Journal of Cellular Biochemistry 63:37–50 (1996)

Oncogene Alterations in Primary, Recurrent, and Metastatic Human Bone Tumors

Franca Pompetti, Paola Rizzo, Richard M. Simon, Boris Freidlin, Daphne J. Mew, Harvey I. Pass, Piero Picci, Arthur S. Levine, and Michele Carbone

Department of Pathology, University of Chicago, Chicago, Illinois 60637 (P.R., M.C.), Section on DNA Replication, Repair, and Mutagenesis, National Institute of Child Health and Human Development, Bethesda, Maryland 20892 (F.P., A.S.L., M.C.), Biometric Research Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20892 (R.M.S.), Emmes Corporation, Bethesda, Maryland 20892 (B.F.), Thoracic Oncology Section, National Cancer Institute, Bethesda, Maryland 20892 (D.J.Y.M., H.I.P.), Laboratorio di Oncologia, Istituti Ortopedici Rizzoli, 40121 Bologna Italy (F.P., P.P.)

Abstract We investigated the structure and the expression of various oncogenes in three of the most common human bone tumors—osteosarcoma (36 samples from 34 patients), giant cell tumor (10 patients), and chondrosarcoma (18 patients)—in an attempt to identify the genetic alterations associated with these malignancies. Alterations of RB and p53 were detected only in osteosarcomas. Alterations of c-myc, N-myc, and c-fos were detected in osteosarcomas and giant cell tumors. Ras alterations (H-ras, Ki-ras, N-ras) were rare. Chondrosarcomas did not contain any detectable genetic alterations. Our results suggest that alterations of c-myc, N-myc, and c-fos oncogenes occur in osteosarcomas, in addition to those previously described for the tumor suppressor genes RB and p53. Moreover, statistical analyses indicate that c-fos alterations occur more frequently in osteosarcoma patients with recurrent or metastatic disease.

Key words: osteosarcoma, chondrosarcoma, GCT, oncogene alterations

Recent progress in molecular genetics has revealed a consistent set of genetic alterations in various human cancers, possibly reflecting multistep tumor development and progression [Carbone and Levine, 1990; Fearon and Vogelstein, 1990]. Analysis of the genetic changes in primary tumor cells may therefore improve the clinician's ability to judge the prognosis in individual patients and to identify, at the outset, those patients whose tumors are most likely to metastasize rapidly and that require more aggressive therapies [Paterson et al., 1991; Berns et al., 1992; Esrig et al., 1994]. Specific genetic alterations might also be associated with enhanced susceptibility or resistance to radiotherapy and/or chemotherapy [Vande Woude et al., 1990; Brennan et al., 1991; Loewe et al., 1993; Fan et al., 1994; Baldini et al., 1995]. Thus, a detailed knowledge of the genetic alterations consistently associated with each tumor type might permit improvements in the design of effective therapeutic approaches for individual cancer patients, as well as improve the precision of diagnosis.

Bone tumors, predominantly osteosarcomas (OS), account for more than 10% of all malignancies in adolescents [Bode and Levine, 1982; Baldini et al., 1995]. OS occur mainly in patients between 10 and 25 years of age, and their survival is dependent upon an early diagnosis in order to completely eradicate the tumor before it metastasizes. Giant cell tumors (GCT) are usually seen in patients over 20 years of age, and these patients have a much better prognosis than patients with OS. Chondrosarcomas usually occur in patients between 30 and 60 years of age. These tumors also have a much better prognosis than OS, but are resistant to chemotherapy.

Compared to some other human malignancies, for which there is now a large fund of knowledge of the molecular alterations associated with tumor development (such as colon and

Received December 15, 1995; accepted March 28, 1996.

Address reprint requests to Michele Carbone, University of Chicago, Department of Pathology, MC3083, Room S-342, 5841 South Maryland Avenue, Chicago, IL 60637.

breast cancer), little is known about the genetic alterations that lead to the development of human bone tumors. Several groups have reported chromosomal deletions and/or structural rearrangements of the RB and p53 antioncogenes [Friend et al., 1986; Masuda et al., 1987; Weichselbaum et al., 1988; Toguchida et al., 1988; Romano et al., 1989; Miller et al., 1990; Mulligan et al., 1990; Wunder et al., 1991; Toguchida et al., 1992] in human OS. However, these reports have not generally distinguished among primary, recurrent, and metastatic tumors (exposed or not exposed to chemotherapy). Moreover, the possibility that alterations of the cellular proto-oncogenes, e.g., myc, fos, and ras, contribute to the development of bone tumors has not been investigated.

The limited knowledge we have about the molecular alterations associated with human osteosarcomas is related to the relatively rare occurrence of these tumors (approximately 2,000 cases per year in the US), and to the difficulty of obtaining samples suitable for molecular studies. Following the diagnosis of osteosarcoma, patients are usually treated with high doses of chemotherapy that results in close to 99% of tumor necrosis. When the tumor is surgically resected it is almost completely necrotic and cannot be used for molecular studies. Therefore, the only sample suitable for molecular studies is the limited amount of biopsy tissue obtained at the time of diagnosis. These biopsies contain a very small amount of tumor tissue which often is just sufficient for histology, and frequently no remaining tissue is available for molecular studies.

In this study we analyzed the structure and/or the expression of the tumor suppressor genes RB and p53, and of the proto-oncogenes c-myc, N-myc, c-fos, H-ras, Ki-ras, and N-ras, in biopsy samples from 23 untreated primary OS (obtained at the earliest detectable stage of tumor development), nine secondary OS (local recurrences or metastases all of which had been previously treated), and four primary cell cultures derived from four different OS. We also investigated the structure and/or expression of these genes in two other bone malignancies: GCT (10 primary lesions) and chondrosarcomas (10 primary lesions, five secondary lesions, and three primary cell cultures derived from three different chondrosarcomas) (Table I). Because of the limited amount of tissue available, in some samples only some of the genes listed above were analyzed (Table II).

MATERIALS AND METHODS

Tissues

Human bone tumor samples were obtained from primary lesions, or from metastases and local recurrences (Table I). Regions of tumors shown by histological examination (frozen section at the time of surgery) to contain a high proportion of neoplastic cells (more than 90%) were isolated and frozen in liquid nitrogen. The amount of tumor tissue available, usually minimal, represented the limiting factor in all of the analyses described below.

DNA Isolation and Southern Blot Analysis

DNA isolation, gel electrophoresis, nucleic acid blotting, and hybridization were carried out according to standard procedures [Sambrook et al., 1989]. Muscle tissue from these patients, when available, or normal bone tissue (or human placenta from unselected individuals), were used as controls. cDNA hybridization probes for the RB gene were kindly provided by R. Bernards. These probes were obtained from the 4.7 kb RB cDNA by EcoRI digestion, which yields two fragments of 0.9 and 3.8 kb, respectively [Friend et al., 1986]. The p53 cDNA probe was obtained from E. Appella. The c-myc, N-myc, and c-fos probes were obtained from Lofstrand Inc. Assessment of DNA amplification or deletion was based on qualitative evaluation of densitometric measurements of the gels. Densitometry was performed using the program Image, Version 1.33 (National Technical Information Service), running on an Apple Macintosh IIci equipped with a Sierra Scientific MS4030 High Resolution Video Camera and Data Translation QuickCapture DT2255 Frame Grabber Board.

p53 PCR-SSCP Studies

Oligonucleotide primer pairs were synthesized on a model 392 DNA synthesizer (Applied Biosystems, Foster City, CA) for exons 5–6, 7, and 8–9, as follows:

Exons 5–6 sense 5′ TTC CTC TTC CTG CAG TACT

antisense 5′ AGT TGC AAA CCA GAC CTC A

Exon 7 sense 5′ GTG TTG TCT CCT AGG TTG GC

antisense 5′ CAA GTG GCT CCT GAC CTG CA

Exons 8–9 sense 5′ CCT ATC CTG AGT AGT GGT AA

antisense 5′ CCA AGA CTT AGT ACC TGA AG.

Genomic DNA (100 ng), 0.5 μl α-^{32}P dCTP (6000 Ci/mmol, 10 Ci/ml) 1.5 mM $MgCl_2$, 100 mM dNTPs, 5 μl 10X PCR buffer (Perkin Elmer Cetus, Norwalk, CT), 2.5 U Taq DNA polymerase (Amplitaq, Perkin Elmer Cetus), and 1.0 μM of each primer was amplified in a final volume of 50 μl. The samples were initially denatured at 97°C for 2 min, then cycled 35 times at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, on a model 9600 Thermocycler (Perkin Elmer Cetus). Final extension conditions were 72°C for 7 min followed by a 4°C hold. 2 μl of the amplification mixture were diluted 10-fold in stop solution (20 mM EDTA, 96% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated at 80°C for 2 min, and placed immediately on ice. 2 μl of the diluted sample were loaded onto an MDE gel (HydroLink, AT Biochem, Inc., Malvern, PA) with or without 10% glycerol. Electrophoresis was performed at 6–8 Watts overnight. The gels were dried on filter paper under vacuum at 70°C for 2 h and exposed to X-ray film at −80°C overnight with an intensifying screen.

Polymerase chain reaction and oligomer hybridization assay. Genomic DNA from the tumors (100 ng–1 μg) was amplified in a total volume of 100 μl by means of the Gene Amp PCR Reagent Kit (Perkin Elmer Cetus). PCR was run in the DNA Thermal Cycler (Perkin Elmer Cetus, thermal cycler 480). Primers for amplification of H-ras, Ki-ras, and N-ras were obtained from Clontech Laboratories (Palo Alto, CA). The templates were denatured for 5 min at 95°C, followed by 35 cycles of PCR with 1 min incubations at 56°C (annealing), 1 min at 74°C (extension), and 1 min at 95°C (denaturation). PCR products were electrophoresed on a 2% agarose gel to test the efficiency of amplification: 20 μl of the reaction mixture were then denatured and blotted onto a nylon filter. The DNAs were cross-linked to the filter using the UV-Stratalinker 2400 (Stratagene, La Jolla, CA). The blots were hybridized with ^{32}P-5′-end labeled 20 mt oligonucleotides corresponding to the wild-type and mutant sequences at each nucleotide position of a particular codon. Codons 12, 13, and 61 of the H-ras, Ki-ras, and N-ras genes were analyzed for the presence of mutations. The blots were washed according to the conditions recommended by Clontech and the filters were autoradiographed at −70°C.

RNA Isolation and Northern Blot Analyses

RNA isolation, gel electrophoresis, blotting, and hybridization were carried out according to standard procedures [Sambrook et al., 1989]: 6 μg of total RNA were electrophoresed in a denaturing formamide/formaldehyde agarose gel, transferred onto Sure blot membranes (Oncor, Gaithersburg, MD), and hybridized with ^{32}P labeled probes.

Protein Isolation and Western Blot Analysis

Crude protein extracts from tumor samples were prepared by mincing and homogenizing the samples according to standard procedures [Cance et al., 1990]. Proteins were immunoprecipitated by specific antibodies, electrophoresed through 8% SDS/polyacrylamide gels and transferred onto nitrocellulose membranes. The immunocomplexes were visualized by alkaline phosphatase-conjugate anti-mouse IgG (Bio-Rad, Hercules, CA) and a chemiluminescent substrate (Tropix, Bedford, MA). A protein extract obtained from proliferating human osteocytes was used as a positive control in these immunoprecipitations. Protein extracts from Saos-2 (RB negative) and Hela (RB positive) cells were used as additional controls.

RESULTS

Tumor Suppressor Gene Analyses

We first investigated whether RB and p53 alterations were present in our bone tumor samples. Southern blots of tumor cell DNA digested with Hind III were examined for gross DNA alterations. These analyses could only be performed on those samples for which the amount of material was sufficient to yield adequate amounts of genomic DNAs. Using the 0.9 kb and 3.8 kb DNA probes [Friend et al., 1986] which represent the 4.7 kb RB transcript, we analyzed DNA from 28 OS, seven GCT, and seven chondrosarcomas. We further digested the 0.9 kb fragment to obtain a 675-bp EcoRI-HpaI fragment, which we used as a probe. This procedure removes a GC-rich region that produces a high background upon hybridization [Harbour et al., 1988]. In OS sample 10, densitometric analyses revealed a 70% decrease in intensity of the 7.0, 6.0, and 1.5 kb bands when hybridized with the 0.6 kb probe, suggesting that an inter-

TABLE I. Bone Tumor Samples Studied

Case number	Age at diagnosis (years)	Sex	Location of primary tumor	Tumor type	Source of tumor sample
1	14	M	Ilium	Osteosarcoma	B
2	72	M	Tibia	Osteosarcoma	B
4	30	F	Ischium	Osteosarcoma	R, T
5	14	M	Femur	Osteosarcoma	B
6	47	M	Hip	Osteosarcoma	B
7	24	M	Femur	Osteosarcoma	B
8*	24	M	Lung	Osteosarcoma	M (L), T
9	13	M	Femur	Osteosarcoma	B
10	13	M	Femur	Osteosarcoma	R, T
11	20	M	Femur	Osteosarcoma	B
12	12	M	Humerus	Osteosarcoma	M (S), T
13	13	F	Ilium	Osteosarcoma	B
14	25	M	Femur	Osteosarcoma	B
15	12	F	Tibia	Osteosarcoma	B
16	20	F	Femur	Osteosarcoma	B
17	6	F	Femur	Osteosarcoma	M (L)
18**	13	F	Humerus	Osteosarcoma	B and M (L)
19	45	F	Ilium	Giant cell tumor	B
20	27	F	Humerus	Giant cell tumor	B
21	24	M	Femur	Giant cell tumor	B
22	51	M	Femur	Giant cell tumor	B
24	32	M	Fibula	Giant cell tumor	B
25	25	F	Ilium	Giant cell tumor	B
26	60	M	Tibia	Chondrosarcoma	B
27	55	M	Humerus	Chondrosarcoma	B
28	32	M	Femur	Chondrosarcoma	B
29	68	F	Ilium	Chondrosarcoma	B
30	23	F	Femur	Osteosarcoma	B
31	46	M	Tibia	Chondrosarcoma	B
32	70	F	Femur	Chondrosarcoma	R
33	10	M	Femur	Osteosarcoma	B
34	12	M	Femur	Osteosarcoma	B
35	11	M	Humerus	Osteosarcoma	B
36	11	M	Femur	Osteosarcoma	B
37	18	M	Femur	Osteosarcoma	B
38	22	M	Femur	Osteosarcoma	B
39	62	F	Ilium	Osteosarcoma	B
40	28	M	Femur	Osteosarcoma	B
41	24	F	Humerus	Giant cell tumor	B
42	20	M	Humerus	Giant cell tumor	B
43	35	M	Humerus	Giant cell tumor	B
44	39	M	Femur	Giant cell tumor	B
48	48	F	Ilium	Osteosarcoma	M (L), T
50	21	M	Femur	Osteosarcoma	B, T
51	55	M	Femur	Osteosarcoma	R
52	39	F	Femur	Osteosarcoma	R, T
53	29	M	Femur	Osteosarcoma	B, T
54	52	M	Ilium	Chondrosarcoma	B
55	77	F	Femur	Chondrosarcoma	B
56	62	M	Ischium	Chondrosarcoma	R
57	42	M	Ilium	Chondrosarcoma	R
58	65	M	Femur	Chondrosarcoma	R
59	24	F	Ilium	Chondrosarcoma	B
60	51	F	Femur	Chondrosarcoma	B, T

TABLE I. (continued)

Case number	Age at diagnosis (years)	Sex	Location of primary tumor	Tumor type	Source of tumor sample
61	43	F	Femur	Chondrosarcoma	B
62	80	M	Ilium	Chondrosarcoma	R
64***	14	M	Femur	Osteosarcoma	B
66***	17	F	Femur	Osteosarcoma	B, T
68***	16	F	Humerus	Osteosarcoma	Unknown
69***	6	M	Femur	Osteosarcoma	M (L)
70***	52	M	Ilium	Chondrosarcoma	B
71***	80	M	Ilium	Chondrosarcoma	R
72***	49	F	Ilium	Chondrosarcoma	B

*Case number 8 represents lung metastatic tissue from case number 7.
**For case number 18 both primary biopsied tumor and lung metastatic tumor tissues were available.
***Case numbers 64–72 represent cell lines derived from the indicated sources. A total of 34 Os, 18 chondrosarcomas, and 10 GCT patients were studied (note that the total number of Os specimens was 36, because samples 7 and 8 were from the same patient, and two specimens were available for patient sample 18). Table Key: B, Biopsy from the primary untreated tumor; M, Metastatic tumor tissue; R, Recurrence; T, Sample obtained from patient previously treated with chemotherapy; L, Lung metastasis; S, subcutaneous metastasis.

TABLE II. Number of Samples of Each Tumor Analyzed for Each Type of Oncogene and Numbers of Alterations Detected*

Oncogene	Osteo-sarcomas	Giant cell tumors	Chondro-sarcomas
Rb	28 (3)	7 (0)	7 (0)
p53	34 (5)	10 (0)	18 (0)
c-myc	34 (4)	10 (6)	18 (0)
N-myc	23 (3)	9 (1)	5 (0)
Ras	17 (1)	6 (0)	6 (0)
c-fos	17 (7)	9 (3)	3 (0)

*The table summarizes the number of samples with each tumor studied for each oncogene. In parentheses are indicated the number of alterations detected.

nal hemizygous deletion of this genomic fragment may have occurred (Fig. 1B); no alterations of the RB gene were detected in this sample using the 3.8 kb probe (Fig. 1A). Of interest, control DNA extracted from grossly normal adjacent muscle tissue (this sample was obtained by open biopsy) revealed a 70% decrease in intensity of the 7.0 kb band when hybridized with the 0.6 kb probe (Fig. 1B). It appears possible that this child inherited a mutated RB gene (hemizygous deletion) and that additional RB alterations occurred in the tumor cells (deletions of the 6.0 and 1.5 kb RB fragments recognized by the 0.6 kb probe). However, this child did not have an history of retinoblastoma (children with inherited RB deletions usually first develop retinoblastomas, and later osteosarcomas). Sample 11 showed a decrease of intensity in all of the bands, as measured by densitometric analysis, when hybridized with the 3.8 kb probe (Fig. 1A), and a decrease of the 7 kb and 6 kb bands following hybridization with the 0.6 kb probe (Fig. 1B). The other bone tumor samples examined did not contain any alteration of RB detectable by Southern blot analysis (Tables II and III, and Fig. 1A and B).

RB expression was examined in tumor protein extracts by Western blotting using monoclonal antibodies specific for the RB protein (see Materials and Methods for details) in 23 OS, seven chondrosarcomas, and six GCTs. Protein extracts obtained from OS sample 10 did not contain any detectable RB protein, and OS sample 69 contained a lower molecular weight band immunoreactive to the anti-RB which may correspond to a truncated RB protein (Fig. 2). The other bone tumor samples studied contained detectable levels of RB protein (Fig. 2). Thus, three of the 28 OS studied (11%) contained RB alterations; RB appeared normal in all of the GCT (seven samples) and chondrosarcomas (seven samples) studied.

The same Southern blots used for RB hybridization studies were stripped and hybridized with a ^{32}P-labeled p53 human cDNA probe (i.e., the same samples analyzed for RB alterations [see above] were analyzed for p53 structural alterations). Structural alterations of the p53 gene were found in OS sample 1, in which we detected an abnormal band of approximately 3.0 kb (Fig. 3A). However, the normal restriction pattern of the p53 gene was also detectable in this sample, suggesting that one normal allele was still present in the tumor cell population.

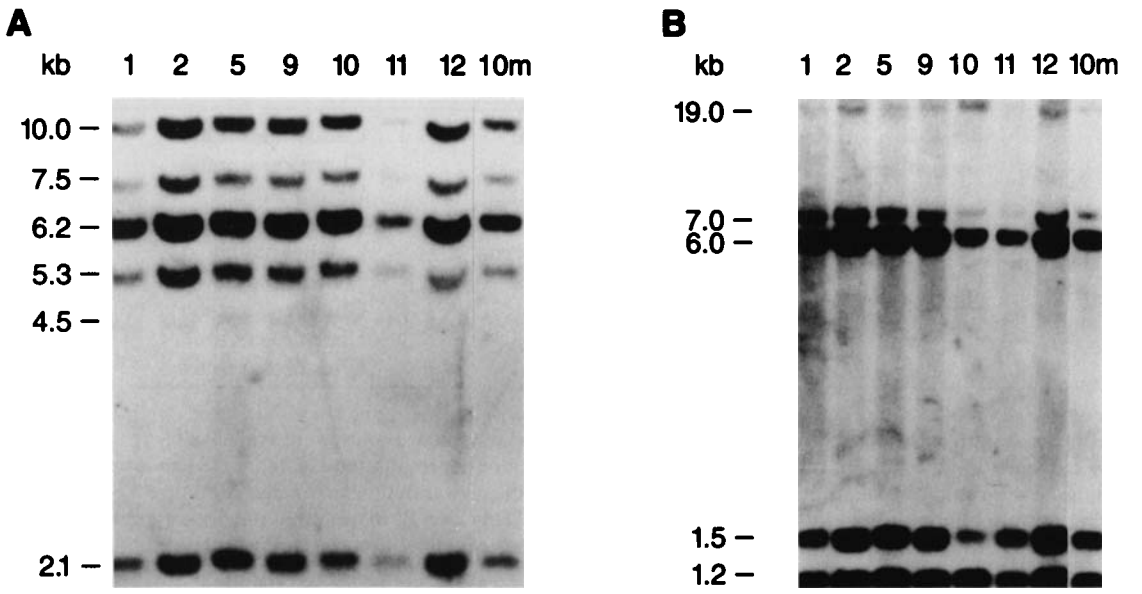

Fig. 1. Representative Southern blot analysis of DNA from different osteosarcoma samples hybridized to probes specific for RB. The samples studied are indicated; see Table I for proper identification of these samples. 10 μg of Hind III-digested DNA were electrophoresed through a 0.8% agarose gel, blotted on a charged nylon membrane (Gene Screen Plus), and hybridized sequentially with a ^{32}-P-labeled probe representing the 3.8 kb fragment of the RB cDNA (**A**), and with the 0.6 kb fragment of the RB cDNA (**B**). OS sample 10 shows a normal hybridization pattern following hybridization with the 3.8 kb probe (A), but a 70% decrease in intensity of the 7.0, 6.0, and 1.5 kb bands following hybridization with the 0.6 kb probe (B). The DNA extracted from grossly normal muscle tissue from this child (sample 10 m) also shows a 70% decrease of the 7.0 kb band (B), suggesting that this child may have inherited an hemizygous deletion of RB. DNA alterations are also seen in OS sample 11, which shows a decrease in intensity in all of the five bands when using the 3.8 kb probe (A), and of the 7 kb and 6 kb bands when using the 0.6 kb probe (B).

DNA from OS sample 12 showed a 60% decrease of the 2.5 kb p53 band (Fig. 3A). The same sample revealed the appearance of an extra band of approximately 6.5 kb after digestion with BglII restriction enzyme (Fig. 3B). OS sample 18 showed a normal p53 pattern after Hind III digestion, but a deletion was apparent after digestion with either BamHI or BglII (Fig. 3B). For this sample both primary tumor and metastatic tumor tissues were available. Interestingly, they contained different alterations of p53: The metastasis retained an apparently normal p53 allele, but no normal allele was present in the original tumor. Either two independent mutational events occurred or a second p53 mutation occurred in the original tumor after it metastasized. Thus, three out of 28 OS studied (11%) contained detectable p53 alterations by Southern blot hybridization (Fig. 3, and Table II). These experiments indicated that 11% of the OS comprising this study had either RB (samples 10, 11, and 69) or p53 (samples 1, 12, and 18) alterations (Table III). Previous Southern blot studies have detected approximately 50% of OS with p53 alterations [Toguchida et al., 1992]. Samples containing p53 point mutations without loss of heterozygosity may appear normal by Southern blot [Toguchida et al., 1992]. Thus, it appeared possible that some of the samples we studied contained point mutations of p53 that were not detected by Southern blot. PCR-SSCP is a reliable method of detecting p53 mutations. When exposed to electrophoresis in nondenaturing polyacrylamide gels, single strands of DNA differing by as little as one base pair will have different conformations and migration patterns [Greenblatt et al., 1994]. SSCP analyses of exons 5–6, 7, and 8–9 were performed on genomic DNA isolated from all of the 64 DNA samples analyzed in this study (these analyses could be performed on all of the samples because of the minimal amount of DNA required). Five samples demonstrated altered mobility: alterations were confirmed in samples 1 (exon 5–6), 12 (exon 7), and 18 (exon 5–6); in addition altered electrophoretic mobility was detected in DNAs from samples 2 (exon 7, Fig. 4), and 8 (exon 5). Therefore, five of the DNAs from 34 patients with OS (14.7%) and none of the DNAs from chondrosarcomas and GCT contained detectable p53 alterations (Table III).

TABLE III. Oncogene Alterations Detected in the Bone Tumors Studied

Case number	Tumor type	Oncogene alterations	Disease-free interval#
1	Osteosarcoma	@c-myc amplification (16×) and overexpression (3×); p53 rearrangement	14 months
2	Osteosarcoma	p53 mutation	39 months
4	Osteosarcoma	c-myc amplification (×3) and overexpression (×3)	0 months
5	Osteosarcoma	N-ras mutation codon 61	20 months
7 and 8	Osteosarcoma	c-fos overexpression (×8), p53 mutation (sample 8 only)	0 months
9	Osteosarcoma	c-myc rearrangement	18 months
10	Osteosarcoma	RB deletion, no protein expression; N-myc (×100) and c-fos (×4) overexpression	11 months
11*	Osteosarcoma	RB deletion; N-myc (25×) and c-fos (3×) overexpression	15 months
12	Osteosarcoma	p53 rearrangement; c-fos overexpression (4×)	0 months
17	Osteosarcoma	c-myc (7×) and N-myc (4×) overexpression	18 months+
18	Osteosarcoma	p53 rearrangement	30 months
19	GCT	c-myc amplification (3×); c-fos (6×) overexpression	24 months+
20	GCT	c-myc amplification (3×); c-fos (3×) overexpression	24 months+
21	GCT	c-myc overexpression (3×)	22 months+
22	GCT	c-myc (3×) and N-myc (8×) overexpression	36 months+
25	GCT	c-myc (3×) and c-fos (3×) overexpression	20 months+
33	Osteosarcoma	c-fos overexpression (3×)	4 months+
34	Osteosarcoma	c-fos overexpression (3×)	4 months+
43	GCT	c-myc overexpression (3×)	6 months+
69	Osteosarcoma	RB truncated protein	0 months

#Disease-free interval after sampling; @, in parenthesis is indicated the degree of gene amplification/overexpression as determined by densitometry (see Material and Methods); *, lung metastasis present at diagnosis; +, the patient did not yet relapse.

Proto-Oncogene Analyses

Using the polymerase chain reaction amplification followed by oligonucleotide hybridization assays (see Materials and Methods), we analyzed codons 12, 13, and 61 of the Ki-ras, N-ras, and H-ras proto-oncogenes. These codons show a number of activating mutations in several human cancers [Bos, 1989]. None of the GCTs and chondrosarcomas studied exhibited any detectable Ras alteration. Among the OS only one sample (sample 5) contained a detectable mutation, which was found in the codon 61 of N-ras (Fig. 5, Table II). Because these studies suggested that mutations of the ras family oncogenes are a rare finding in human bone tumors, we limited our investigation on ras to the first tumors studied; namely 17 OS, six GCTs, and six chondrosarcomas.

The c-myc and N-myc oncogenes are mutated in various pediatric tumors [Wasson et al., 1990]. However, the possibility that patients with OS also carry c-myc or N-myc alterations has not been investigated. We therefore examined the structure (DNA) and the expression (RNA) of c-myc and N-myc in our bone tumor samples. Among OS, c-myc was amplified (Fig. 6) and overexpressed in two of the 34 Os patients studied (samples 1 and 4; for the amount of amplification/overexpression as determined by densitometry, see Table III). A third sample (sample 9) contained an amplified c-myc (Fig. 6); not enough material was available to study c-myc expression. Additional experiments confirmed c-myc amplification in samples 1, 4, and 9. These confirmatory experiments also revealed that the amplification detected in sample 9 resulted from the rearrangement of c-myc, because two bands of close molecular weight were detected after a prolonged run in agarose gels. RNA studies revealed c-myc overexpression in OS sample 17 (Fig. 7C), but no abnormality was detected by Southern blot analysis in the DNA (not shown). Amplification, but apparently normal expression, was detected in two of the 10 GCTs studied (samples 19 and 20, Table III). In four other GCTs samples (samples 21, 22, 25, and 43), c-myc appeared overexpressed in the absence of detectable DNA alterations (Table III). Thus c-myc appeared altered in 11% of the OS, and 60% of the GCTs studied. We also investigated the structure of c-myc in 18 chondrosarcomas, and the expression of c-myc in RNAs derived

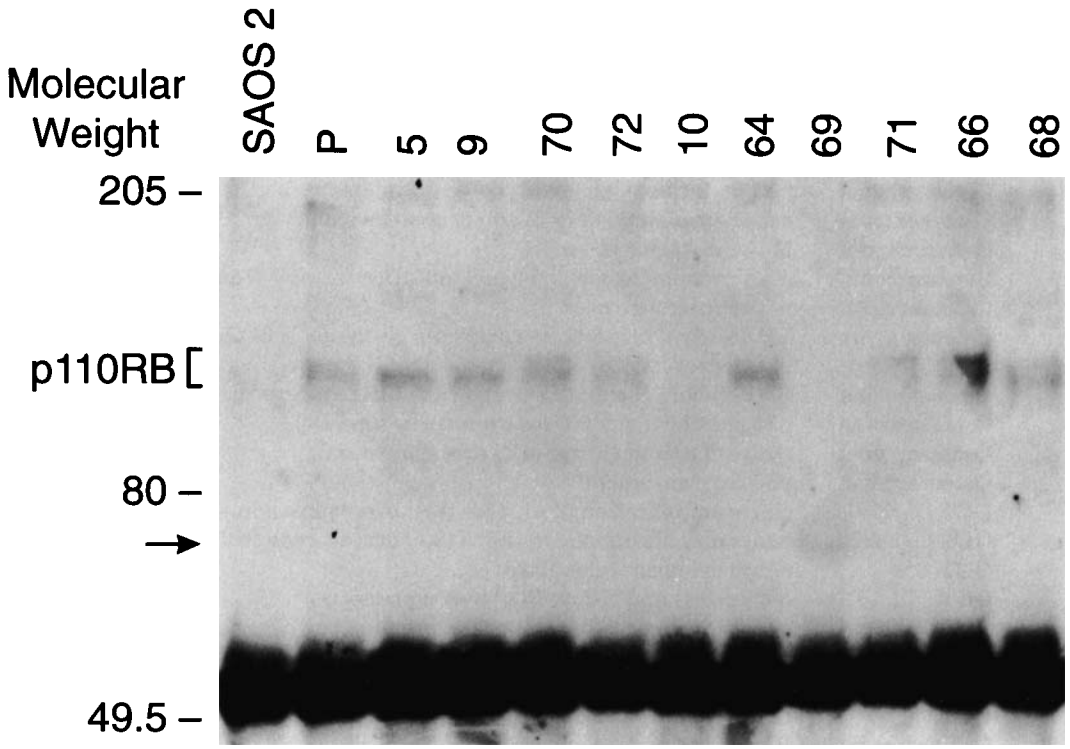

Fig. 2. Representative immunoblot detection of RB protein. Tumor cell lysates were derived from the indicated bone tumor samples (Table I); SAOS-2, negative control; P, placenta (positive control). Lysates were immunoprecipitated using the anti-RB PMG3-245 (Pharmigen), transferred onto nylon membranes and visualized by chemiluminescence. Note the presence of a low molecular weight RB-immunoreactive protein in sample 69 (*arrow*) and the absence of detectable RB protein in sample 10. Also, it is possible to discriminate the different phosphorylated forms of RB (doublets) in samples 66, 70, and 71. Molecular Weight: $\times 10^3$. Two additional experiments confirmed the presence of the lower molecular weight band in sample 69, and the absence of detectable RB protein in sample 10.

from five chondrosarcomas. None of the chondrosarcoma samples studied contained detectable alterations of c-myc (not shown).

Finally, we analyzed the structure of the N-myc proto-oncogene in 23 OS samples and N-myc expression in 12 of them. Three of the OS samples studied (sample 17, Fig. 7A; and samples 10–11, Fig. 7B) appeared to overexpress N-myc, in the absence of detectable N-myc DNA amplification (Table III). Interestingly, two of these samples (samples 10 and 11) contained deletions of RB (Fig. 1), and sample 17 overexpressed c-myc (Fig. 7C) (Table III). N-myc appeared normal by Southern blot studies of DNAs from six GCTs (not shown) and it was overexpressed in one of the nine GCT (11%) RNAs studied (sample 22, which also overexpressed c-myc, Table III). N-myc appeared normal in DNAs from five chondrosarcomas (Table II) and in RNAs from three of these samples (samples 25, 29, and 62, Fig. 7A).

The fos oncogene has been detected in two mouse sarcoma viruses, the FBJ-MSV and the FBR-MSV [Verma, 1986]. Although the two viruses contain structurally different forms of the fos gene, they both induce OS-like tumors in newborn mice [Verma, 1986]. Also, fos expression specifically interfered with normal bone development in transgenic mice [Ruther et al., 1987]. Therefore, it appeared possible to us that fos alterations could occur during bone tumor development. c-fos was overexpressed in seven of the eighteen OS samples we studied (39% of the Os samples), corresponding to 17 different patients (41% of the OS patients), (samples 7 and 8, 10, 11, 12, 33, and 34) (Fig. 7D; Table III). Of these, sample 12 also contained a rearranged p53 (Fig. 3, and Table III), and samples 10 and 11 revealed both RB deletions (Fig. 1; Table III) and N-myc overexpression (Fig. 7B, and Table 2). Samples 7 and 8 were obtained from the primary tumor (sample 7) and metastatic lung tumor (sample 8) of the same patient: both samples overexpressed c-fos, but only sample eight contained a mutated p53 (Table III). This

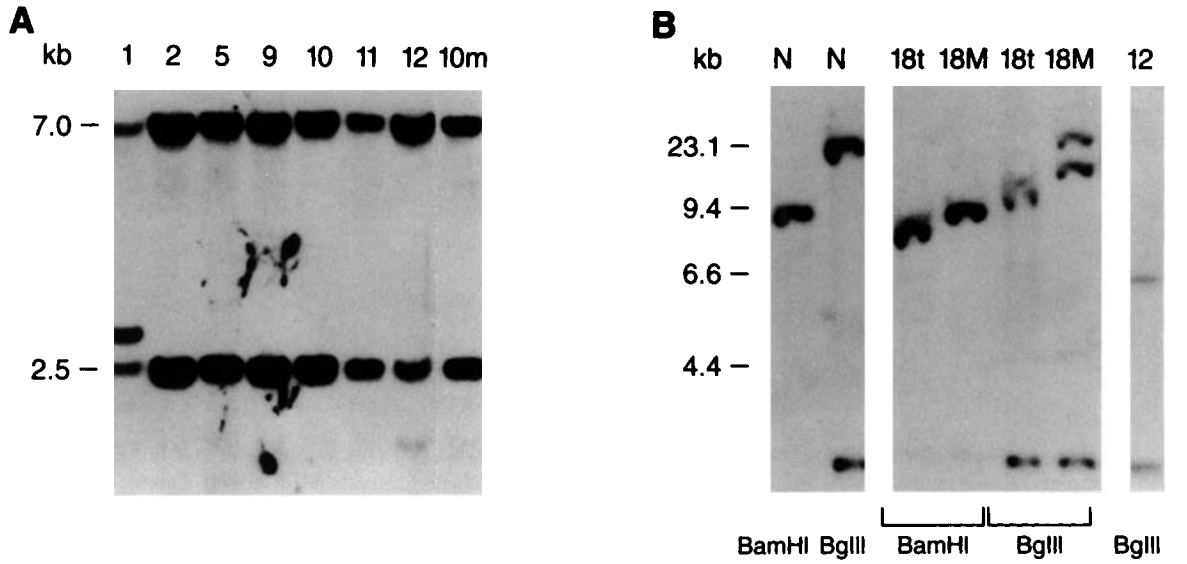

Fig. 3. Representative Southern blot analysis of DNA from different osteosarcoma samples hybridized to a probe specific for p53. **A:** The same blot shown in Figure 1 was stripped according to the membrane manufacturer's instructions and hybridized with the p53 cDNA. An abnormal 3.0 kb band is seen in OS sample 1; the presence of a normal but less intense p53 restriction pattern in the same sample suggests that one normal allele is still present. OS sample 12 shows a 60% decrease of the 2.5 kb band. **B:** DNAs were digested with the indicated restriction enzymes and hybridized with p53 DNA. The unusual migration of the DNAs (i.e., some bands appear U-shaped) may result from their high molecular weight. N, control DNA from placenta, shows the normal restriction pattern. OS sample 12 shows an abnormal restriction pattern following digestion with BglII, confirming the p53 alterations detected with the Hind III restriction enzyme (A). OS sample 18 shows an abnormal restriction pattern following hybridization with both BamHI and BglII. This sample did not show alterations when using the Hind III restriction enzyme. 18t corresponds to DNA from the primary tumor, 18M corresponds to DNA from metastatic tumor. Note that different alterations of p53 are seen in these two DNA samples, suggesting either two independent mutational events or further rearrangements of p53 in the primary tumor following metastasis (see text).

may suggest that in this particular tumor, c-fos alteration was an early event and that p53 mutation was associated with tumor progression and metastasis. Among GCT, three out of the nine tumors studied (33%) overexpressed c-fos (Table III). Interestingly, all of the GCTs that overexpressed c-fos (samples 19, 20, 25) also contained c-myc abnormalities (Table III). Alterations of c-fos were not detected in the three chondrosarcomas studied (Table II).

Statistical Analyses

The exact log-rank test, with or without stratification for histologic type of tumor, was used to evaluate the correlation between effect of gene alterations on clinical disease-free interval measured from the time of tumor sampling. Patients with unresectable metastases at the time of tumor sampling were considered to have a disease-free-interval of zero. All significance levels are of the two-sided type. We first analyzed the subset of 32 OS patients with follow-up data. For c-fos there were six patients with

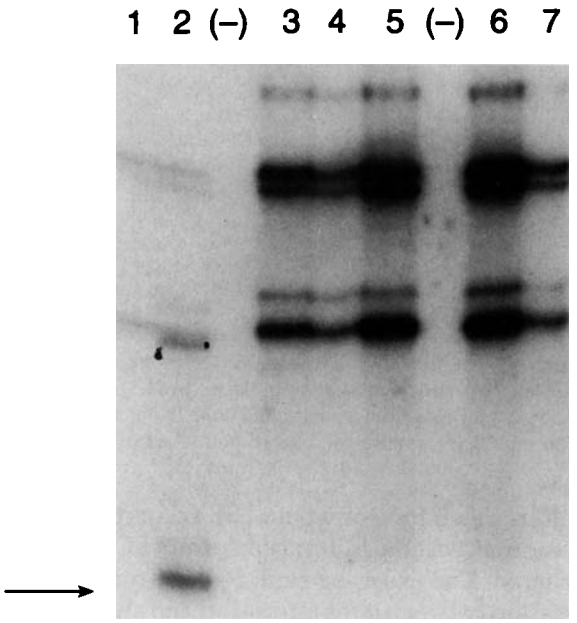

Fig. 4. Representative SSCP analysis of p53 mutations in osteosarcomas (exon 7, for technical details see Material and Methods). Electrophoretic mobility shift is present in DNA from sample 2 (*arrow*).

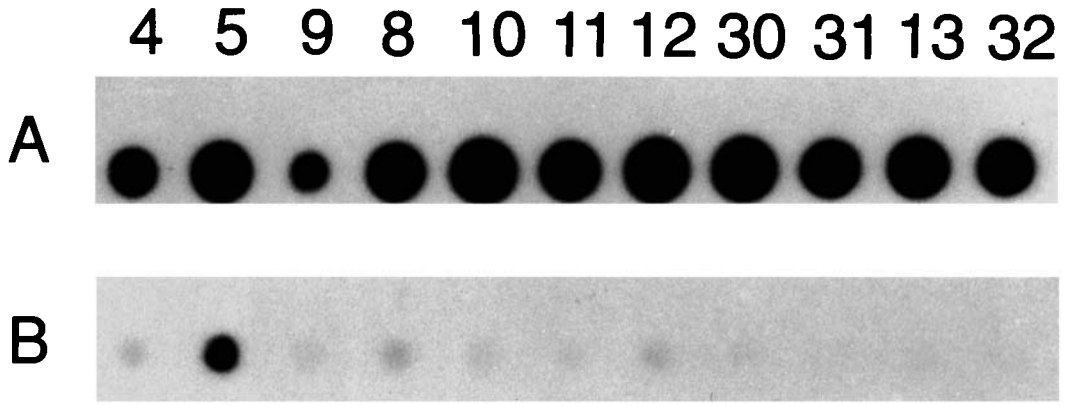

Fig. 5. Representative dot blot hybridization using probes specific for N-ras. DNAs from the indicated bone tumor samples (Table I) were amplified by PCR, spotted onto nylon filters and hybridized with a ^{32}-P labeled oligonucleotide designed to detect wild type N-ras (**A**) and mutations in codon 61 of N-ras (**B**). Sample 5 hybridized with this oligonucleotide (B), indicating that a mutation has occurred.

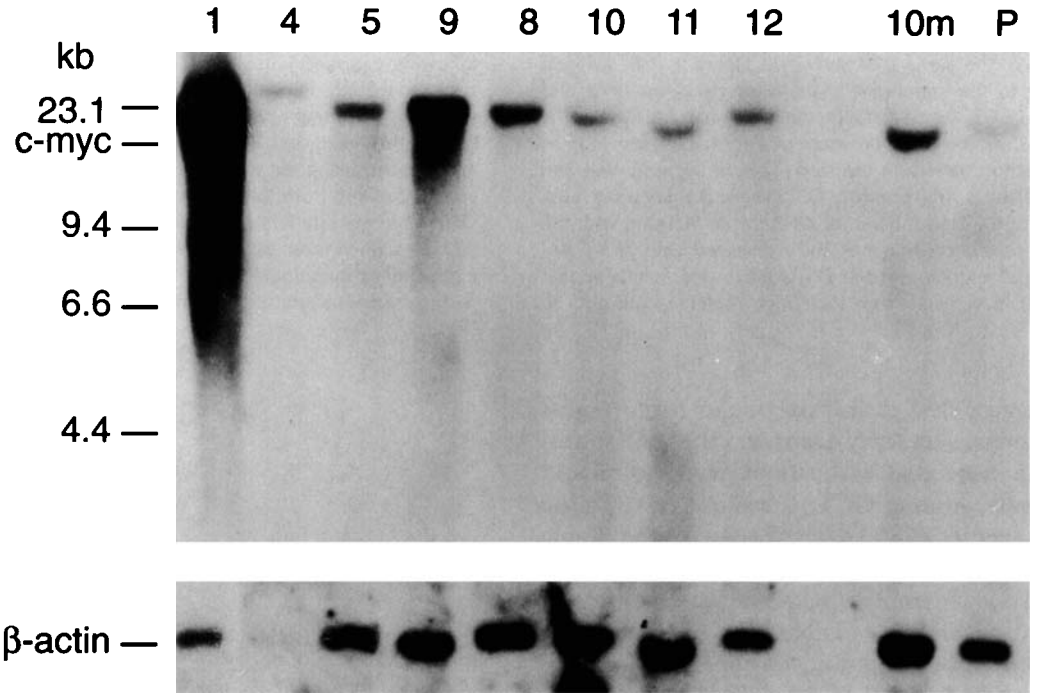

Fig. 6. Representative Southern blot hybridization using a probe specific for c-myc. The samples studied are indicated, see Table I for proper identification of these samples. 10 μg of Eco RI digested DNAs from the indicated bone tumor samples were run on a 0.8% agarose gel, blotted onto charged nylon membranes and hybridized sequentially with a ^{32}-P-labeled c-myc probe and a B-actin probe (control). The samples studied are indicated at the top of the gel. 10 m, muscle tissue from sample 10; P, placenta. Densitometric studies indicated that in samples 1, 4, and 9 c-myc was amplified (see also Table II).

alterations, four of whom had recurred, and 10 patients without alterations, four of whom recurred. The exact log-rank *P*-value for the association between c-fos alteration and shorter time to recurrence was .072. For RB, there were three patients with alterations, all of whom had recurred, and 23 patients without alterations, 11 of whom had recurred. The exact log-rank p-value was .12. There were four recurrences among the five patients with p53 alterations and 12 recurrences among the other 27 patients. The log-rank *P*-value was .38. Results for c-myc and N-myc did not approach statistical significance. Including patients with GCT and using the exact log-rank test stratified by histologic type did not change the significance levels be-

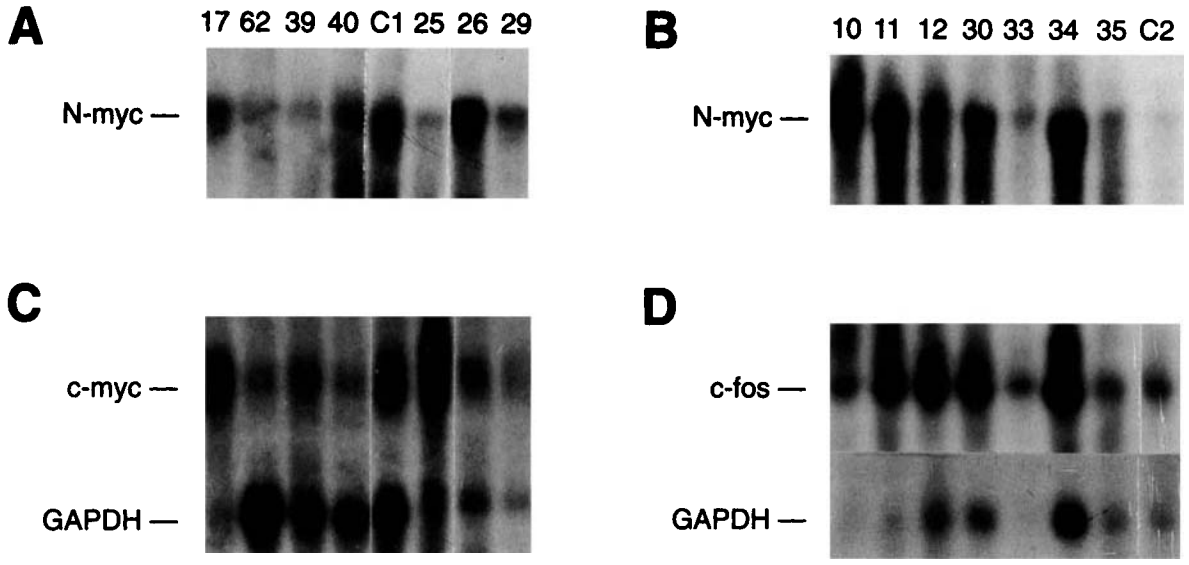

Fig. 7. Representative Northern blot hybridizations of RNAs from the indicated bone tumor samples using probes specific for N-myc, c-myc, and c-fos. 5 μg (or less if not enough material was available) were run on a formaldehyde denaturing gel, blotted onto charged nylon membranes and hybridized with a ^{32}-P labeled N-myc probe (**panels A and B**); **panel C,** the same blot shown in panel A was washed and hybridized with c-myc and GAPDH (control) probes; **panel D,** the same blot shown in panel B was washed and hybridized with c-fos and GAPDH probes. C1 and C2 represent RNAs derived from two different aneurysmal cysts (controls, two different samples were used because of the limited amount of RNAs available). Densitometric analysis revealed that N-myc was overexpressed in samples 10, 11, and 17; c-myc was overexpressed in samples 17 and 25; c-fos was amplified in samples 10, 11, 33, and 34 (see Table II).

cause none of these additional patients had recurred. We did not include patients with chondrosarcoma because no gene alterations were detected among those patients.

We also performed an exact log-rank analysis for the association between c-fos alteration and time-to-recurrence for OS patients stratified by risk group. Patients who had metastases or a recurrence at or before the time of tumor sampling were considered poor risk. For the six patients with c-fos alterations, five were poor risk and one good risk at the time of tumor sampling. For those without c-fos alterations, only two were poor risk and eight were good risk. The *P*-value for prognostic effect of c-fos adjusted for risk group did not approach statistical significance ($P = .56$). We also performed a log-rank test of the prognostic effect of RB alteration adjusted for risk strata. The three OS patients with RB alterations all were in the poor risk group; the *P*-value for RB adjusted for risk group did not approach significance ($P = .99$). There were an insufficient number of good risk patients for separate analyses.

We next evaluated the difference between the incidence of c-fos alterations in poor-risk OS patients (5/8) compared to good risk patients (1/9). In these analyses we included patients for whom follow-up was not available. These analyses suggested that c-fos alterations tended to occur more frequently in patients with recurrent or metastatic disease (poor risk patients). The difference was statistically significant ($P < .05$) by a Fisher exact test. This suggests that OS patients with c-fos alterations may have more aggressive tumors. The incidence of RB alterations in poor risk patients (3/17) compared to good risk patients (0/11) was not statistically significant but the statistical power is limited by the small number of cases with RB alterations. The same was true for the incidence of p53 alterations, 3/5 for poor risk patients compared to 7/28 for good risk patients ($P = .13$).

DISCUSSION

We found that 13 of the 34 OS patients studied (38%) and six of the 10 GCTs studied (60%), contained some detectable genetic alteration. Ideally, we would have liked to screen RB, p53, C-fos, c-myc, N-myc, Ki-ras, H-ras, and N-ras in all of the bone tumor samples studied. As expected, this was not possible because of the often very limited amount of tissue available for molecular studies. For obvious ethical reasons, the amount of tissue available for our studies could

not be increased. Given these limitations, our study provides new and important information about the molecular alterations that may be associated with human bone tumors.

Chondrosarcomas did not contain any detectable alteration of RB, p53, c-myc, N-myc, Ki-ras, H-ras, or N-ras. However, while the DNA status of p53 and c-myc was studied in all of the 18 chondrosarcoma samples (c-myc RNA expression in 5 of 18), Rb was studied only in seven samples (DNA and protein expression), and only six chondrosarcomas were studied for ras mutation, five for c-fos expression, and five for N-myc structure (the expression of N-myc was studied in three of these five samples) (Table II). Therefore, absence of detectable oncogene alterations might simply reflect the relatively small number of chondrosarcomas studied for Rb, ras, c-fos, and N-myc. Of interest, to the best of our knowledge, oncogene alterations in chondrosarcomas have not been previously reported, and Wunder et al. [1991], did not find any alteration of RB in six chondrosarcomas they studied. These results suggest that the genetic alterations found in OS and GCTs may be rare in chondrosarcomas. The absence of p53 mutations in chondrosarcomas was particularly unexpected given the reported association of p53 mutations with resistance to chemotherapy [Loewe et al., 1993; Fan et al., 1994]. Patients with chondrosarcomas are not treated with chemotherapy because these tumors are resistant to it. Our results suggest that genes other than p53 may be responsible for the peculiar resistance to chemotherapy of chondrosarcomas.

We confirmed that RB and p53 abnormalities occur in human osteosarcomas; however our results indicate that alterations of these genes are not a common characteristic of these tumors. Also, RB and p53 alterations do not appear to correlate with prognosis or with resistance/sensitivity to therapy. More importantly, our results demonstrate for the first time alterations of the oncogenes c-myc, N-myc, and c-fos in human OS and GCTs. Therefore, genetic alterations involving the cellular tumor suppressors RB and p53, as well as the cellular oncogenes fos and myc, may both contribute to the development of some OS and GCTs. In our study, several patients (seven OS and four GCT, Table III) contained more than one genetic alteration. On the other hand, several samples did not contain any detectable alteration. This suggests that the development of bone tumors is not necessarily associated with concomitant tumor suppressor gene/oncogene alterations. Some patients may be more susceptible than others to develop genetic alterations, and among these patients, some may have more aggressive tumors that require a different therapeutic approach (see below). Other possibilities exist; for example, oncogenes not investigated in this study could be altered. Furthermore, some patients may be infected by viruses that may render the cell genome more susceptible to develop genetic abnormalities. De Mattei et al. [1995] reported that 50% of human bone tumors contain BK virus sequences. However, BK sequences were also detected in normal human tissues, thus the significance of these findings awaits further experiments.

Simian virus 40 (SV40) is a DNA tumor virus that induces mesotheliomas, lymphomas, ependymomas, choroid plexus tumors, sarcomas, and osteosarcomas in rodents [Diamandopoulous, 1972; Carbone et al., 1989; Cicala et al., 1992; Cicala et al., 1993]. SV40 has been recently associated with 50% or more of human mesotheliomas, ependymomas, and choroid plexus tumors [Bergasagel et al., 1992; Carbone et al., 1994; Carbone et al., 1995]. Recently, we investigated a variety of human tumors for SV40-like sequences [Carbone et al., in press]. The only tumors that tested positive for SV40 sequences were human bone tumors, specifically OS (43 out of 131 different OS studied), GCT (4/6), and rarely other sarcomas [Carbone et al., in press]. The OS samples included 31 of the 36 samples investigated in this report for genetic alterations, and six of the 10 GCT. Of these, 25 out of 31 OS samples, and four out of six GCT, contained SV40-like sequences. Given the ability of the SV40 small t antigen to activate the c-fos promoter and stimulate AP-1 activity [Frost et al., 1994], it would be of interest to study if the presence of SV40-like sequences in human bone tumors contributes to the overexpression of c-fos. In addition, it is well known that the expression of the SV40 large T antigen may cause a variety of genetic abnormalities in the host cell [for a review see Ray, 1995]. The possibility that the expression of SV40 tumor antigens contributes to the development of genetic abnormalities in human bone tumors should be investigated.

Identifying genetic alterations in human bone tumors is important because of obvious potential clinical applications. Although the outcome

of osteosarcoma has improved since the addition of chemotherapy to surgery, systemic relapses still occur in 40 to 50% of cases [Baldini et al., 1995]. The ability to predict the course of osteosarcoma by identifying patients with more aggressive disease which will not respond to conventional chemotherapy would help guide treatment. These patients could be treated at the outset with high doses of myeloablative chemotherapy followed by bone marrow transplant, or agents that reverse the multidrug-resistance phenotype, such as verapamil and cyclosporine, could be added to their therapy from the beginning. Our finding that c-fos alterations occur more frequently in poor-risk OS patients may suggest that these patients represent appropriate candidates for more aggressive therapies. However, additional studies specifically designed to investigate the association between c-fos alterations and prognosis in OS patients are needed before concluding that this association has clinical relevance. Very recently, Baldini et al. [1995] reported that increased levels of P-glycoprotein in osteosarcomas were associated with more aggressive tumors that did not respond to conventional chemotherapy. If our findings are extended, and those of Baldini et al. [1995] are confirmed, it may become possible to identify by molecular analyses osteosarcoma patients that require more intensive therapeutic approaches, and at the same time, spare the rigors of intensive therapy for those with less aggressive disease.

ACKNOWLEDGMENTS

This work was supported in part by grant number 7-50386 from the DeMuth Memorial Cancer Research Fund, and by a gift from Pfizer Inc. to M.C. This work was also supported in part by a gift from the Passis family.

REFERENCES

Baldini N, Scotlandi K, Barbanti-Brodano G, Manara MC, Maurici D, Bacci G, Bertoni F, Picci P, Sottili S, Campanacci M, Serra M (1995): Expression of P-glycoprotein in high grade osteosarcomas in relation to clinical outcome. N Engl J Med 333:1380–1385.

Bergsagel DJ, Finegold MJ, Butel JS, Kupsky WJ, Garcea R (1992): DNA sequences similar to those of simian virus 40 in ependymomas and choroid plexus tumors of childhood. N Engl J Med 36:988–993.

Berns EMJ, Klijn JGM, van Putten WLJ, van Staveren IL, Portengen H (1992): c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. Cancer Res 52:1107–1113.

Bode U, Levine AS (1982): The biology and management of osteosarcoma. In: Levine AS (ed): "Cancer in the Young." New York: Masson Publishing USA Inc., pp. 575–614.

Bos JL (1989): Ras oncogenes in human cancer: A review. Cancer Res 49:4682–4689.

Brennan J, O'Connor T, Makuch RW, Simmons AM, Russell E, Linnoila RI, Phelps RM, Gadzar AF, Ihde DC, Johnson BE (1991): Myc family DNA amplification in 107 tumors and tumor cell lines from patients with small cell lung cancer treated with different combination chemotherapy regimens. Cancer Res 51:1708–1712.

Cance WG, Brennan MF, Dudas ME, Huang C-M, Cordon-Cardo C (1990): Altered expression of the retinoblastoma gene product in human sarcomas. N Engl J Med 323:1457–1462.

Carbone M, Lewis AM, Jr., Matthews BJ, Levine AS, Dixon K (1989): SV40 small t antigen mutants induce true histiocytic lymphomas in hamsters. Cancer Res 49:1565–1571.

Carbone M, Levine AS (1990): Oncogenes, antioncogenes, and the regulation of cell growth. TEM 1:248–253.

Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio M, Mew DJY, Levine AS, Procopio A (1994): SV40-like sequences in human pleural mesothelioma. Oncogene 9:1781–1790.

Carbone M, Rizzo P, Pass HI (1995): Association of simian virus 40 with rodent and human mesotheliomas. In: Barbanti Brodano G, Bendinelli M, Friedman H (eds): "DNA Tumor Viruses: Oncogenic Mechanisms." New York and London: Plenum Press, pp 75–90.

Carbone M, Rizzo P, Procopio A, Giuliano M, Pass HI, Gebhardt MC, Mangham C, Hansen M, Malkin DF, Bushart G, Pompetti F, Picci P, Levine AS, Bergsagel JD, Garcea R: SV40-like sequences in human bone tumors. Oncogene (in press).

Cicala C, Pompetti F, Nguyen P, Dixon K, Levine AS, Carbone M (1992): SV40 small t deletion mutants preferentially transform mononuclear phagocytes and B-lymphocytes in vivo. Virology 190:475–479.

Cicala C, Pompetti F, Carbone M (1993): SV40 induces mesotheliomas in hamsters. Am J Pathol 142:1524–1533.

De Mattei M, Martini F, Corallini A, Gerosa M, Scotland K, Carinci P, Barbanti-Brodano G, Tognon M (1995): High incidence of BK virus large T antigen coding sequences in normal human tissues and tumors of different histotypes. Int J Cancer 61:756–760.

Diamandopoulous GT (1972): Leukemia, lymphoma, and osteosarcoma induced in Syrian golden hamster by SV40. Science 176:173–175.

Esrig D, Elmajian D, Groshen S, Freeman JA, Stein JP, Chen SC, Nichols PW, Skinner DJ, Jones PA, Cote RJ (1994): Accumulation of nuclear p53 and tumor progression in bladder cancer. N Engl J Med 331:1259–1264.

Fan S, El-Deiry WS, Bae I, Freeman J, Bhatia K, Fornace AJ, Jr, Magrath I, Kohn KW, O'Connor PM (1994): p53 mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 54:5824–5830.

Fearon ER, Vogelstein B (1990): A genetic model for colorectal tumorigenesis. Cell 61:759–767.

Friend SH, Bernards R, Rogeji S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP (1986): A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643–646.

Frost JA, Alberts AS, Sontag E, Guan K, Mumby MC, Feramisco JR (1994): Simian virus 40 small t antigen cooperates with mitogen activated kinases to stimulate AP-1 activity. Mol Cell Biol 14:6244–6252.

Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994): Mutations in the p53 tumor suppressor gene; cluses to cancer etiology and molecular pathogenesis. Cancer Res 54:4855–4878.

Harbour JW, Lai S-L, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ (1988): Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science 241:353–357.

Loewe SW, Ruley EH, Jacks T, Housman DE (1993): p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74:957–967.

Masuda H, Miller C, Koeffler HP, Battifora H, Cline MJ (1987): Rearrangement of the p53 gene in human osteogenic sarcomas. Proc Natl Acad Sci USA 84:7716–7719.

Miller CW, Abdulkarim A, Tsay C, Slamon D, Ishizaki K, Toguchida J, Yamamuro T, Lampkin B, Koeffler HP (1990): Frequency and structure of p53 rearrangements in human osteosarcoma. Cancer Res 50:7950–7954.

Mulligan LM, Matlashewski GJ, Scrable HJ, Cavenee WK (1990): Mechanisms of p53 loss in human sarcomas. Proc Natl Acad Sci USA 87:5863–5867.

Paterson MC, Dietrich KD, Danyluk J, Paterson AHG, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA, Slamon DJ, Fourney RM (1991): Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 51:556–567.

Ray FA (1995): Simian virus 40 large T antigen induces chromosome damage that precedes and coincides with complete neoplastic transformation. In: Barbanti Brodano G, Bendinelli M, Friedman H (eds): "DNA Tumor Viruses: Oncogenic Mechanisms." New York and London: Plenum Press, pp 15–26.

Romano JW, Ehrhart JC, Duthu A, Kim CM, Appella E, May P (1989): Identification and characterization of a p53 gene mutation in a human osteosarcoma cell line. Oncogene 4:1483–1488.

Ruther U, Garber C, Komitowski D, Muller R, Wagner EF (1987): Deregulated c-fos expression interferes with normal bone development in transgenic mice. Nature 325:412–416.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning, second edition." Cold Spring, NY: Cold Spring Harbor Laboratory Press, Vol 1. pp 6.2–6.62.

Toguchida J, Ishizaki K, Sasaki MS, Ikenaga M, Sugimoto M, Kotoura Y, Yamamuro T (1988): Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. Cancer Res 48:3939–3943.

Toguchida J, Yamaguchi T, Ritchie B, Beauchamp RL, Dayton SH, Herrere GE, Yamamuro T, Kotoura Y, Sasaki MS, Little JB, Weichselbaum RR, Ishizaki K, Yandell DW (1992): Mutation spectrum of the p53 gene in bone and soft tissue sarcomas. Cancer Res 52:6194–6199.

Vande Woude GF, Schulz N, Zhou R, Paules RS, Daarl, Yew N, Oskarsson M (1990): Cell cycle, oncogenes, and antineoplastic drugs. Accomplishments in Cancer Res 1:129–143.

Verma IM (1986): Proto-oncogene fos: A multifaceted gene. Trends Genet 2:93–96.

Wasson JC, Saylors III RL, Zelter P, Friedman HS, Bigner SH, Burger PC, Bignell DD, Look AT, Douglass EC, Brodeur GM (1990): Oncogene amplification in pediatric brain tumors. Cancer Res 50:2987–2990.

Weichselbaum RR, Beckett M, Diamond A (1988): Some retinoblastomas, osteosarcomas, and soft tissue sarcomas may share a common etiology. Proc Natl Acad Sci USA 85:2106–2109.

Wunder JS, Czitrom AA, Kandel R, Andrulis IL (1991): Analysis of alterations in the retinoblastoma gene and tumor grade in bone and soft-tissue sarcomas. J Natl Cancer Inst 83:194–200.