Molecular Analysis of *p53, MDM2,* and *H-ras* Genes in Osteosarcoma and Malignant Fibrous Histiocytoma of Bone in Patients Older than 40 Years

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Some investigators have reported that the histological features of osteosarcoma (OS) arising in elderly patients are different from those in younger patients; however, a molecular biologic study of OS in elderly patients has not been documented. In this study, 23 cases of OS (15 osteoblastic and 8 MFH-like types) and 18 cases of MFH of bone in patients 40 years of age or older were analyzed for mutation of the *p53* gene, amplification of the *MDM2* gene, and mutation of the *H-ras* gene, using formalin-fixed paraffin-embedded materials. We also examined the expression of p53, MDM2, and p21WAF1 protein immunohistochemically and assessed the proliferative activities using the monoclonal antibody MIB-1. p53 immunoreactivity was recognized in 5 of 23 OS cases (22%), whereas *p53* gene mutations were also detected in 5 of 23 OS cases (22%; osteoblastic [4/15; 27%] and MFH-like [1/8; 18%] types) and in 4 of 18 cases of MFH of bone (22%). There was a statistically significant correlation between p53 immunoreactivity and *p53* mutation status in OS ($P = .0482$). All those cases of osteoblastic OS and MFH of bone that had *p53* mutations, with the exception of one case of MFH of bone that had a silent mutation, showed aggressive biologic behavior (dead of disease within 12 mo), in contrast to the MFH-like OS cases (alive without disease at 22 mo). Three cases of OS (13%) and three cases of MFH of bone (17%) showed immunoreactivity for MDM2. As for gene alteration, three cases of OS (13%) and 3 cases of MFH of bone (17%) demonstrated *MDM2* amplification. *MDM2* amplification showed a significant correlation with the expression of MDM2 protein in OS ($P = .0344$). p21WAF1 expression was detected in three cases of OS (13%) and in six cases of MFH of bone (33%). *MDM2* alteration and *p21WAF1* expression were not observed in any of the cases of MFH-like OS. MIB-1-LI showed a statistically significant correlation with p53 immunoreactivity and MDM2 immunoreactivity in OS ($P = .0307$ and $P = .0358$, respectively). *H-ras* mutation at Codons 12 and 13 was not recognized in any of the cases of OS or MFH of bone. In conclusion, although treatment differences during the time of study make it difficult to compare survival analysis, in the current study, *p53* mutation in osteoblastic OS and MFH of bone in elderly patients seemed to be closely associated with the progression of the tumor, which was not the case in MFH-like OS. Furthermore, *MDM2* alteration and *p21WAF1* expression were demonstrated only in osteoblastic OS and MFH of bone, whereas they were not recognized in MFH-like OS. Although the number of patients in this analysis was small, it would appear that MFH-like OS may have some characteristic biologic aspects when compared with osteoblastic OS and MFH of bone in elderly patients.

KEY WORDS: H-ras, Malignant fibrous histiocytoma of bone, MDM2, Osteosarcoma, p53.

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Osteosarcoma (OS) is a primary malignant bone tumor that frequently arises in younger patients, occurring less commonly in adults or the elderly (1–3). In contrast, malignant fibrous histiocytoma (MFH) of bone is a malignant bone tumor that frequently arises in adults and the elderly. In the case of OS arising in elderly patients, MFH-like type has been noted more frequently than osteoblastic type (2, 4). In addition, Naka *et al.* (4) reported that patients with MFH-like OS had a favorable survival compared with patients with osteoblastic OS or

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MFH of bone and that the proliferative activity in MFH-like OS tended to be lower than in osteoblastic OS or MFH of bone, using Ki-67 as the proliferative marker.

p53 gene mutation has been investigated extensively in mesenchymal tumors, and it has been suggested that the alterations of *p53* play a potential role in tumorigenesis and tumor progression. In OS, Yokoyama *et al.* (5) reported that most cases with *p53* mutation were associated with recurrence or metastatic lesions, suggesting the possibility that *p53* mutation plays an important role in tumor progression. The *MDM2* gene codes protein with the ability to bind to *p53* and inhibits the transcriptional activity of *p53* (6). Gene amplification of *MDM2* has been described as the pathway of tumorigenesis or tumor progression in various sarcomas (6–8). In OS, gene amplification of *MDM2* was found to be correlated with recurrence or metastasis in some previous studies (5, 9, 10), but not in others (11).

H-ras gene mutation has been investigated in various soft-tissue sarcomas (12, 13). With regard to OS, *K-ras* and *N-ras* gene mutations have been found in previous studies (5, 14), however *H-ras* gene mutation has not been detected (15).

Although OS arising in elderly patients has been analyzed in several reports clinicopathologically, the immunohistochemical and molecular biological findings have not been analyzed in detail. In the present study, we analyzed the immunohistochemical and molecular abnormalities of *p53, MDM2,* and *H-ras* in OS and MFH of bone arising in patients ≥40 years of age.

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MATERIALS AND METHODS

Two hundred and forty-seven patients with osteosarcoma (OS) and 72 patients with malignant fibrous histiocytoma (MFH) of bone were registered in the bone tumor files of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Japan between 1965 and 1999. Formalin-fixed, paraffin-embedded tissue from a total of 23 cases of OS in patients aged ≥40 years (15 osteoblastic and 8 MFH-like types), and 18 cases of MFH of bone in patients within the same age group were used for immunohistochemical and molecular analyses. The number of evaluated histologic slides ranged from 3 to 12, with a mean of 5 slides per case. We defined osteoblastic OS as a tumor with a prominent lacelike osteoid formation produced by rounded or polygonal atypical cells (Fig. 1A). Almost all the osteoblastic OS cases demonstrated predominantly or entirely osteoblastic lesions. MFH-like OS was defined as a tumor with prominent MFH-like areas that consisted of a mixture of pleomorphic fibroblast-like spindle-shaped and histiocyte-like round cells arranged in a storiform pattern, together with a focal tumor osteoid formation, where the osteoblastic areas never exceeded 50% of the entire lesion (Fig. 1B–C).

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Furthermore, we defined MFH of bone as a tumor with a proliferation of atypical polygonal to short spindle cells in a storiform pattern, but with no tumor osteoid formation (Fig. 1D). Diagnoses were based on hematoxylin-eosin (HE) staining, with sampling specimens being decalcified when needed. Immunohistochemical studies using the streptavidin-biotin peroxidase (SAB) method were performed when it was necessary to rule out other lesions. Survival data were available for 21 of the OS cases (14 osteoblastic and 7 MFH-like types) and 17 of the cases of MFH of bone, with a follow-up ranging from 3 to 130 (median, 35.6) months in OS and 2 to 128 (median, 30.7) months in MFH of bone. We assessed the correlation between the clinicopathologic factors (gender, histological subtype, histological grading, and mitotic rate) and the results of both immunohistochemical and molecular analyses.

Immunohistochemistry

Immunohistochemical analysis was performed using mouse IgG monoclonal antibodies against Ki-67 (MIB-1; 1:100, Immunotech, Marseille, France), p53 (PAb1801, 1:100, Oncogene Science, New York, NY), MDM2 (IF2; 1:40, Oncogene Research Products, Cambridge, MA), and p21/WAF1 (EA10; 1:100, Oncogene Research Products). PAb1801 reacts with both mutant and wild-type human p53 protein, and IF2 recognizes an epitope in the amino terminal portion of human mdm-2 protein that corresponds to the p53-binding site.

Four-micron-thick histologic sections were cut, mounted on glass slides coated by 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked by methanol containing 3% H_2O_2 for 30 minutes. For staining with the above antibodies, specimens were pretreated with citrate buffer (0.01 mol/L citric acid: pH, 6.0) four times, each time for 5 min at 100° C in a microwave oven. Sections were incubated with the primary antibody at 4° C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan).

The sections were then finally reacted in a 3,3' diaminobenzidine, peroxytrichloride substrate solution, counterstained with methyl green or hematoxylin, and then mounted. As for p53, MDM2 and WAF1, when ≥10% of the nuclei in the tumor cells were stained, it was interpreted as a positive result. The MIB-1–labeling index (MIB-1-LI) was determined as

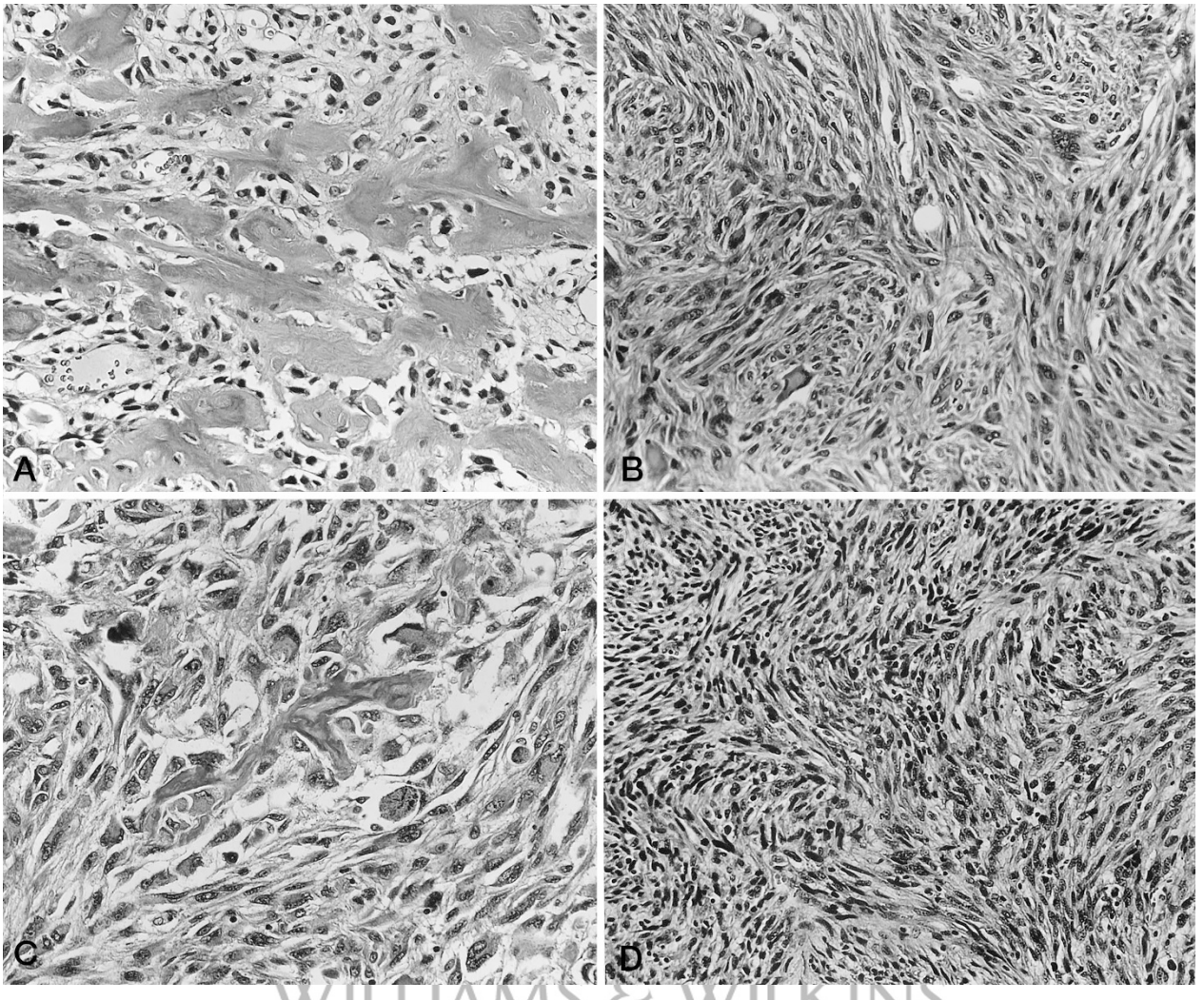

FIGURE 1. **A,** osteoblastic OS (Case OS8). The lesion is composed of atypical round to polygonal cells with a lace-like osteoid formation (200×). **B,** MFH-like OS (Case OS17). Microscopically, the majority of the lesion is characterized by MFH-like areas consisting of a mixture of fibroblast-like spindle-shaped cells in a storiform pattern and histiocyte-like round to polygonal cells (150×). **C,** tumor osteoid formation is evident focally in MFH-like areas (200×). **D,** MFH of bone (Case MFH11). Histologically, atypical polygonal to short spindle cells can be seen proliferating in a storiform pattern. No tumor osteoid was observed within examined specimens (150×).

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the percentage of positive cells by counting the positively stained nuclei in ≥1,000 tumor cells.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism for p53

Genomic DNA was purified using standard proteinase K digestion and phenol–chloroform extraction methods. The obtained DNA was subjected in a total volume of 5 μL containing 10 mM Tris–HCL (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 25 mM of dNTP, 0.25 U *Taq* DNA polymerase, and 0.2 μM of each of the primers. Mutations of the *p53* gene were examined from exons 5 to 9. The sequences of the primers are summarized in Table 1. PCR was carried out using a programmable thermal cycler (PTC-100TM: MJ Research Inc., Watertown, MA) for 40 cycles after the first denaturation at 95° C for 1 minute (95° C for 1 min, 66° C for 1 min, and 72° C for 2 min). PCR products were electrophoresed through 2.0% agarose gel with ethidium bromide. The DNA band was purified using a SUPREC tube (TAKARA Biomedicals, Japan) and the products were reamplified for 25 cycles. Five microliters of the reamplified products was diluted 1:1 in loading buffer (94% formamide, 10 mg bromphenol blue, and 0.05% xylene cyanol). Each sample was denatured at 96° C for 5 min and chilled on ice, then a total of 6 μL of the samples was applied onto gel containing 12.5% acrylamide (GenePhor, Amersham Pharmacia Biotech, Uppsala, Sweden). Single-strand conformation polymorphism was performed using a DNA fragment analyzer (GenePhor, Amersham Pharmacia Biotech) at 600 V, 25 mA, 15 W, and 5° C, for 120 minutes. The bands were visualized by a DNA Silver Staining Kit (GenePhor, Amersham Pharmacia Biotech).

TABLE 1. Primers Used for *p53* Mutation, *MDM2* Amplification, and *H-ras* Mutation Analysis

	Primer	Direction	Nucleotide Sequence
p53	Exon 5	Forward	5′-CTCTTCCTGCAGTACTCCCCTGC-3′
		Reverse	5′-GCCCCAGCTGCTCACCATCGCTA-3′
	Exon 6	Forward	5′-GATTGCTCTTAGGTCTGGCCCCTC-3′
		Reverse	5′-GGCCACTGACAACCACCCTTAACC-3′
	Exon 7	Forward	5′-GCTTGCCACAGGTCTCCCCAAG-3′
		Reverse	5′-AGGCTGGCAAGTGGCTCCTGAC-3′
	Exon 8	Forward	5′-TGGTAATCTACTGGGACGGA-3′
		Reverse	5′-GCTTAGTGCTCCCTGGGGGC-3′
	Exon 9	Forward	5′-GCCTCTTTCCTAGCACTGCCCAAC-3′
		Reverse	5′-CCCAAGACTTAGTACCTGAAGGGTG-3′
MDM2		Forward	5′-GGTTGGATCAGGATTCAGTT-3′
		Reverse	5′-GAGTTCTTGTCCTTCTTCAC-3′
PAH		Forward	5′-ATGCCACTGAGAACTCTTT-3′
		Reverse	5′-GAGTTCTTGTCCTTCTTCAC-3′
H-ras	First-round amplification	Forward	5′-GGAGACCCTGTAGGAGGACCC-3′
		Reverse	5′-TCTATAGTGGGGTCGTATTCGTCC-3′
	Second-round amplification for codons 12 and 13	Forward	5′-TGAGGAGCGATGACGGAAT-3′
		Reverse	5′-ATGGTCAGCGCACTCTTGCCCTC-3′

TABLE 2. Clinicopathological Data in Osteosarcoma (OS) and Malignant Fibrous Histiocytoma (MFH) of Bone

Case (subtype)	Gender	Age (y)	Location	Treatment	Grade	MR	Follow-Up
			OS (23 cases)				
OS1 (OB)	M	54	Maxilla	Resection (post. CT)	4	9	DOD7M
OS2 (OB)	M	47	Proximal tibia	Resection	3	19	DOD3M
OS3 (OB)	M	46	Proximal tibia	Amputation	4	16	DOD6M
OS4 (OB)	M	49	Distal femur	Disarticulation (post. CT)	4	11	DOD26M
OS5 (OB)	M	57	Proximal femur	Amputation	4	22	AWD76M
OS6 (OB)	F	41	Proximal femur	Curettage	4	12	NA
OS7 (OB)	F	62	Rib	Resection	4	24	DOD24M
OS8 (OB)	F	61	Ilium	Resection	4	14	DOD14M
OS9 (OB)	M	58	Ilium	NA	4	15	DOD9M
OS10 (OB)	M	59	Distal femur	Amputation	4	10	DOD8M
OS11 (OB)	F	44	Distal femur	Resection (post. CT)	3	17	AWD42M
OS12 (OB)	F	55	Vertebra	Resection	4	18	DOD34M
OS13 (OB)	M	66	Skull	Resection	4	13	DOD4M
OS14 (OB)	M	41	Skull	Resection	4	9	DOD18M
OS15 (OB)	M	48	Distal tibia	Resection	4	15	DOD4M
OS16 (MFH-like)	F	43	Distal femur	Resection (post. CT)	4	12	AWD130M
OS17 (MFH-like)	F	48	Distal femur	Resection	3	10	AWD22M
OS18 (MFH-like)	M	44	Proximal femur	Curettage (amputation)	3	13	DOD51M
OS19 (MFH-like)	F	63	Proximal femur	Resection (amputation)	3	5	AWD128M
OS20 (MFH-like)	M	69	Distal femur	Amputation	3	7	DOD16M
OS21 (MFH-like)	F	69	Proximal femur	Resection (post. CT)	3	16	NA
OS22 (MFH-like)	F	77	Ilium	NA	3	11	DOD24M
OS23 (MFH-like)	F	67	Proximal tibia	Resection (amputation)	4	10	AWD102M
			MFH of bone (18 cases)				
MFH1	M	50	Tibia	Resection	4	12	DOD10M
MFH2	M	73	Femur	Pre. RT (resection)	3	11	DOD2M
MFH3	M	60	Ilium	Pre. RT (resection)	3	14	DOD4M
MFH4	F	84	Sacrum	Resection	4	12	DOD15M
MFH5	M	41	Femur	Curettage	2	10	NA
MFH6	F	46	Ilium	Curettage	4	19	DOD12M
MFH7	F	43	Tibia	Curettage (amputation)	4	26	DOD11M
MFH8	M	49	Femur	Resection	3	13	DOD17M
MFH9	F	75	Femur	Curettage	4	18	DOD26M
MFH10	M	61	Tibia	NA	4	21	DOD128M
MFH11	M	52	Tibia	Resection	4	20	DOD12M
MFH12	M	72	Tibia	Curettage (amputation)	3	12	DOD90M
MFH13	F	42	Vertebra	NA	4	10	AWD46M
MFH14	M	48	Sacrum	Curettage (post. RT)	3	17	DOD50M
MFH15	M	42	Scapula	Resection	4	10	DOD39M
MFH16	M	75	Sternum	Resection	4	7	DOD38M
MFH17	F	45	Femur	Resection (post. RT)	3	10	AWD19M
MFH18	M	69	Vertebra	Resection (post. RT)	4	18	DOD3M

OB, osteoblastic; DOD, died of disease; AWD, alive without disease; NA, not available; pre., preoperative; post., postoperative; CT, chemotherapy; RT, radiotherapy; MR, mitotic rate; M, months.

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Differential PCR for *MDM2*

The differential PCR method for detecting amplification of *MDM2* was based on a modification of a reported method using *PAH* as the internal control (16). DNA samples were added to a PCR mix with a total volume of 25 μL containing 10 mM Tris-HCl (pH, 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.5 U *Taq* DNA polymerase, and 0.5 μM of each of

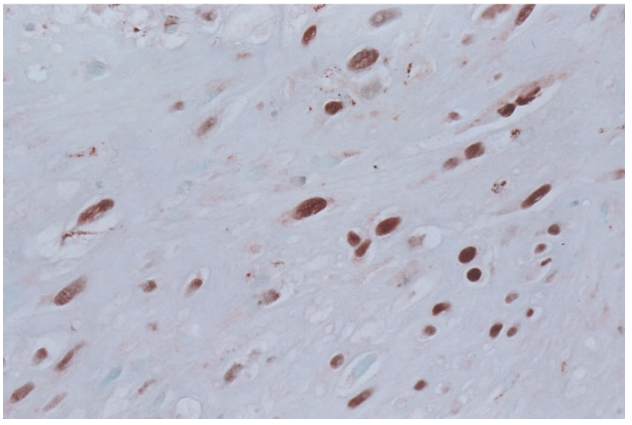

FIGURE 2. p53 immunoreactivity is visible in osteoblastic OS, which also reveals *p53* mutation (Case OS15; 400×).

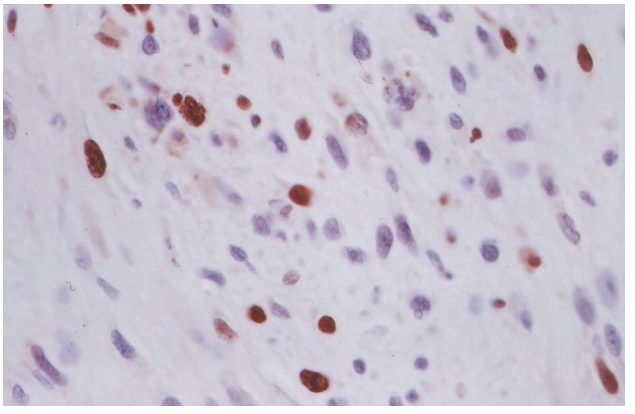

FIGURE 3. MDM2 immunoreactivity can be noted in osteoblastic OS, which also shows the expression of p53 protein (Case OS13; 400×).

the primers. The sequences of the primers are summarized in Table 1. PCR was carried out for 30 cycles after the first denaturation at 95° C for 1 minute (94° C for 1 min, 50° C for 1 min, and 70° C for 1 min). DNA of the SA-1 cell line (American Type Cell Collection, Rockville, MD), which is known to show seven-fold amplification of the MDM2 gene by Southern blot analysis, was used as a positive control. After the amplification, 10 μL of PCR products were electrophoresed through 3.0% agarose gel with ethidium bromide, and the intensities of the DNA products were quantified by National Institutes of Health (NIH) Imaging software, Version 1.56. Comparing the ratio of the intensities of the *MDM2* and *PAH* PCR products for the samples with positive SA-1 cells (seven-fold), the degree of *MDM2* amplification was analyzed. Samples showing more than two-fold amplification were judged as showing positive results.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) for *H-ras*

We used the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) procedure to detect *H-ras* gene mutations at codons 12 and 13 with strategy primers as reported (17). The sequences of the primers are summarized in Table 1. PCR was carried out using a programmable thermal cycler for 35 cycles (95° C for 1 min, 59° C for 1 min, and 72° C for 2 min). Then, 5 μL of the amplified products that had been diluted 100 times were reamplified by means of nested PCR for 30 cycles (92° C for 15 s, 55° C for 15 s, and 72° C for 30 s). Codon 12 mutations could be detected due to a naturally occurring *Hpa*II site (CCGG) that is lost when the mutation occurs. *Hpa*II digests the 71-bp amplified fragment into two fragments (41 bp and 30 bp), thereby revealing the presence of the normal allele, whereas the mutant allele remains within the undigested 71-bp fragment. With regard to codon 13 mutations, nested primers were used to create a new restriction site for *Hph*I (GGTGA) by changing a T for an A in the second position of codon 14. *Hph*I digests the same 71-bp amplified fragment into two fragments (58-bp and 13-bp), thereby revealing the presence of the normal allele, whereas the mutant allele remains within the undigested 71-bp fragment. The DNA bands were an-

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TABLE 3. Correlation between *p53* Mutation, p53 Immunoreactivity, MDM2 Immunoreactivity, and Clinicopathologic Parameters in Osteosarcoma

Parameter	p53 Mutation		*P* Value[a]	p53 IHC		*P* Value[a]	MDM2 IHC		*P* Value[a]
	−	+		−	+		−	+	
Sex									
Female (n = 12)	10	2	.6404	12	0	.0137[b]	11	1	.5901
Male (n = 11)	8	3		6	5		9	2	
Histological subtype									
Osteoblastic (n = 15)	11	4	.6214	10	5	.1221	12	3	.5257
MFH-like (n = 8)	7	1		8	0		8	0	
Mitotic rate (per 10HPF)									
<15 (n = 14)	11	3	>.9999	12	3	.3428	11	3	.3530
≧15 (n = 9)	7	2		6	2		9	0	
Histological grading									
III (n = 8)	7	1	.6130	7	1	.6130	8	3	>.9999
IV (n = 15)	11	4		11	4		12	0	

IHC, immunohistochemistry; HPF, high-power field.
[a] Fisher's exact test.
[b] Statistically significant.

alyzed by 3% agarose gel electrophoresis, stained with ethidium bromide, and then photographed.

DNA Sequencing

Aberrantly migrating bands of *p53* were excised from the SSCP gel, and the amplified product was purified by Microcon centrifugal filter devices (Millipore, Bedford, MA). After purification, direct sequencing was carried out by the dideoxy chain termination method using a Perkin Elmer ABI Prism 310 sequence analyzer (Applied Biosystems, Foster City, CA). The primers used for direct sequences were the same sense and anti-sense primers used for the PCR-SSCP in *p53*.

Statistical Analysis

Fisher's exact test was used to evaluate the association between two dichotomous variables. The difference in the MIB-1-LI between two groups was estimated by Mann–Whitney *U* test. A *P* value of $<.05$ was considered to indicate statistical significance.

RESULTS

Clinical and Histological Findings

Clinicopathologic data for the patients with OS or MFH of bone are summarized in Table 2. There were 11 males and 12 females with OS (M–F ratio, 11:12; osteoblastic [M–F ratio, 3:2] and MFH-like [M–F ratio, 1:3] types), whereas there were 12 males and 6 females with MFH of bone (M–F ratio, 2:1). Although OS arose at various sites, and although the most common site was distal femur (6 cases), followed by proximal tibia (3 cases), this meant that only 9 cases (39%) were found in the bones around the knee. There was no therapeutic history of preoperative chemotherapy or radiotherapy in OS. After the surgery, adjuvant chemotherapy was performed in five OS cases (three osteoblastic and two MFH-like types). Two OS cases had no follow-up and in two more cases the treatment was unknown. In MFH of bone, preoperative radiotherapy was performed in two cases; however, these cases were not considered to be under the influence of radiotherapy histologically.

The histological grade of malignancy was assessed according to the criteria of Dahlin and Unni (18). In OS, 15 cases were found to be Grade 4 (13 osteoblastic and 2 MFH-like types), whereas the other 8 cases were Grade 3 (2 osteoblastic and 6 MFH-like types); however, in MFH of bone, 11 cases were Grade 4, 6 cases were Grade 3, and the other one case was Grade 2. The mitotic rate was classified as high in 9 of the 23 OS cases (9/23; 39%; 8 osteoblastic and one MFH-like types) and in 8 of the 18 MFH of bone cases (7/18; 39%) with $\geq$15/10 high-power fields.

Immunohistochemistry

Five of the 23 cases of OS (22%; osteoblastic [5/15; 33%] and MFH-like [0/8] types) and 8 of the 18 cases of MFH of bone (44%) demonstrated nuclear accumulation of *p53* protein (Fig. 2). There was a significantly positive correlation between p53 immunoreaction and gender in OS (M–F ratio, 5:0, $P = .0137$; Table 3).

Three of the 23 cases of OS (13%; osteoblastic [3/15; 20%] and MFH-like [0/8] types) and 3 of the 18 cases of MFH of bone (17%) showed immunoreaction for MDM2 (Fig. 3). Coexpression of p53 and MDM2 was observed in two cases in OS (9%), both cases being osteoblastic type (2/15; 13%). No correlation was observed between MDM2 immunoreaction and the other clinicopathologic parameters (Table 3).

Three of the 23 cases of OS (13%; osteoblastic [3/15; 20%] and MFH-like [0/8] types) and 3 of the 18 cases of MFH of bone (33%) demonstrated positive immunoreaction for *p21/WAF1*. No correlation was observed between p53 immunoreaction or the

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TABLE 4. Correlation between p53 Immunoreactivity, *p53* Mutation Status, MDM2 Immunoreactivity, and p21/WAF1 Immunoreactivity

Variable	p53 IHC: OS (Osteoblastic OS)		*P* Value[a]	p53 IHC: MFH of Bone		*P* Value[a]
	−	+		−	+	
p53 mutation						
−	16 (9)	2 (2)	.0482[b]	9	5	.2745
+	2 (1)	3 (3)	(.0769)	1	3	
MDM2 IHC						
−	17 (9)	3 (3)	.1073	10	5	.0686
+	1 (1)	2 (2)	(.2418)	0	3	
p21/WAF1 IHC						
−	16 (8)	4 (4)	.5392	7	5	>.9999
+	2 (2)	1 (1)	(>.9999)	3	3	

IHC, immunohistochemistry; OS, osteosarcoma; MFH, malignant fibrous histiocytoma.
[a] Fisher's exact test.
[b] Statistically significant.

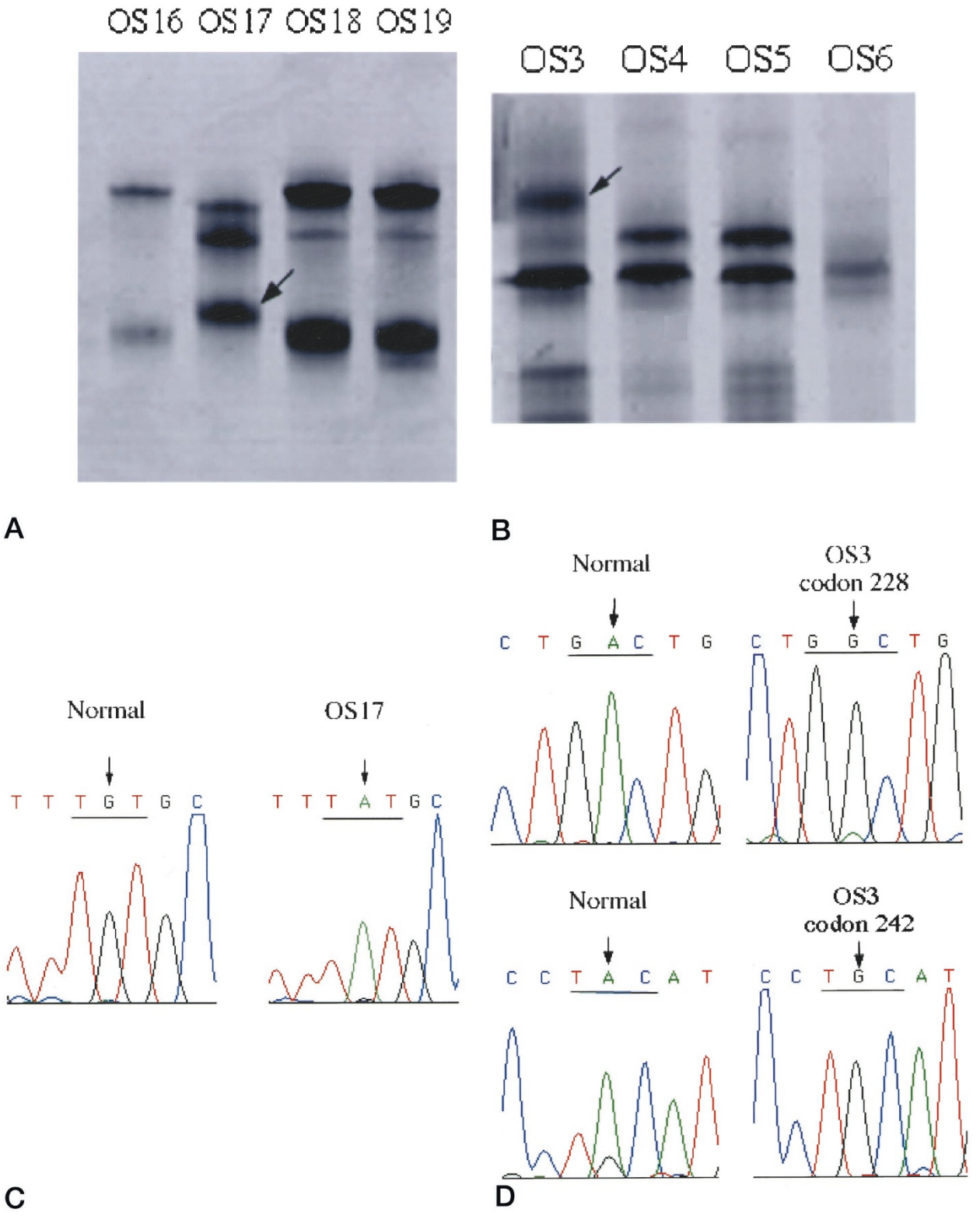

FIGURE 4. PCR-SSCP analysis at Exon 8 (**A**) and Exon 7 (**B**). Abnormal shifted bands are evident in Case OS17 (MFH-like OS; **A**) and in Case OS 3 (osteoblastic OS; **B**), respectively. Direct DNA sequencing of Exon 8 in Case OS17 (**C**) and of Exon 7 in Case OS3 (**D**). (**C,** Case OS17, Codon 275, TGT to TAT; **D,** Case OS3, Codons 228 and 242, GAC to GGC and TAC to TGC, respectively).

accumulation of MDM2 and p21 protein (Table 4). *p21/WAF1* expression had no statistically significant correlation with *p53* mutation in OS or MFH of bone ($P = .5392$, $P > .9999$, respectively, Fisher's exact test). Co-assessment of *p21/WAF1* and *p53* subgroups showed that *p21/WAF1+/p53−* patients seemed to have a better survival rate compared with other groups; however, the difference was not

TABLE 5. *p53* Mutation in Osteosarcoma (OS) and Malignant Fibrous Histiocytoma (MFH) of Bone

Case	Exon	Codon	Base Change	Amino Acid	p53 IHC	Follow-Up
OS1 (OB)	7	245	GGC–TGC	Gly–Cys	+	DOD7M
OS3 (OB)	7	228	GAC–GGC	Asp–Gly	−	DOD6M
		242	TAC–TGC	Cys–Tyr		
OS13 (OB)	7	240	AGT–GGT	Ser–Gly	+	DOD4M
OS15 (OB)	9	317	CAG–CGG	Gln–Arg	+	DOD4M
OS17 (MFH-like)	8	275	TGT–TAT	Cys–Tyr	−	AWD22M
MFH1	8	279	GGG–CAG	Gly–Glu	+	DOD10M
MFH11	5	143	CAG–CGG	Gln–Arg	−	DOD12M
MFH14	5	168	CAC–CAT	His–His	+	DOD50M
MFH18	6	216	GTG–ATG	Val–Met	+	DOD3M

IHC, immunohistochemistry; DOD, died of disease; AWD, alive without disease; OB, osteoblastic; M, months.

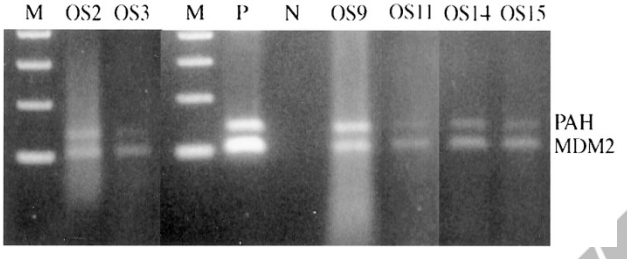

FIGURE 5. Differential PCR for OS cases. *MDM2* amplification is visible in Cases OS3, OS11, and OS15. In these cases, the intensities of *MDM2* are more than 2-fold those of the concordant *PAH* gene. *MDM2* immunoreactivity was also recognized in Cases OS3 and OS15. P, positive control; N, negative control.

significant. In two cases of MFH of bone in which preoperative radiotherapy was given, the overexpression of these proteins was not recognized.

p53 Mutation in Exons 5–9

p53 gene mutations were detected in 5 of the 23 cases of OS (21%; osteoblastic [4/15; 27%] and MFH-like [1/8; 13%] types) and in 4 of the 18 cases of MFH of bone (22%) by molecular biological analysis (Fig. 4A–D). As for the type of base substitution, all five cases were missense mutations (four osteoblastic and one MFH-like type) in OS, whereas three cases were missense mutations and one case was a silent mutation in MFH of bone (Table 5). One case in osteoblastic OS showed two mutations at codons 228 and 242. Four mutations were transition (three osteoblastic and one MFH-like types) and one mutation was transversion among five mutations of OS, whereas all four cases were transition in MFH of bone. All patients with *p53* mutation in osteoblastic OS and MFH of bone, including one osteoblastic OS case, in which postoperative chemotherapy were given died within 12 months, except for one case with missense mutation of *p53* gene in MFH of bone. There were three osteoblastic OS cases with both p53 immunoreaction and concordant *p53* mutation, in addition to three cases of MFH of bone (two missense and one silent mutations). There was a statistically correlation between p53 immunoreactivity and *p53* mutation status in OS arising in patients ≥40 years of age ($P = .0482$), but not in MFH of bone in the same group (Table 4). According to the subtype of OS, precise statistical analysis could not be assessed between p53 immunoreaction and p53 mutation or prognosis because of the small number of cases. No positive relationship was detected between *p53* mutation and clinicopathologic parameters (Table 3).

MDM2 Amplification

Three of the 23 cases of OS (13%; osteoblastic [3/15; 20%] and MFH-like OS [0/8] types) and 3 of the 18 cases of MFH of bone (17%) showed *MDM2* amplification (Fig. 5). *MDM2* amplification had a significantly positive correlation with MDM2 immunoreactivity only in the OS cases ($P = .0344$), and not in the MFH of bone cases (Table 6). There was no correlation between *p53* mutation and *MDM2* amplification in OS or MFH of bone. Two cases with osteoblastic OS showed both *p53* mutation and *MDM2* amplification. Amplification of the

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TABLE 6. Correlation between MDM2 Immunoreactivity and *MDM2* Amplification

MDM2 IHC	*MDM2* Amplification (OS)		*P* Value[a]	*MDM2* Amplification (MFH)		*P* Value[a]
	−	+		−	+	
−	19	1	.0344[b]	14	1	.0564
+	1	2		1	2	

IHC, immunohistochemistry; OS, osteosarcoma; MFH, malignant fibrous histiocytoma
[a] Fisher's exact test.
[b] Statistically significant.

MDM2 gene also showed no correlation with clinicopathologic parameters (Table 3).

H-ras Mutation Status

In this study, H-ras mutation at codons 12 and 13 was not detected in any of the 23 OS cases (0/23; 0%) nor in any of 18 cases of MFH of bone (0/18; 0%).

MIB-1–Labeling Index

MIB-1-LI ranged from 10.1 to 36.8 (mean, 19.6) in OS (osteoblastic type: mean, 21.1; MFH-like type: mean, 16.6), whereas it ranged from 13.9 to 39.6 (mean, 24.6) in MFH of bone. The MIB-1-LI of MFH-like OS was significantly lower than that of MFH of bone ($P = .0055$, Fisher's exact test). The OS cases with a high MIB-1-LI tended to have a poor prognosis, but the difference was not significant. MIB-1-LI was positively correlated with p53 immunoreactivity and MDM2 immunoreactivity in OS ($P = .0307$ and $P = .0358$, respectively; Table 7). In MFH of bone, p53 immunoreactivity was correlated with high MIB-1-LI ($P = .0410$; Table 7). No correlation was recognized between MIB-1-LI and *p53* mutation, *MDM2* amplification or p21WAF1.

DISCUSSION

OS is the most common primary malignant bone tumor, frequently affecting the region around the knee in younger patients, but occurring less commonly in elderly patients. In fact, it has been reported that only approximately 10% of patients with OS are ≥60 years of age (1). Histological features in elderly OS cases are different from those in younger cases, with MFH-like OS occurring more commonly than osteoblastic type (1, 2). As for the prognosis according to the subtype of OS, Naka *et al.* reported that MFH-like OS showed a more favorable prognosis than osteoblastic OS with regard to 5-year survival (4). In contrast, some reports stated MFH-like OS was frequently seen in younger patients. Moreover, Dahlin *et al.* proposed that MFH-like OS falls within the spectrum of conventional OS, but this hypothesis is still controversial (3).

In previous reports of OS, the expression of p53 protein was recognized in 15 to 72% of OS cases (9, 19–22). Lanardo *et al.* (9) reported that p53 immunoreactivity was observed to be more likely to occur in elderly patients than in younger patients (27%). On the other hand, Naka *et al.* (4) revealed that there was no difference regarding the expression of p53 protein between patients of ≥40 years of age (25%) and those of <40 years of age (24%). In addition, with regard to the subtype of OS, they reported that p53 immunoreaction was not observed in MFH-like OS (0/6, 0%); however, it was rather frequently observed in osteoblastic OS (66.7%). These data, including our results, suggest that *p53* mutations may play a less important role in MFH-like OS than in osteoblastic OS from an immunohistochemical aspect.

p53 mutations were found in 15 to 31% of OS cases in previous studies (5, 19, 23–27). Mutations of the *p53* gene in patients of <40 years of age have been reported in 13% (4/30; 24) and 16% (6/38; 13), whereas among those of ≥40 years of age they were seen in 20% (1/5) (24) and 14% (1/7) (14). As for the subtype of OS with *p53* mutation, osteoblastic type was more common than other subtypes. However, *p53* mutations were not detected in MFH-like OS in the previous studies. Taking the current findings into consideration together with previous reports of immunohistochemical and mutation analysis of

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TABLE 7. Correlation between MIB-1-LI and Immunohistochemical Expression of p53, MDM2, and p21WAF1, or Mutation Status of *p53* and *MDM2* Amplification

Variable	MIB-1-LI: OS (Mean ± SD)	*P* Value[a]	MIB-1-LI: MFH of Bone (Mean ± SD)	*P* Value[a]
p53 IHC				
	26.8 ± 7.3	.0307[b]	29.1 ± 8.5	.0410[b]
	17.6 ± 6.9		21.0 ± 4.2	
MDM2 IHC				
+	28.6 ± 0.9	.0358[b]	29.6 ± 10.3	.2604
−	18.2 ± 7.6		23.6 ± 6.9	
P21WAF1 IHC				
+	18.1 ± 9.1	.5839	27.7 ± 8.9	.2611
−	19.8 ± 7.9		23.1 ± 6.6	
p53 mutation				
+	23.2 ± 7.4	.1797	26.0 ± 10.9	.8318
−	18.6 ± 7.9		24.2 ± 6.8	
MDM2 amplification				
+	22.6 ± 9.6	.6481	22.0 ± 7.2	.5147
−	19.1 ± 7.8		25.1 ± 7.7	

IHC, immunohistochemistry.
[a] Mann-Whitney U test.
[b] Statistically significant.

p53, MFH-like OS dose not seem to be greatly concerned with *p53* gene mutation. In fact, in our series, *p53* mutation was observed in only one of the 8 MFH-like OS cases, whereas 4 of 15 osteoblastic OS cases demonstrated *p53* gene alteration.

The correlation between *p53* nuclear accumulation or its mutation and prognosis in sarcoma is controversial. Although Kawai *et al.* (28) showed that the nuclear immunoreaction of p53 protein was correlated with poor prognosis in various soft-tissue sarcomas, other reports showed no association (19). In the current study, *p53* immunoreaction demonstrated no association with prognosis in OS, indicating that it was less correlated with tumor progression in elderly patients. On the other hand, *p53* mutation was observed to be more common in recurrent or metastatic lesions, and it has been reported that the mutation of *p53* may have some correlation with tumor progression (5). In our study, although the number of patients was small, all those cases of osteoblastic OS and MFH of bone that had *p53* mutations, with the exception of one case of MFH of bone that had a *p53* silent mutation, demonstrated progressive behavior (dead of disease [DOD], within 12 months), in contrast to the cases of MFH-like OS (alive without disease [AWD], 22 months). Although substantial improvement in chemotherapy and differences in treatment during the very large time interval covered by this study make it difficult to compare survival analysis between the two subtypes of OS, in MFH-like OS, *p53* mutation may be less correlated with the progression of the tumor than it is in osteoblastic OS or MFH of bone.

Gene amplification of *MDM2* has been described as the pathway of tumorigenesis or tumor progression in various sarcomas (6–8). *MDM2* amplification in OS has been detected in 0 to 27% of cases (5, 6, 9–11, 25, 29). Some investigators have reported that *MDM2* amplification was recognized more frequently in metastatic or recurrent OS, indicating that *MDM2* amplification may be correlated with tumor progression (5, 9, 10). In our study, *MDM2* amplification had no relationship with progression either in OS or in MFH of bone. Yokoyama *et al.* (5) reported that *MDM2* amplification and *p53* mutation coexisted in 2 of 17 OS cases in progressive tumors. In the current study, the coexistence of *p53* mutation and *MDM2* amplification was observed in two cases of osteoblastic OS with both patients dying within 6 months (DOD at 4 and 6 mo). Although most studies of *MDM2* amplification have been conducted in younger patients, there was a report that one of four OS cases among patients of ≥40 years of age had *MDM2* amplification (25%) (10). In the current study, *MDM2* amplification was recognized in 3 of 23 cases of OS (13%). As for the subtype of OS, *MDM2* amplification was detected in only osteoblastic cases (3/15, 20%), and not in MFH-like OS cases. These results would seem to indicate that *MDM2* gene alteration shows different patterns in the different subtypes of OS.

p21 protein product is believed to block cyclin and cyclin-dependent kinase (CDK) complex activity and prevents the passage of cycling cells from G1 to the S phase in a p53-dependent or p53-independent manner (30–32). In this study, p21WAF1 expression did not show any significant correlation with p53 immunoreactivity or *p53* mutation either in OS or in MFH of bone. McClelland *et al.* (33) demonstrated that a subgroup of p21+/p53− patients had good survival characteristics with regard to breast cancer. In the current study, this subgroup had no association with survival or MIB1-LI either in OS or in MFH of bone. In all MFH-like OS cases, the expression of p21 protein was not detected, and MFH-like OS showed a different pattern when compared with osteoblastic OS or MFH of bone.

Concerning the study of OS, *K-ras* gene mutation was detected in 2 of 17 cases (5), and *N-ras* gene mutation was seen in one of 28 cases (14). In contrast, *H-ras* gene mutation has not been found in OS (15). In our series, *H-ras* gene mutation was not detected in any of the cases of OS. Bohle *et al.* (12) reported that *H-ras* gene mutation was detected in 9 of 32 soft tissue MFH cases (28%), whereas Yoo *et al.* (13) described it in 6 of 27 soft tissue MFH cases (22%). In the current study, MFH of bone had no *H-ras* gene mutation. These results suggest that MFH of bone may have some different molecular aspects from MFH of soft tissue.

The MIB-1 monoclonal antibody has been used prominently as an indicator of proliferating activity in malignant tumors, and as a prognostic factor in some malignant tumors (34–36). Lanardo *et al.* (9) revealed that a significantly positive correlation was not found between p53 immunoreactivity and the proliferative rate as assessed by MIB-1-LI in OS. In the current study, MIB-1-LI had a significant relationship with *p53* and *MDM2* immunoreactivity in all the histologic subtypes of OS. *p53* and *MDM2* immunoreactivity may be considered as useful factors of proliferative activity in OS arising in elderly patients.

In conclusion, *p53* mutation was recognized in osteoblastic OS, MFH-like OS, and MFH of bone in elderly patients. Although the number of patients in this study was small, those cases of osteoblastic OS and MFH of bone that had *p53* mutations had progressive tumors, in contrast to the MFH-like OS cases. In addition, the pattern of *MDM2* alteration and p21WAF1 expression in MFH-like OS was different from that in osteoblastic OS and MFH of bone. It would thus seem likely that MFH-like OS has some characteristic biologic aspects when compared with osteoblastic OS and MFH of bone in

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elderly patients, however, a further detailed analysis of a large number of cases, including cases in younger patients is necessary.

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