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# Association of Loss of Heterozygosity at the p53 Locus with Chemoresistance in Osteosarcomas

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**Although the osteosarcoma is considered to be among the most chemosensitive malignancies and preoperative chemotherapy is commonly applied, an appreciable proportion of cases are in fact quite insensitive. Predictive markers for chemosensitivity are therefore desirable in order to develop effective treatment strategies. Thirty-two cases of conventional osteosarcomas treated at the Cancer Institute Hospital, Tokyo, were analyzed. The sensitivity to preoperative chemotherapy was investigated with reference to loss of heterozygosity (LOH) at the 17p13 (*p53*) and 13q14 (*Rb*) loci and expression of the cell-cycle associated proteins, p53, Rb, p21/Waf-1, mdm-2 and Ki-67, as detected immunohistochemically. LOH was detected by analyzing polymerase chain reaction products at marker microsatellite loci. The efficacy of chemotherapy was evaluated both radiologically and histologically. LOH at *p53* or *Rb* loci was seen in 54% (13/24) and 58% (14/24) of cases, respectively. Only 15% of osteosarcomas with LOH at the *p53* locus were sensitive to preoperative chemotherapy, as compared to 64% of tumors without such loss ($P<0.05$). A similar but much less distinct tendency was observed with LOH at the *Rb* locus. No relationship was evident between chemosensitivity and immunohistochemical staining patterns for p53, Rb, p21/Waf-1, mdm-2 or Ki-67. The results suggest that *p53* gene deletion, but not the other parameters investigated, may be useful for predicting chemoresistance of osteosarcomas.**

Key words: Osteosarcoma — Chemosensitivity — p53 — Rb — Loss of heterozygosity

Having reliable markers to predict response to chemo- and/or radiotherapy is very important, not only to facilitate choice of the most effective therapeutic modality or drug type for individual tumors, but also to avoid potential therapy-related complications and delays in appropriate treatment. Recent investigations have shown that the *p53* and/or *Rb* gene status may be related to the relative sensitivity/resistance of tumor cells to drugs and/or radiation.[1–15] Most of the data, however, were obtained from *in vitro* experiments and the number of clinical studies in this area is as yet quite limited, with considerable inconsistency between findings. Clearly, many more clinical studies are needed to confirm the utility of these markers.

The present investigation addresses this problem by concentrating attention on the chemosensitivity of osteosarcomas with reference to loss of heterozygosity (LOH) at 17p13 (*p53*) and 13q14 (*Rb*) loci and, in addition, expression of the cell-cycle associated proteins, p53, Rb, p21/Waf-1, mdm-2 and Ki-67. The osteosarcoma was chosen as a model tumor because 1) it is known to show a high frequency of *p53* and/or *Rb* gene deletions,[16–21] 2) it is thought to be among the malignancies most sensitive to chemotherapy and, therefore, preoperative application of therapeutic agents is common in order to localize the primary lesion and also to prevent distant metastasis, and 3), in our experience, an appreciable proportion of cases are in fact quite insensitive to chemotherapy in terms of both radiographic and histological findings.

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## PATIENTS, MATERIALS AND METHODS

**Patients and materials** A total of thirty-two conventional osteosarcomas, undergoing preoperative chemotherapy without radiation and then surgical resection at the Cancer Institute Hospital, Tokyo from 1979 to 1994, were investigated. Clinical details are summarized in Table I. The average age at diagnosis was 16.4 years (from 6 to 58 years); the primary lesions were located in the femur in 24 cases, the tibia in 7 cases and the humerus in 1 case. Clinical stages of the patients at admission, determined according to the American Joint Commission on Staging and End Result Studies,[22] were 2B in 24 cases and 3 in 8 cases. Histological grades of these cases[23] were grade 3 in 23 cases and grade 4 in 9 cases. The histological subtypes were osteoblastic in 25 cases, chondroblastic in 2, fibroblastic in 3 and telangiectatic in 2. All received neoadjuvant chemotherapy according to the modified Rosen T10 protocol as described previously.[24] The principal drugs used were methotrexate (MTX) and/or cisplatin (CDDP), given in combination to 10 patients, and singly to 12 and

Table I. Sensitivity to Chemotherapy and Other Clinical Information

| Case | Sex | Age | Site | Size (cm) | Clinical stage | Histol. grading | Histol. subtype | Drugs used | Response to therapy | | | Mat. | LOH | | Immunohistochemistry | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Radiol. | Histol. | Course (days) | | *p53* | *Rb* | p53 | Rb | p21 | mdm2 | Ki-67 |
| 1 | M | 6 | Femur | 14×6×5 | 2B | 4 | OB | MTX | − | − | Alive (9y3m) | L | + | + | − | + | − | − | − |
| 2 | F | 8 | Femur | 15×9×8 | 3 | 3 | OB | MTX+CDDP | − | − | Dead (1y0m) | R | + | + | − | + | − | − | + |
| 3 | M | 8 | Tibia | 3×16×3 | 3 | 3 | OB | MTX | − | − | Dead (2y4m) | L | NI | − | + | + | + | + | ND |
| 4 | M | 9 | Femur | 8×7×25 | 2B | 3 | OB | MTX | − | + | Dead (1y9m) | L | + | NI | ++ | + | + | − | + |
| 5 | M | 9 | Femur | 5×5×8 | 3 | 3 | TE | CDDP | − | − | Dead (8m) | B | NI | + | − | + | − | − | ND |
| 6 | M | 9 | Femur | 11×6×5 | 2B | 4 | OB | MTX+CDDP | − | − | Alive (1y10m) | R | NI | NI | − | − | − | − | + |
| 7 | F | 10 | Femur | 6×8×15 | 3 | 4 | OB | MTX | − | − | Dead (11m) | B | + | + | + | + | − | + | ND |
| 8 | M | 10 | Femur | 4×4×4 | 2B | 3 | OB | MTX+CDDP | − | − | Dead (1y7m) | L | + | + | − | − | + | − | + |
| 9 | M | 10 | Femur | 9×7×4 | 2B | 3 | OB | CDDP | + | + | Alive (3y11m) | B | NI | − | − | + | − | − | ND |
| 10 | F | 11 | Femur | 25×5×5 | 2B | 3 | OB | MTX | − | ND | Alive (1y11m) | B | NI | − | − | + | − | − | + |
| 11 | M | 11 | Femur | 16×7 | 2B | 3 | OB | MTX+CDDP | + | + | Alive (13y7m) | B | − | − | − | + | + | + | ++ |
| 12 | F | 12 | Femur | 13×4 | 2B | 4 | OB | CDDP | − | − | Dead (7m) | B | − | NI | − | − | + | − | + |
| 13 | F | 13 | Femur | 6×6×11 | 2B | 3 | OB | CDDP | − | − | Dead (1y0m) | L | − | + | − | − | + | − | ++ |
| 14 | M | 13 | Tibia | 21×6×6 | 3 | 4 | OB | MTX+CDDP | − | − | Alive (11m) | B | + | − | − | − | + | + | ++ |
| 15 | F | 14 | Femur | 12×8×9 | 2B | 4 | FB | MTX | − | − | Alive (3y6m) | B | + | + | − | − | − | − | + |
| 16 | M | 14 | Hum. | 10×6×6 | 2B | 3 | OB | CDDP | − | − | Dead (2y6m) | B | + | − | ++ | − | − | − | + |
| 17 | M | 14 | Femur | 7×4×4 | 2B | 3 | OB | MTX | − | − | Alive (3y6m) | L | + | + | − | + | − | − | + |
| 18 | M | 14 | Femur | 6×6×21 | 2B | 4 | OB | MTX+CDDP | + | + | Alive (4y10m) | B | − | − | − | − | + | + | + |
| 19 | M | 14 | Femur | 9×4×5 | 2B | 3 | OB | MTX+CDDP | + | − | Alive (3y7m) | B | − | − | ++ | − | + | − | + |
| 20 | F | 15 | Femur | 5×8×5 | 2B | 3 | TE | CDDP | ++ | + | Alive (10y1m) | B | − | + | ++ | − | + | + | + |
| 21 | M | 16 | Femur | 15×5 | 2B | 3 | OB | MTX | + | + | Alive (13y7m) | B | − | + | − | + | − | − | ND |
| 22 | M | 16 | Tibia | 13×6×5 | 2B | 3 | OB | MTX+CDDP | − | + | Alive (2y4m) | B | − | − | − | + | − | − | + |
| 23 | M | 17 | Tibia | 15×9×6 | 2B | 3 | OB | MTX | − | + | Alive (13y7m) | L | − | − | − | + | − | − | + |
| 24 | M | 18 | Femur | 12×8×9 | 2B | 4 | OB | MTX+CDDP | + | ++ | Dead (1y8m) | R | + | NI | − | + | + | + | + |
| 25 | M | 18 | Femur | 8×7×6 | 2B | 3 | OB | MTX | + | − | Dead (1y8m) | B | + | + | − | − | + | − | ++ |
| 26 | M | 19 | Femur | 6×13×5 | 3 | 3 | OB | CDDP | − | − | Dead (1y4m) | B | NI | NI | − | − | − | − | + |
| 27 | M | 19 | Tibia | 4×3×3 | 2B | 3 | CB | MTX | + | ++ | Dead (1y8m) | B | NI | NI | − | − | − | − | + |
| 28 | M | 21 | Tibia | 4×4×8 | 3 | 3 | OB | MTX+CDDP | − | − | Dead (1y2m) | L | + | + | − | − | + | − | + |
| 29 | M | 26 | Femur | 5×4×4 | 2B | 3 | OB | CDDP | + | − | Dead (3y10m) | B | NI | + | − | − | − | − | + |
| 30 | M | 26 | Femur | 11×5 | 2B | 4 | FB | MTX | − | − | Dead (1y11m) | B | − | NI | + | − | + | − | + |
| 31 | M | 47 | Femur | 28×9×8 | 3 | 3 | FB | CDDP | − | − | Dead (3m) | B | + | + | + | + | + | − | + |
| 32 | M | 58 | Femur | 8×5×5 | 2B | 3 | CB | CDDP | ++ | ++ | Dead (3y1m) | B | − | NI | ++ | + | + | − | + |

Hum, humerus; Histol., histological; OB, osteoblastic; CB, chondroblastic; FB, fibroblastic; TE, telangiectatic; MTX, methotrexate; CDDP, cisplatin; Mat., materials; B, biopsy; R, resection; L, lung metastasis; Radiol., radiological; ND, not determined; NI, non informative. p53 expression: higher positivity between two antibodies. Survival: post-diagnosis day.

10, respectively. The mean dose of MTX was 22.6 g/m$^2$ and that of CDDP was 201.1 mg/m$^2$.

For the DNA analysis, 24 samples of primary lesions (21 by biopsy and 3 at operation) and 8 of lung metastases were available, 21 having been obtained before and 11 after chemotherapy. All the primary lesions were removed by means of ablative surgery or limb salvage procedures and formalin-fixed for histological evaluation of the response to chemotherapy.

**Radiological evaluation of the effects of chemotherapy** With all the patients, a radiogram was taken every week during chemotherapy and angiographs just before and 1 week after the termination of chemotherapy. The efficacy of the preoperative chemotherapy was assessed essentially according to the criteria of Smith *et al.*[25] and Chuang *et al.*[26] based on radiographic and angiographic changes: the response was graded into progressive disease (PD), no change (NC), partial response (PR) or complete response (CR) categories. The "PD" and "NC" cases were patients who demonstrated an increase in tumor bulk, and a minimal or no response, respectively. The "CR" cases demonstrated both a decrease in tumor bulk or increased ossification radiographically and decrease in blood flow angiographically within 10 days. The "PR" group comprised patients who first demonstrated these changes after more than 10 days or only one of them. The evaluations were independently performed by two orthopedists (S. M. & N. K.). In this text, PD and NC are referred to as chemoresistant (−) and PR and CR as chemosensitive (+ or ++).

**Histological evaluation of the effects of chemotherapy** All the patients underwent surgical treatment within 10 days after the termination of the chemotherapy. Both longitudinal and cross sections were made for each sample so as to obtain the maximum cut tumor surfaces. In each case, tissue blocks were made for histological examination from both cut faces to allow thorough assessment. The average number of examined blocks per case was

120. Histological evaluation of the chemotherapeutic effects was carried out essentially according to Hiruta's criteria,[27] which are modified from those of Picci *et al*.[28]: Microscopically, regions showing necrosis, hemorrhage, fibrosis and granulation tissue, with or without sparse degenerative tumor cells, were diagnosed as "effective" and regions occupied by abundant viable cancer cells as "ineffective." Each case was classified into one of three categories according to the percentage, effective region: <70%, resistant (−); 70–90%, moderately sensitive (+); 90%<, remarkably sensitive (++). The histological evaluation was carried out in a blinded fashion by two pathologists ( H. K. & R. M.), independently. The reason why we used Hiruta's criteria rather than the internationally established Huvos system[29] is as follows; with the protocol applied in our hospital, the period of chemotherapy is 8 weeks, in contrast to the 12 weeks of chemotherapy (the total dose is larger by one-third) in the US or western countries. Hiruta *et al*. studied histological parameters with this particular protocol in relation to radiological evaluation and prognosis and came to the conclusion that 70% tumor necrosis, rather than the 90% of Huvos' or Picci's[28] criteria is a better cut-off for chemosensitivity. The adequacy of Hiruta's criteria is supported by the survival rate data in the present study.

**LOH analysis** Tumor or adjacent normal tissue was sampled under a stereomicroscope with sterile needles, from five to eight 10-$\mu$m-thick hematoxylin-stained tissue sections in each case. DNA was then extracted according to the method of Goeltz *et al*.[30]

Microsatellite primers of HSGPOCA3[31] and D13S270[32] were used for analysis of LOH at 17p13 and 13q14, respectively.

HSGPOCA3

sense: 5′-CCTTCTGGGCCCTTTCAATGGAG-AA-3′

antisense: 5′-CAACCTGTGCCTACTGCTCCAACT-T-3′.

D13S270

sense: 5′-AGTGCCTGGGTATGAACGTG-3′

antisense: 5′-CTGGAAATGCCTTGGAAGGA-3′.

The polymerase chain reaction (PCR) was run in a total volume of 50 $\mu$l, with 5 $\mu$l of extracted DNA, 1 $\mu$l of primer mix (20 $\mu M$ each primer), 2 $\mu$l of deoxynucleotide triphosphates (2.0 m$M$ of each deoxynucleotide triphosphate, Toyobo, Osaka), 0.5 $\mu$l (5 units/$\mu$l) of *Taq* DNA polymerase (Boehringer-Mannheim GmbH, Germany) and 5 $\mu$l of 10×PCR buffer (Boehringer-Mannheim GmbH). Standard DNA amplification conditions were as follows: 33 cycles of denaturation for 1 min at 94°C, 1 min of annealing at 54°C (HSGPOCA3) or 57°C (D13S270) and 2 min of extension at 72°C. The final extension step at 72°C was lengthened to 7 min. PCR products were electrophoresed on agarose gels (3% Nusieve GTG, FMC Bioproducts, Rockland, ME) and photographed. Normal DNA samples polymorphic at a given locus were considered informative, allowing identification of LOH, whereas homozygotes were considered as non-informative. LOH was considered to occur when a clear reduction in the intensity of one of the two alleles in the tumor DNA was evident on visual examination.

**Immunohistochemistry** After deparaffinization, rehydration, and antigen retrieval by microwave treatment in sodium citrate buffer,[33] 4 $\mu$m paraffin-embedded sections of tumors were exposed to the first antibodies at 4°C overnight. Two antibodies to p53 protein were applied: *DO-7* (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) at a 1:400 dilution and *Rsp53* (Nichirei Corp., Tokyo) at a 1:250 dilution. Antibodies for RB, p21/Waf-1 and mdm2 protein were respectively RB (*NCL-RB-1*, Novocastra Laboratories Ltd.) at a 1:50 dilution, p21/Waf-1 (*Waf-1/Ab-1*, Oncogene Science, New York, NY) at a 1:100 dilution and mdm2 (*mdm2/Ab-1*, Oncogene Science) at a 1:100 dilution. All tumors were also stained with Ki-67 (*MIB-1*, Immunotech, France), a mouse monoclonal antibody against a cell proliferation antigen, at a 1:50 dilution, as above but without microwave treatment. Second antibodies of biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) were used for *DO-7*, *NCL-RB-1*, *Waf-1*/*Ab-1*, *mdm2*/*Ab-1* and *MIB-1*. Biotinylated anti-rabit IgG was used for *Rsp53* (Vector Laboratories Inc.). The streptoavidin-biotin-peroxidase (SAB) system (Histofine SAB-PO(M) kit; Nichirei Corp.) was used to visualize the binding of *DO-7* and *Rsp53*. The avidin-biotin-peroxidase system (Vectastain ABC kits, Vector Laboratories Inc. ) was used to visualize the binding to RB, p21/Waf-1, mdm-2 and Ki-67. Hematoxylin was used as the counterstain. For negative controls, non-immunized serum was substituted for the first antibodies. Immunoreactions for each protein were scored as follows; more than 50% of tumor cells positive (++), less than 50% positive (+), all cells negative (−).

**Statistical analysis** Fisher's exact test[34] was used to evaluate associations between *p53* or *Rb* LOH, or the immunohistochemical parameters and chemosensitivity. Kaplan-Meier plots[35] and the log-rank test[36] were used to evaluate the association of *p53* or *Rb* LOH with survival.

## RESULTS

The results of radiological and histological evaluation of sensitivity to preoperative chemotherapy are summarized in Table I, together with other clinical information. With case 10, histological examination could not be performed since Pasteur's operation had been carried out. Of the 32 cases radiologically evaluated, CR, PR, NC and PD were 2 (6.2%), 9 (28.1%), 11 (34.3%) and 10 (31.2%), respectively, with a total of 11 (34.3%) cases

being sensitive. Histological evaluation for chemotherapy was performed for 31 cases, 20 (64.5%), 8 (25.8%) and 3 (9.7%), respectively, being assigned to the resistant (−), moderately sensitive (+), remarkably sensitive (++) groups, giving 11 (35.4%) sensitive cases. No difficulty was encountered with either the radiological or the histological evaluations of sensitivity since there were no borderline cases resulting in differences in diagnosis between the examiners. Eight cases were classified as sensitive from both radiological and histological evaluations. However, in 3 cases there was a discrepancy between the two evaluations: of three histologically moderately sensitive cases, two were NCs and one PD, radiologically. There was no distinct correlation between chemosensitivity and histological features (malignant grade or subtype) of tumors. Radiologically or histologically sensitive cases were all in stage 2B. In contrast, of 21 resistant cases, 8 (38.0%) were in stage 3.

**Relationship between LOH at the *p53* or *Rb* loci and sensitivity to chemotherapy** Among the 32 cases, 24 were heterozygous for HSGPOCA3 (*p53*) and/or D13S270 (*Rb*) loci, *p53* LOH being found for 13 of the 24 (54%) and *Rb* LOH for 14 of the 24 (58%), respectively. Representative figures showing LOH of *p53* and *Rb* loci are illustrated in Fig. 1. As demonstrated in Fig. 2, a and b, radiologically and histologically chemosensitive osteosarcomas only accounted for 2/13 (15%) and 2/13 (15%) of the *p53* LOH-positive cases, respectively. In sharp contrast, the figures for LOH-negative cases were 6/11 (55%) and 7/11 (64%), respectively. Thus, *p53* LOH-positive cases were significantly more resistant to preoperative chemotherapy than those without LOH by histological evaluation ($P$<0.05, Fisher's exact test). Similarly,

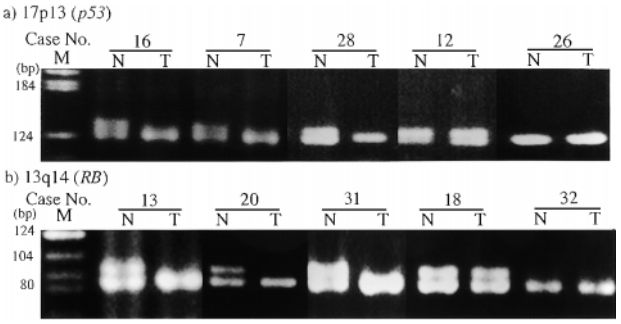


Fig. 1. LOH at HSGPOCA3 (17p13, *p53*) and D13S270 (13q14, *RB*) loci. PCR products were electrophoresed on a 3% agarose gel and photographed. a) 17p13 (*p53*) locus. Cases 16, 7 and 28 are LOH-positive, 12 is LOH-negative and 26 is non-informative. b) 13q14 (*RB*) locus. Cases 13, 20 and 31 are LOH-positive, 18 is LOH-negative and 32 is non-informative. M, Markers, PBR322/*Hae*III fragment; N, normal tissue; T, tumor tissue; LOH, loss of heterozygosity.

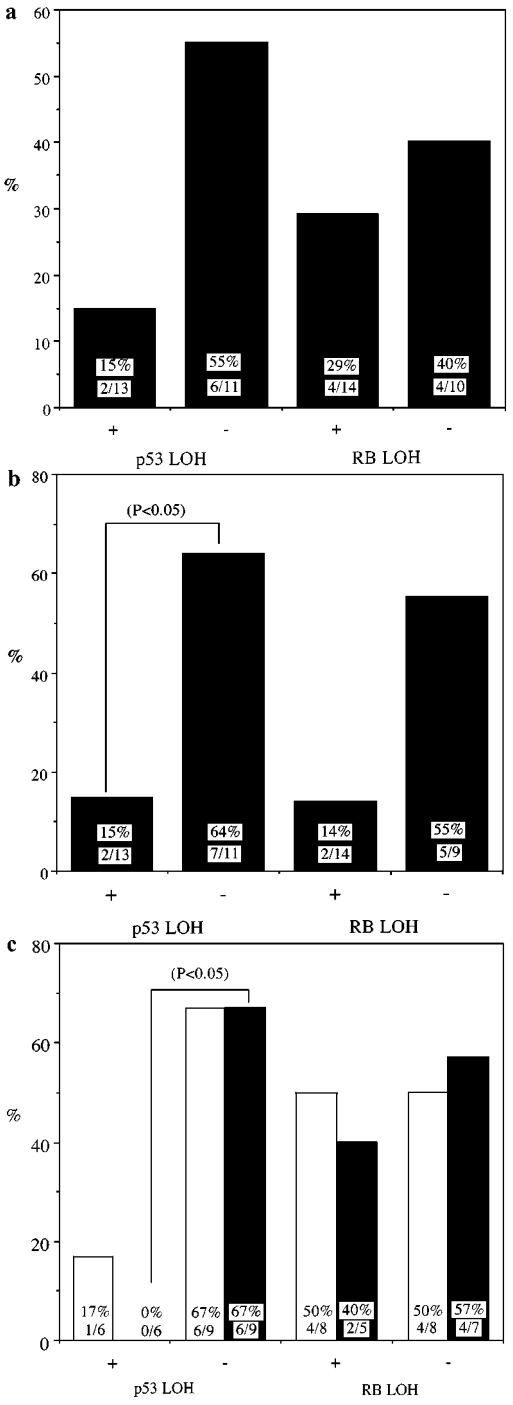


Fig. 2. Relationship between LOH at *p53* or *RB* loci and sensitivity to preoperative chemotherapy. a, Radiological evaluation. b, Histological evaluation: The differences between LOH-positive and -negative cases for the *p53* locus is statistically significant ($P$<0.05, Fisher's exact test). c, Results with biopsy materials: Statistically significant differences exist only in relation to the *p53* LOH status on histological evaluation ($P$<0.05, Fisher's exact test). □ Radiological evaluation, ■ histological evaluation.

chemosensitive *Rb* LOH-positive cases made up 4/14 (29%) and 2/14 (14%), and *Rb* LOH-negative 4/10 (40%) and 5/9 (55%), by radiological or histological evaluation, respectively (Fig. 2, a and b). With the *Rb* locus, no statistically significant association of LOH with resistance to preoperative chemotherapy was found. Among these LOH-analyzed cases, 19 were informative for both *p53* and *Rb* loci. Of these, 1/9 (11%) and 0/9 (0%) of the tumors LOH-positive for both *p53* and *Rb* were chemosensitive by radiological and histological evaluation, respectively. For cases LOH-negative for both, the figures were 3/5 (60%) and 4/5 (80%), respectively. No *p53* LOH-positive but *Rb* LOH-negative cases were chemosensitive, while 2/3 (67%) *p53* LOH-negative but *Rb* LOH-positive cases were chemosensitive by both radiological or histological evaluation.

When the materials obtained by biopsy before chemotherapy were selectively analyzed, in order to exclude possible modification of LOH status by the treatment, the frequencies of *p53*-LOH-positive cases showing radiological or histological chemosensitivity were 1/6 (17%) and 0/6 (0%), whereas the figures for negative cases were 6/9 (67%) and 6/9 (67%), respectively (Fig. 2c), the differences by histological examination being statistically significant ($P<0.05$, Fisher's exact test). In contrast, 4/8 (50%) and 2/8 (25%) of *Rb*-LOH-positive cases showed chemosensitivity, the difference from the 4/8 (50%) and 4/7 (57%), respectively, of *Rb*-LOH-negative cases not being statistically significant (Fig. 2c). The relationship between *p53*-LOH status and histological response is presented in Table II.

**Relationship between p53, Rb, p21/Waf1, mdm2 or Ki-67 protein expression and sensitivity to chemotherapy** A representative picture of p53 immunostaining is presented as Fig. 3. The immunohistochemical data are summarized in Table III. No significant relationship between any of these data and chemosensitivity was noted.

**Relationship between LOH at the *p53* or *Rb* loci and protein expression** Among LOH-positive cases at the *p53* locus, positive staining with *DO-7* and *Rsp53* was observed in 4 (30.7%) and 2 (14.3%) cases, respectively. Among the LOH-negative cases, the figures were 2 (18.2%) and 3 (27.2%), respectively. In cases with LOH at the *Rb* locus, negative staining with Rb was observed in 7 (50.0%). In LOH(−) cases, negative staining was observed in 6 (60.0%). No statistically significant relationship between LOH at the *p53* or *Rb* loci and status of p53 or Rb expression was found.

**Correlation between LOH at the *p53* and *Rb* loci and survival** The prognostic significance of LOH at the *p53* and *Rb* loci was analyzed using Kaplan-Meier survival curves (Fig. 4, a and b). LOH(−) cases had a sig-

Table II. *p53*-LOH Status and Chemosensitivity[a)]

| Grade of chemosensitivity | Number of cases | |
|---|---|---|
| | LOH-positive | LOH-negative |
| − | 11(6) | 4(3) |
| + | 1(0) | 6(5) |
| ++ | 1(0) | 1(1) |
| Total | 13(6) | 11(9) |

*a*) Histological evaluation. Numbers in parentheses indicate numbers of biopsy materials.

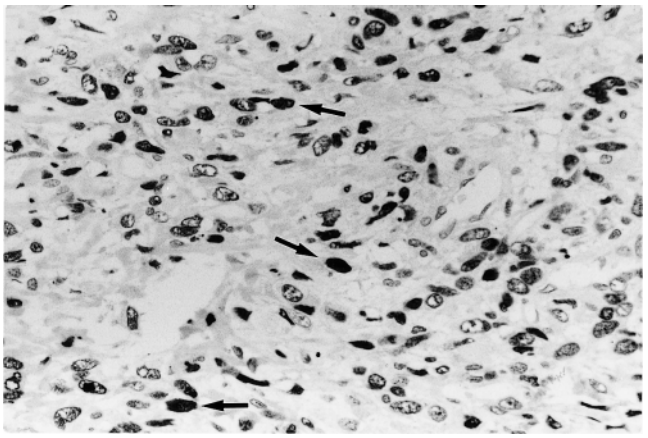

Fig. 3. Representative picture of immunohistochemichal staining for p53 protein (Case 20). Over 50% of the osteosarcoma cell nuclei are positively stained in this figure (arrows) (×400; Ab, DO-7).

Table III. Sensitivity to Chemotherapy Relative to p53, Rb, p21(Waf1), mdm-2 and Ki-67 Immunostaining

| | p53 | | Rb | | p21(Waf1) | | mdm-2 | | Ki-67 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | + | − | + | − | + | − | + | − | ++ | + |
| Radiological evaluation | 3/9 (33%) | 8/23 (35%) | 6/17 (35%) | 5/15 (33%) | 7/16 (44%) | 4/16 (25%) | 4/7 (57%) | 7/25 (28%) | 2/4 (50%) | 8/22 (36%) |
| Histological evaluation | 3/9 (33%) | 8/22 (36%) | 3/17 (18%) | 8/14 (57%) | 6/16 (38%) | 5/15 (33%) | 4/7 (57%) | 7/24 (29%) | 1/4 (25%) | 8/21 (38%) |

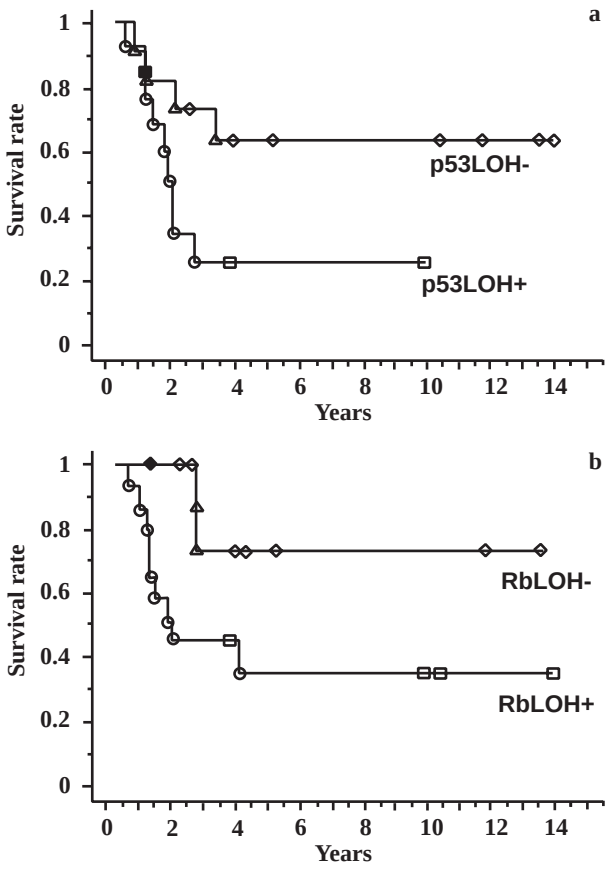


Fig. 4. Survival curves for *p53* (a) and *Rb* (b) LOH-positive and -negative cases. ○ and △ died; ◇ and □ observation ended. Significantly shorter survival rates for LOH-positive cases are evident both for *p53* and *Rb* ($P<0.05$; log-rank test). All survivors were event-free at the end of this study. One patient (◆ ■) died during preparation of the manuscript with lung metastasis.

nificantly more favorable outcome than LOH(+) cases ($P<0.05$: log-rank test). Concerning survivors, patient 14 with lung metastasis died during preparation of this manuscript. All the other patients are currently event-free. No significant correlation was observed between p53 or Rb protein expression and survival.

## DISCUSSION

The present investigation revealed a good correlation between *p53* gene deletion (LOH) in osteosarcomas and their chemoresistance, especially when their sensitivity to therapy was estimated on a histological basis. A similar but much less distinct tendency was observed for the *Rb* gene status. On the other hand, no correlation was found between p53, Rb, p21/Waf-1, mdm-2 or Ki-67 protein accumulation in nuclei and chemosensitivity/resistance.

There have already been many studies revealing that inactivation of *p53* or *Rb* genes in malignancies is associated with reduced chemo- and/or radiosensitivity.[1–15] Most of them were, however, carried out using cultured cells, transplanted tumors in nude mice or knockout mice, or with hematopoietic disease, and the number of clinical studies, especially with solid tumors, is as yet quite limited. To our knowledge, there have been only 5 such studies so far, one with osteosarcomas,[21] one with colorectal cancers,[37] another with breast cancers[38] and 2 with ovarian tumors.[7, 14] In the only clinical study of osteosarcomas, LOH at the *Rb* gene locus was found to be a predictive factor for poor prognosis but not for chemosensitivity.[21] Concerning the ovarian cancer cases, one revealed a reduction of sensitivity to cisplatin with acquisition of *p53* mutations or protein accumulation,[14] whereas the other demonstrated no relationship between *p53* gene mutation and the response to primary chemotherapy.[7]

The present results are essentially in line with previous observations *in vitro*[1–6, 9–11, 13, 15] or *in vivo*.[7, 8, 12, 14, 21] They differ, however, from earlier reports regarding the effects of *p53* and/or *Rb* gene alteration only in cases with LOH, and not in cases with point mutations of these genes only. Since *p53*-mutated tumors are known to undergo progression with loss of the normal *p53* allele,[39–42] the present results might be important in indicating a difference in chemosensitivity between two phases of carcinogenesis which are not clearly observed in *in vitro* experiments. In the literature, 86–91% of LOH-positive colonic cancers showed a point mutation in the remaining allele.[40] In the present results, the proportion of chemosensitive cases was considerably lower than those in previous reports, in which about 70% were classified into the sensitive category.[21, 29] This discrepancy may principally be attributable to the difference in the length of chemotreatment, 4 to 8 weeks in our series and 12 weeks in others.[21, 29] While the present clinical investigation of osteosarcomas only covered a study population of 32, the fact that this type of malignancy is relatively rare, limited to about 200 cases per year in Japan, and that they were all treated and examined in one hospital by the same physicians and pathologists with established procedures, may allow us to regard the findings as significant.

There was a slight discrepancy between histological and radiological evaluations of the effects of chemotherapy in our series of cases. One contributory factor could have been the difference in the timing; the radiological evaluation was mainly based on the reduction in lesion size and blood supply along with calcification during the therapy, whereas the histological examination was performed on resected material.

The frequencies of LOH in *p53* genes, at around 50%, in the present study were about the same as in other reports.[17, 19] The figures for accumulation of p53 protein in the nuclei and loss of Rb protein expression were, however, low in comparison with some previous descriptions.[20, 43, 44] The reason for this is not clear, although differences in the quality of the material in terms of antigen preservation, antibodies used and criteria for positive judgment might have been responsible factors.

In conclusion, in the present series of osteosarcomas, *p53*-LOH-positive cases demonstrated a higher degree of resistance to chemotherapy than their LOH-negative counterparts. LOH at *p53* or *Rb* loci was also associated with a significantly poor prognosis, in accordance with previous reports for osteosarcomas,[19, 20] as well as for other malignancies.[45, 46] With increase in the available information on the significance of tumor cell derangement at the genetic level, such as LOH of the *p53* gene, we may anticipate a greater ability to predict chemosensitivity or resistance.

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