional activity of the gene must be considered, as is the case with some gene rearrangements in Burkitt lymphomas involving the first noncoding exon of *MYC* (23, 24). Interestingly, the first exon of the p53 gene is also noncoding (10) but shows evolutionary conservation (25). Furthermore, mutations produced in 5′-coding sequences of the murine p53 gene give rise to stable protein products and can extend the life span of recipient cells (11).

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