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Expression of p53 Protein, PCNA, and Ki-67 in Osteosarcomas of Bone

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Expressions of p53 protein, a product of the tumor suppressor gene were studied in osteosarcomas relating to various prognostic factors. Thirty-four osteosarcomas were investigated immunohistochemically with a monoclonal antibody clone PAb240, which recognizes a common conformational epitope of mutant p53 proteins and another clone PAb1801, which reacts with both wild- and mutant-type p53 proteins. The results were compared with expressions of proliferating cell nuclear antigen (PCNA) and Ki-67 providing a simple method for the assessment of growth fractions of tumors. PAb240 stained nuclei and cytoplasm of tumor cells in 8 of 34 osteosarcomas(23.5 %), whereas PAb1801 reacted in all 34 osteosarcomas(100 %). Fifteen tumors(44.1 %) showed positivity for PAb1801 in more than half of the tumor cells. Twelve patients were alive and thirteen were dead. Tumors from 9 patients(75 %) who survived revealed only focal positive immunoreactions with PAb1801 and tumors from 6 patients(46.1 %) who died revealed diffuse reactions. Twelve cases(35.3 %) showed a high PCNA index(>40 %) and fibroblastic osteosarcomas revealed the highest PCNA positivity. Twenty-two cases(64.7 %) revealed a very low Ki-67 index(less than 10 %) and Ki-67 index showed a good correlation with PCNA positivity (r=0.6247). Expressions of both wild-and mutant-type p53 protein, PCNA, and Ki-67 were not correlated with other clinical or pathological parameters.

Key Words : *p53 protein, PCNA, Ki-67, Immunohistochemistry, Osteosarcoma*

INTRODUCTION

The etiology of human osteosarcoma remains unknown. Recent studies have elucidated several genetic alterations that occur during the development of osteosarcoma(Masuda et al., 1987 ; Toguchida et al., 1989 ; Miller et al., 1990). The normal p53 gene possesses tumor suppressor activity, and a mutant form exerts a dominant oncogenic function during in vitro transformation(Eliyasu et al., 1989 ; Finlay et al., 1989). The human p53 gene is located on the short arm of chromosome 17, a frequent site of allele loss in osteosarcomas(Toguchida et al., 1989 ; Yamaguchi et al., 1992 ; Ueda et al., 1993). Among several tumor suppressor genes identified, the p53 gene has been characterized best, and its mutation has been shown

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to be associated with many types of common human malignancies, such as colon, lung, liver, esophagus, and breast cancers(Bartek et al., 1991 ; Hollstein et al., 1991 ; Purdie et al., 1991 ; Walker et al., 1991 ; Porter et al., 1992). In the field of bone tumors, major structural alterations of the p53 gene have been shown in about 20 % of osteosarcomas(Masuda et al., 1987 ; Miller et al., 1990). Little is known concerning point mutations of the p53 gene in osteosarcomas.

Monoclonal antibody clone PAb1801 is specific to human p53, either wild- or mutant-type, which cannot distinguish an overexpression of mutant forms(Walker et al., 1991 ; Dobashi et al., 1993 ; Ueda, et al., 1993). Immunohistochemically a recently established monoclonal antibody, PAb240, which recognizes a common conformational change of mutant form, enables the selective detection of mutant p53 protein(Gannon et al., 1990 ; Ueda et al., 1993).

Immunostaining with the monoclonal antibodies PCNA and Ki-67 provides a simple method for the assessment of growth fractions of tumors(Kamel et al., 1991 ; Rosa et al., 1992 ; Rieger et al., 1993 ; Tuccari et al., 1993). PCNA is an auxilliary protein of DNA polymerase delta, and is expressed in the late G1, S, G2, and M phases of the cell cycle(Betta et al., 1993 ; Haapasalo et al., 1993 ; Gasparini et al., 1994 ; Korolopoulou et al., 1994 ; Sasaki et al., 1994). Experimental data suggest that PCNA expression is mainly restricted to S-phase(Sasaki et al., 1994). Ki-67 is a mouse monoclonal antibody which recognizes a nuclear antigen expressed in all phases of the cell cycle except G0 and early G1(Kamel et al., 1991 ; Rosa et al., 1992 ; Rieger et al., 1993 ; Tuccari et al., 1993).

In this study, we examined the expressions of p53 protein in 34 osteosarcomas using two different monoclonal antibodies. The contribution of p53 gene mutation to the development of osteosarcomas was evaluated. The results were correlated with markers of the cell cycle, expression of PCNA and Ki-67.

MATERIALS AND METHODS

Osteosarcomas

Thirty-four osteosarcomas were studied. All of these cases were obtained from 1982 to 1994 in the Department of Pathology, Kyung Hee University Hospital, Seoul, Korea. The clinical profiles were searched for age, sex, and site. Information about survival was available for 25 cases. All the histologic slides were reviewed by the authors and classified according to their histologic subtypes and Broder's histologic grading (grades I-IV). According to the conventional histologic classification of osteosarcomas, 16 cases were osteoblastic type, 8 chondroblastic, 9 fibroblastic, and one was parosteal osteosarcoma. Presence of necrosis was also evaluated.

Monoclonal antibodies

The two antibodies to p53 protein were mouse monoclonal antibodies. Clone PAb240 (Pharmingen, San Diego, CA) recognizes a common conformational epitope of the mutant type. However it does not react to wild-type p53 protein. Clone PAb1801 (Pharmingen, San Diego, CA), specific to human p53, reacts with either wild- or mutant-type.

Anti-PCNA (PC10, DAKO, Carpinteria, CA) and anti-Ki-67 clone MIB-1 (Immunotech S.A., Marseille, France) were monoclonal mouse antibodies.

Immunohistochemistry

Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method(DAKO LSAB Kit, Carpinteria, CA). Briefly, 6-μm paraffin sections were adhered to silanized slides (DAKO, Carpinteria, CA) and dried. After the deparaffinization and rehydration, the tissue sections were incubated for five minutes with 3 % hydrogen peroxide and blocking reagent. After microwave antigen retrieval in citrate buffer, the sections were exposed to the primary antibodies (1 : 200 dilution of anti-p53 proteins and 1 : 100 dilution of anti-Ki-67) for 30 minutes at 37℃. The slides for anti-PCNA(1 : 100 dilution) were omitted in this microwave procedure. After washing with TRIS-buffered saline(DAKO, Carpinteria, CA), biotinylated link antibody was applied for 15 minutes followed by streptavidin peroxidase for an additional 10 minutes. Color development was performed with substrate-chromogen (3-amino-9-ethylcarbazole) solution for 10 minutes.

For positive controls, paraffin sections of colon adenocarcinoma that showed a positive reaction both with PAb240 and PAb1801 were used. Sections from normal tonsil were used for positive controls of anti-PCNA and anti-Ki-67. Non-neoplastic components on the same section, such as fibroblasts and endothelial cells, which are supposed to be unstained,

were regarded as internal negative controls in each case.

Staining evaluation

Immunoreactions for p53 proteins were scored as follows : three positive(+++), more than 50 % of tumor cells are positive ; two positive(++), 10 to 50 % of tumor cells are positive ; one positive(+), less than 10 % of tumor cells are positive ; negative(-), tumor cells are negative.

PCNA positivity and Ki-67 index were calculated by determining the number of PCNA- and Ki-67-positive cells among the total number of tumor cells over 300 in high power fields, respectively.

Statistics

The Pearson correlation coefficients were calculated to study the correlation between analysis variables. One-way analysis of variance (ANOVA) was carried out to study the difference in the p53 positivity, PCNA-positivity, and Ki-67 index between clinico-pathological factors. These analyses were performed with SAS software (Version 6, Cary, USA).

RESULTS

The monoclonal antibody clone PAb240 showed granular positive immunoreactions in nuclei of osteosarcoma cells. The staining reactions were nuc-

Table 1. Clinico-pathologic profiles and expressions of p53 protein in osteosarcomas.

Case	Age/Sex	Location	Subtype	Grade	Necrosis	PAb1801	PAb240	Survival
1	15/F	Tibia	Fibroblastic	4	+	+	−	Death
2	22/F	Ilium	Chondroblastic	3	+	+	+/−	Death
3	15/F	Tibia	Fibroblastic	4	−	+++	++	Death
4	23/M	−	Chondroblastic	3	−	+	+/−	Survival
5	29/F	Tibia	Osteoblastic	3	−	+	+/−	Survival
6	18/M	Femur	Fibroblastic	3	−	++	−	Death
7	7/M	Femur	Osteoblastic	3	−	+++	+/−	Death
8	42/F	Femur	Chondroblastic	2	−	++	++	Survival
9	42/F	Femur	Chondroblastic	2	−	+	−	Survival
10	8/M	Femur	Osteoblastic	3	−	+++	+/−	Death
11	12/M	−	Osteoblastic	4	−	+	−	Death
12	32/F	Femur	Osteoblastic	3	+	+	−	Survival
13	43/F	Femur	Fibroblastic	3	+	+++	+/−	Survival
14	−	−	Chondroblastic	3	−	+++	++	NA
15	18/M	Tibia	Chondroblastic	3	−	+	++	Survival
16	34/F	Maxilla	Fibroblastic	2	−	+++	−	NA
17	11/M	Femur	Osteoblastic	3	−	++	−	Death
18	20/M	Shoulder	Chondroblastic	3	+	+	++	Death
19	32/M	Scapula	Parosteal	1	−	+	−	Survival
20	−	Femur	Osteoblastic	3	+	+++	+/−	Death
21	20/F	Maxilla	Fibroblastic	2	−	+++	−	NA
22	66/M	Femur	Osteoblastic	3	+	+++	−	Death
23	13/F	Femur	Chondroblastic	3	−	++	++	NA
24	53/M	Pubic	Fibroblastic	3	−	+++	++	Death
25	17/F	Humerus	Osteoblastic	4	−	+++	−	Survival
26	16/M	Femur	Osteoblastic	3	−	+	−	Death
27	3/F	Humerus	Osteoblastic	3	−	++	++	NA
28	12/F	Ilium	Osteoblastic	3	−	+++	+/−	NA
29	17/M	Femur	Osteoblastic	4	+	+	−	Survival
30	19/M	Femur	Fibroblastic	3	+	+++	−	NA
31	13/M	Tibia	Osteoblastic	3	−	+	−	Survival
32	13/M	Tibia	Osteoblastic	3	−	+	−	Survival
33	17/M	Fibula	Fibroblastic	3	+	+++	+/−	NA
34	−	−	Osteoblastic	4	−	+++	−	NA

NA ; Not available

lear in pattern in all the instances with variable cytoplasmic staining. The proportion and distribution of positive staining cells in osteosarcoma were variable from tumor to tumor and even within the same tumor. In all the positive cases, p53 expressions were confined to the tumor cell population : blood vessels and some inflammatory cells which constitute tumor stroma were negative. Eight cases out of 34 osteosarcomas (23.5 %) showed positive reactions with PAb240 (Table 1).

All 34 osteosarcomas showed positive immunoreactions with PAb1801(Fig. 1). PAb1801 exhibited both intranuclear and intracytoplasmic granular positive reactions in osteosarcoma cells. Non-neoplastic cells were negative for PAb1801. Staining intensities of PAb1801 were stronger than those of PAb240 in most of the positive tumors. In fifteen tumors(44.1 %), more than half of the tumor cells were positive.

The staining intensities of PAb1801 and PAb240 were variable. There was no significant different staining pattern between the histologic subtypes. The classical osteoblastic osteosarcomas revealed variable staining intensity in tumor cells. Adjoining osteoid and necrotic areas were negative. In chondroblastic osteosarcomas, there was a tendency for intense stainability in the cellular, peripheral areas of chondroid lobules. In fibroblastic osteosarcomas, the spindle tumor cells as well as multinucleated giant cells were positive. Expressions of PAb240 and PAb1801 were not correlated with other clinical or pathological factors such as age, location, grade, and presence of necrosis (Table 1).

Follow-up was available for 25 patients and the mean follow-up period was 60 months. Twelve patients were alive and thirteen were dead. Tumors from 9 patients who survived(75 %) revealed only focal (+) positive immunoreactions with PAb1801 and two(16.6 %) revealed diffuse (+++) reactions. Tumors from 6 patients who died(46.1 %) revealed diffuse (+++) positive immunoreactions with PAb1801 and five(38.4 %) revealed focal (+) reactions. The staining intensities with PAb240 were not different between these two, survival and death groups (Table 1).

PCNA(Fig. 2) immunostaining demonstrated a wide range of positivity from 3 % to 91 % (total mean 39.06 %). The staining pattern was nuclear in all instances. 12 tumors (35.3 %) showed high (>40 %) PCNA grades. Among the histologic subtypes, fibroblastic osteosarcomas revealed the highest PCNA positivity (mean 50.5 %), followed by osteoblastic (mean 39.03 %). The chondroblastic osteosarcomas showed the lowest PCNA positivity (mean 24.42 %, $p=0.0797$). According to the follow-up, the survival group (mean

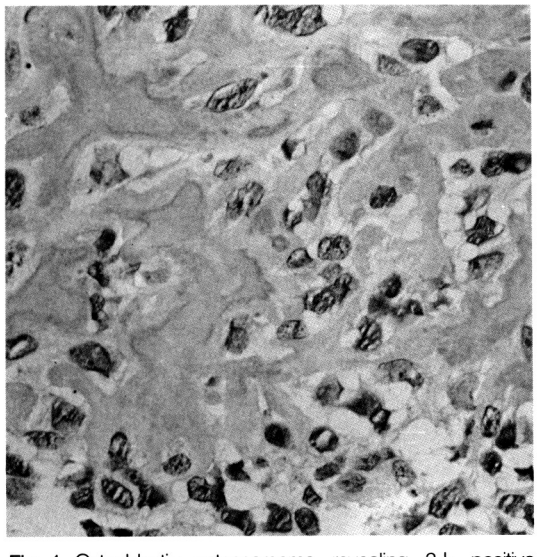

Fig. 1. Osteoblastic osteosarcoma revealing 3+ positive reaction with PAb1801(ABC stain).

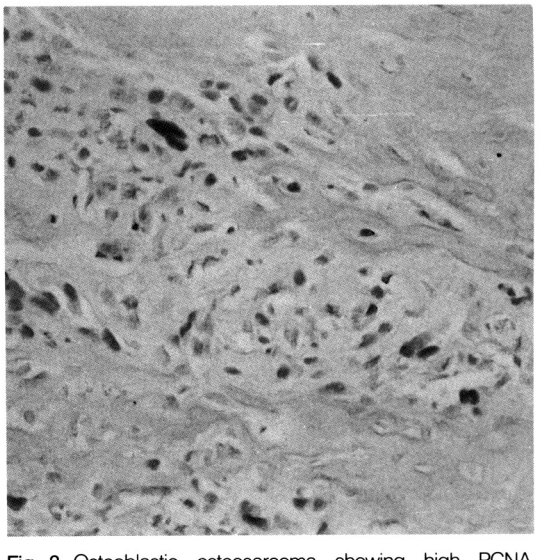

Fig. 2. Osteoblastic osteosarcoma showing high PCNA grade(ABC stain).

Table 2. Expression of PCNA and Ki-67 in relation to various prognostic features.

Prognostic feature	Total number	PCNA(mean %)	Ki-67(mean %)
Age			
Below 20	20	36.21	10.66
21-40	6	34.20	3.00
Over 41	5	52.80	25.75
Site			
Long bone	24	36.41	11.42
Flat bone	4	50.66	27.00
Jaw	2	58.75	3.50
Histologic subtype			
Osteoblastic	16	39.03	11.23
Chondroblastic	8	24.42	4.20
Fibroblastic	9	50.50	16.50
Parosteal	1	39.00	11.00
Histologic grade			
Grade 1	1	39.00	11.00
Grade 2	4	35.87	4.33
Grade 3	23	38.52	11.88
Grade 4	6	44.00	14.40
Necrosis			
None	24	38.18	11.82
Present	10	41.22	10.88
Survival			
Death	13	45.26	11.18
Survival	12	34.40	15.44
PAb1801 reactivity			
1+	14	32.75	8.54
2+	5	23.80	2.50
3+	15	49.92	17.72
PAb240 reactivity			
−	17	39.70	9.42
+/−	9	39.38	15.28
2+	8	37.28	12.00

34.4 %) revealed lower PCNA positivity than the death group (mean 45.26 %), but it was not significant statistically ($p>0.05$). PCNA positivity (mean 49.92 %) in the group of diffuse (+++) PAb1801 positivity was much higher than groups of focal (+) or moderate (++)PAb1801 positivity and it was statistically significant ($p=0.0413$). However, the PCNA positivity was not correlated with PAb240 reaction, age, site, grade, and presence of necrosis (Table 2).

Immunoreactions for Ki-67(Fig. 3) were also nuclear and especially mitotic cells were strongly positive. Ki-67 index ranged from 1 % to 55 % (total mean 11.5 %) and 22 cases (64.7 %) revealed a very low Ki-67 index (less than 10 %). According to the histologic subtypes, the fibroblastic (mean 16.5 %) and osteoblastic types (mean 11.23 %) revealed a higher Ki-67 index than the chondroblastic type (mean 4.2

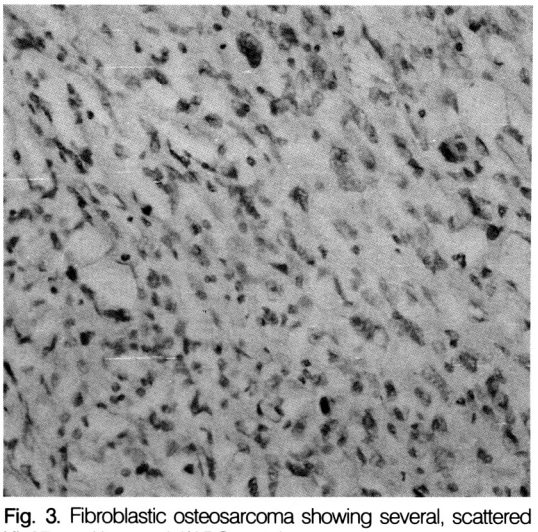

Fig. 3. Fibroblastic osteosarcoma showing several, scattered Ki-67 positive nuclei(ABC stain).

%), but it was not statistically significant ($p>0.05$). The diffuse PAb1801 positive group revealed a higher Ki-67 index than the focal or moderate groups but it was not significant ($p>0.05$). Ki-67 index was not correlated with PAb240 positivity, age, site, grade, presence of necrosis, and survival (Table 2). Ki-67 index showed moderate correlation with PCNA positivity ($r=0.6247$).

DISCUSSION

Gannon et al.(Gannon et al., 1990) have recently established a monoclonal antibody to p53 protein, designated PAb240, which does not react with wild-type p53 protein but recognizes mutant forms of p53 protein resulting from various activating mutations. The current immunohistochemical study using this monoclonal antibody showed that mutant p53 protein is partly(23.5 %) expressed in the nuclei and cytoplasms of osteosarcoma cells. These expressions of mutant p53 protein in osteosarcomas suggest that mutation of p53 gene may be one of the crucial steps in the development of osteosarcomas. These results were compatible with those of several authors investigating the genetic alterations in osteosarcomas with variable frequency using molecular biologic techniques(Masuda et al., 1987 ; Benedict et al., 1988 ; Toguchida et al., 1989 ; Miller et al., 1990 ; Yamaguchi et al., 1992). Comparative studies have reported that PAb1801 and PAb240 stained in a similar pattern and frequency in common human cancers(Marks et al., 1991 ; Purdie et al., 1991 ; Walker et al., 1991 ; Ueda et al., 1993). Of the 34 osteosarcomas reacting positively with PAb1801, 26 did not react with PAb240. This result suggests that PAb1801 positivity results from the increased accumulation of wild-type p53 rather than mutant p53. Differences in reported frequency of p53 overexpression in some tumor types may be attributable to case selection factors, as well as the use of different anti-p53 monoclonal antibodies(Yewdell et al., 1986 ; Bartek et al., 1991 ; Porter et al., 1992 ; Said et al., 1992). Both mutant and wild-type p53 may be present in the nucleus, and mutant p53 may be found also in the cytoplasm. In transformed cell lines, mutant p53 forms oligomeric complexes with other cytoplasmic proteins, greatly enhancing its stability and half-life.

Recently, several monoclonal antibodies have been established against p53 protein(Marks et al., 1991 ; Purdie et al., 1991 ; Walker et al., 1991 ; Ueda et al., 1993). Immunohistochemical evaluation of tumors allows determination of the number of cells with detectable p53, although variable in intensity. It was also clearly evident that there could be differences in the percentage of reactive cells with the different antibodies in individual cancers(Purdie et al., 1991 ; Walker et al., 1991 ; Tuccari et al., 1993 ; Ueda et al., 1993). p53 positivity in human carcinomas varies depending on the type of carcinoma studied(Bartek et al., 1991 ; Hollstein et al., 1991 ; Porter et al., 1992). Among human lung and colon carcinomas(Purdie et al., 1991), about 70 % of cases are positive, while lower percentages have been reported in breast, prostate, and thyroid carcinomas(Walker et al., 1991 ; Ito et al., 1992 ; Dobashi et al., 1993). It also seems possible that there are variations in the frequency of p53 positivity between different sarcomas(Soini et al., 1992 ; Toguchida et al., 1992). In our study the monoclonal antibody PAb1801 revealed 100 % positivity in 34 osteosarcomas with variable intensity. This high rate of positivity has not been reported so far in the literature(Masuda et al., 1987 ; Toguchida et al., 1992 ; Ueda et al., 1993). This could result from the various factors, such as the type of antibody, the dilution factor, and the use of microwave antigen retrieval.

p53 immunocytochemistry has two possibilities of false negatives ; first, the underlying lesion may be not a missense point mutation but a gross deletion which abolishes all p53 protein production. The second possibility is that the point mutation does not stabilize the protein sufficiently for its level to reach detectability by immunocytochemistry. It also has a possibility of false positives ; the mechanism for such non-mutational stabilization is most likely the result of interruption to the normal degradative pathway of p53(Wynford-Thomas, 1992).

The expression of p53 protein was not correlated with any of the clinicopathological factors examined including age, location, histologic subtype, grade, and presence of necrosis in this study. However, of all osteosarcoma patients in this study, nine subjects(75 %) in the survival group revealed only focal positive immunoreactions with PAb1801 and six(46.1 %) in the death group revealed diffuse reactions. This suggests that subjects with strong PAb1801 positivity are likely to have less chance of survival. However, PAb240 positivity was not correlated with the survival data statistically, probably due to a small sample size.

Immunostaining to identify nuclear antigens ex-

pressed throughout the cell cycle provides a convenient way of assessing proliferating kinetics in tumors and assessing prognosis(Rosa et al., 1992 ; Gasparini et al., 1994). PCNA concentrations are maximally increased at the late G1 and S phases of the cell cycle and correlate directly with the rates of cellular proliferation and DNA synthesis(Celis & Celis, 1985 ; Korkolopoulou et al., 1994). Ki-67 nuclear antigen is also associated with cell proliferation and found throughout the cell cycle (G1, S, G2/M phases ; Kamel et al., 1991 ; Rosa et al., 1992 ; Rieger et al., 1993 ; Tuccari et al., 1993). PCNA labelling indices have been shown to correlate with other markers of the cell cycle, e.g., bromodeoxyuridine (BrdU) incorporation, thymidine labelling, and variably with Ki-67 expression(Kamel et al., 1991 ; Rosa et al., 1992 ; Haapasalo et al., 1993 ; Rieger et al., 1993 ; Tuccari et al., 1993).

In this study, PCNA positivity in osteosarcomas ranged from 3 % to 91 % (mean 39.06 %), contrasting with Ki-67 indices ranging from 1 % to 55 % (mean 11.5 %). Expression of PCNA and Ki-67 shows a large discrepancy in index although expressions of two proliferation markers are correlated statistically (r=0.6247). Most of the data so far demonstrated the lower Ki-67 index compared to the PCNA index.

In the literature(Kamel et al., 1991 ; Rosa et al., 1992 ; Rieger et al., 1993 ; Tuccari et al., 1993), the correlation between PCNA and Ki-67 was variable. Some authors(Kamel et al., 1991 ; Rieger et al., 1993) have reported a good correlation between PCNA and Ki-67 data. However in other studies(Rosa et al., 1992 ; Tuccari et al., 1993), no correlation was found between PCNA and Ki-67. This lack of relationship between the two markers may be attributed to a number of factors. The most likely is the marked inter- and intra-tumour heterogeneity reflected in high standard deviation values. The long life of PCNA may lead to detection of cells that have recently left the cell cycle(Rosa et al., 1992). In a study of breast carcinomas a linear relationship between PCNA and Ki-67 immunostaining was absent because the immunoreactivity for Ki-67 appeared less represented and focal(Tuccari et al., 1993).

Among the histologic subtypes, fibroblastic and osteoblastic osteosarcomas reveal higher PCNA and Ki-67 indices than chondroblastic types. This may suggest the lower proliferation capacity in chondroblastic types. Further, the expression of PCNA and Ki-67 is not significantly correlated with variable clinical and pathological variables in our series. Betta et al.(1993) revealed no correlation between the PCNA grade and other prognostic variables, such as tumor size and axillary node status in breast carcinoma. Mercer et al.(Mercer et al., 1991) reported that p53 protein had a regulatory effect on the PCNA gene : the wild type protein selectively down-regulates PCNA mRNA and protein expression probably by inhibiting the function of the PCNA promoter whereas the mutant p53 seems to activate PCNA promoter directly. The weak correlation between p53 and PCNA expression in our series may indicate that in most cases the p53 protein we detected immunohistochemically is of the wild type. In the study of non-Hodgkin's lymphomas p53 immunoreactivity was associated with the proliferation state as expressed by PCNA and c-myc p62 expression, indicating the interrelationship of these three genes(Korkolopoulou et al., 1994).

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