# Apoptosis Detected by in Situ DNA end-extension in Osteosarcomas
## - In relation to p53 and Bcl-2 expression -

Yong-Koo Park, M.D., Moon Ho Yang, M.D. Hye-Rim Park, M.D.*,
Youn Wha Kim, M.D., Juhie Lee, M.D.

Departments of Pathology, College of Medicine, Kyung Hee University and
Hallym University*, Seoul, Korea

— Abstract —

**Objective** : The objective of this study was to compare expression of various proto-oncogenes and rates of apoptosis in osteosarcoma patients. Modulation of apoptosis may influence resistance to chemotherapy and therefore affect the outcome of cancer treatment. Osteosarcoma is one of the most fatal malignancies in young adolescents and investigation of the role of apoptotic cell death is warranted in relation to chemotherapy and tumor outcome.

**Design** : The terminal deoxynucleotidyl transferase to exposed 3' -hydroxyl termini of DNA (TUNEL method) staining method has been applied for the in situ detection of DNA double strand breaks.

**Patients** : Thirty-three osteosarcomas in various stages of differentiation from twenty-nine patients were investigated immunohistochemically for p53, Bcl-2 and TUNEL method for apoptosis.

**Results and conclusion**; We have found that higher level of wild type p53 were correlated with enhanced expression of apoptosis. Increased apoptosis rates were found in cases of negative Bcl-2 expression. In the present study, we have concluded that a significant proportion of osteosarcoma, a tumor in which resistance to chemotherapy often occurs, express high levels of p53 and low levels of Bcl-2. Our data provide further evidence for cross-talk between Bcl-2 and p53 and suggests that these genes are important determinants of drug-induced apoptosis.

**Key Words** : Apoptosis, TUNEL method, Osteosarcoma, Bcl-2, Wild type p53

※ Correspondence author : Yong-Koo Park, M.D.
　　　　　　　　　　　Department of Pathology, Kyung Hee University Hospital,
　　　　　　　　　　　#1 Hoeki-Dong, Dongdaemoon-Ku, Seoul, 130-702, Korea.

## Introduction

Apoptosis is a distinct mode of cell death that is responsible for deletion of cells in normal tissues; it also occurs in specific pathologic conditions. Morphologically, it involves rapid condensation and budding of the cells, with the formation of membrane-enclosed apoptotic bodies containing well-preserved organelles, which are phagocytosed and digested by nearby resident cells. There is no association with inflammation. A characteristic biochemical feature of the process is double strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments[17]. Apoptosis can be found in virtually all untreated malignant tumors[31,38], however there have been few precise quantitative studies[29].

Osteosarcoma is a high-grade malignant tumor which occurs predominantly in the long bones of adolescents and young adults. It is generally accepted that the advent of modern chemotherapy has improved the prognosis in osteosarcoma to about 50-60% 5-year survival[28]. Recent study has suggested that prognosis in osteosarcoma is related to the amount of necrosis seen in the resected specimen after chemotherapy[27]. Apoptosis often is particularly prominent near foci of confluent necrosis, where mild ischemia is likely to be involved in its initiation; this is a known cause of enhancement of apoptosis in non-neoplasitic tissues[12,15].

Recently, it has been discovered that apoptosis can be regulated by the products of certain proto-oncogenes, p53 tumor suppressor gene, and bcl-2 protein[13,14,20,36,39].

In situ end-labeling of fragmented DNA can be used for identifying cells at early stages of apoptosis[19,30]. This in situ end-labeling is a one of the convenient methods to detect apoptosis in paraffin embedded tissue. In the present study, both fragmented and condensed nuclei, as well as apoptotic bodies are readily stained using in situ end labeling technique, enabling differentiation of apoptotic cells from infiltrating lymphocytes or mitotic cells[11].

In this article, we studied expression of apoptosis using terminal deoxynucleotidyl transferase to exposed 3'-hydroxyl termini of DNA (TUNEL method) in osteosarcoma patients and compared the expression rates of apoptosis to the rates of proto-oncogene expression, chemotherapy, necrosis and other histologic and clinical parameters.

## Materials and Methods

Thirty-three osteosarcomas in various stages of differentiation from 29 patients were studied by immunohistochemistry. The tumors were subtyped according to their main component as osteoblastic, chondroblastic, and fibroblastic. We graded the histology of the tumor according to the Broder's classification. Patient's age, sex, survival, and location of the lesion were evaluated. Fifteen patients were receiving preoperatively chemotherapy and we also examined the chemotherapy regimen.

In situ Apoptosis staining All surgical specimens were fixed in 4% neutral buffered formalin and decalcified and embedded in paraffin. 6μm sections on poly-L-Lysine-coated slides (DAKO, Carpinteria, Ca) were deparaffinized, rehydrated, and washed with distilled water. Tissue sections were protein stripped by incubation with 20mg/ml proteinase K (Sigma, St. Louis, MO) for 5 minutes at room temperature, followed by a

distilled water wash. The TUNEL method was used for identification of apoptosis (Apoptag in situ detection kit, Oncor, Gaithersburg, MD). Sections were immersed in equilibration buffer for 10 minutes at room temperature, terminal deoxynucleotidyl transferase(TdT) was added to the sections, and the slides were incubated in a humidified chamber at 37℃ for 1 hour. The reaction was stopped by incubating the slides with stop/wash buffer at 37℃ for 30 minutes. After washing in phosphate buffered saline, the sections were incubated with anti-digoxigenin peroxidase solution for 30 minutes at room temperature. The slides were colorized with deaminobenzidine /$H_2O_2$ solution (0. 2mg/$ml$ diamin- obenzidine tetrachroloride and 0.005 % $H_2O_2$ in 50 mM Tris-HCl buffer), and counterstained with hematoxylin. Tissue sections treated with DNAse I (0. 7mg/$ml$ sodium cacodylate buffer, pH 7.2(Amresco, Solon, OH) for 10 minutes

before treatment with TdT served as positive controls. Samples treated similarly but without enzyme served as negative controls.

We graded the in situ apoptosis staining as follow; none of the cells were positive staining as negative; scattered tumor cells were positive even though morphologically normal looking nucleus as one positive; tumor cells were positive and showed morphologically apoptotic change on their tumor cell nuclei as two positive (Fig. 1).

## Monoclonal Antibodies

The antibody to p53 protein was a mouse monoclonal antibody. Clone Pab 1801(Pharmingen, San Diego, Ca), specific to human p53, reacts with either wild or mutant types. Clone Pab 240(Pharmingen, San Diego, Ca) recognizes a common conformational epitope of the mutant type. However, It does not react to wild-type p53 protein.
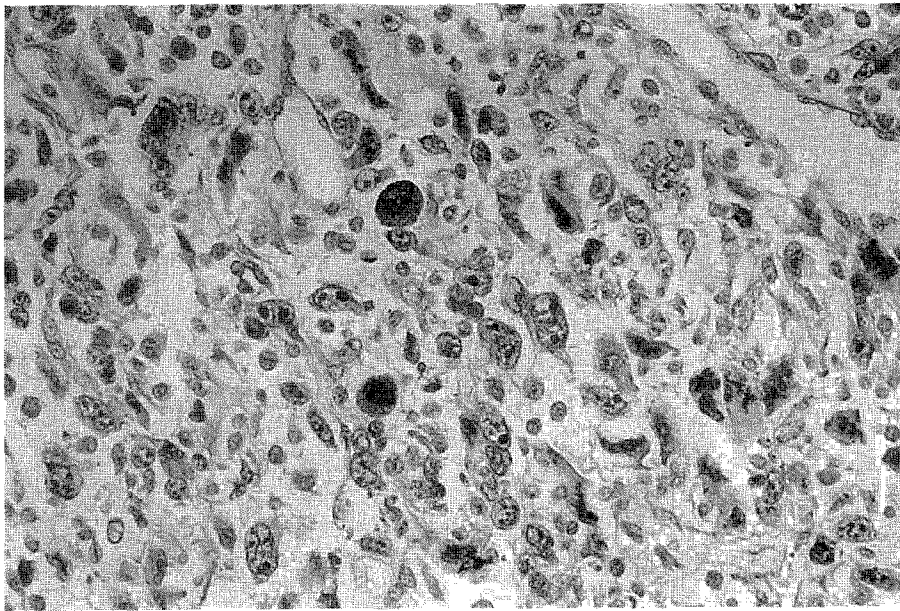


**Fig. 1.** In situ staining of DNA double strand breaks of osteosarcoma counterstained with hematoxylin (×200). Several intesnely stained cells with apoptotic condensed nuclei are present (arrow head).

The antibody to bcl-2 was a mouse monoclonal antibody, IgG class (DAKO, Carpinteria, Ca).

## Immunohistochemistry

Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method (DAKO LSAB kit, Carpinteria, Ca). Briefly, $6\mu m$ paraffin sections were adhered to silanized slides 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 for 30 minutes at 37℃. After washing with Tris buffered saline, 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-ethyl car-

bazole) solution for 10 minutes. Immunoreactions for p53 proteins (Fig. 2) and Bcl-2 (Fig. 3) 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.

## 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 differences in the rates of apoptosis, p53 positivity, bcl-2, and clinico-pathological factors.

## Results

Apoptosis was detected in fifteen cases among 33 osteosarcomas. Among the fifteen cases, six instances revealed grade two posi-
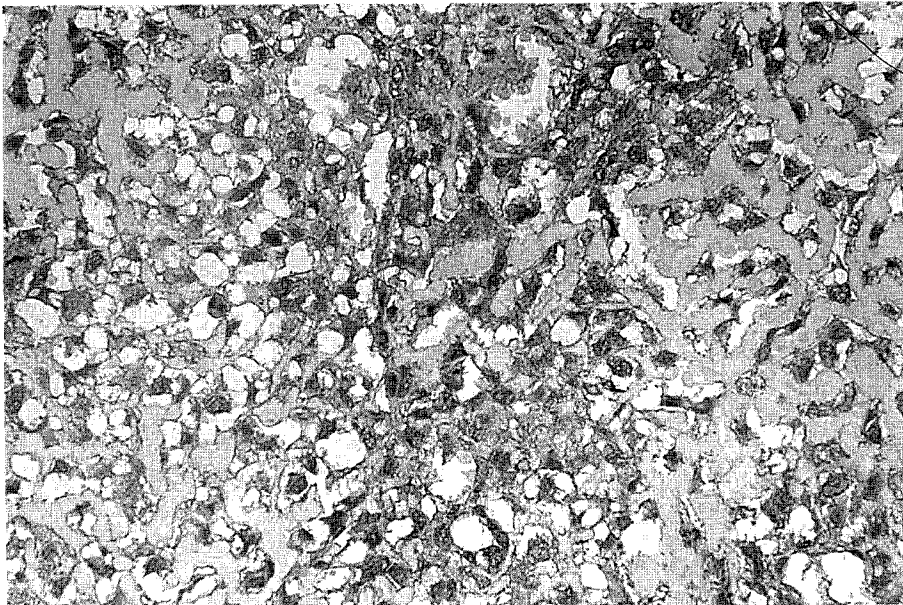


Fig. 2. p53 immunostaining of osteoblastic osteosarcoma counterstained with hematoxylin (×100). Most of the tumor cells showed grade three positive.

tive staining intensity and the remaining nine cases revealed grade one stainability. In some areas, positive apoptotic cells were numerous in the vincinity of the necrotic area. The authors compared the rate of apoptosis with chemotherapy. Among the negative apoptosis, 10 patients had received chemotherapy and seven had not received chemotherapy. Among the five cases of grade one positive apoptosis, four had not received chemotherapy and one had been treated with chemotherapy. Four patients who had been treated with chemotherapy showed grade two apoptosis positivity and the two patients who had not received chemotherapy also showed grade two apoptosis posi-

**Table. 1.** Clinico-pathological profiles and expressions of p53,Bcl-2 and apoptosis in osteosarcomas.

| Case | Age/ sex | Necrosis | H. Sub type | H. Grade | Location | PAB 1801 | PAB 240 | Apopto- sis | Bcl-2 | Chemo- therapy |
|------|----------|----------|-------------|----------|----------|----------|---------|-------------|-------|-----------------|
| 1 | 15/F | Tibia | F | 4 | + | + | - | - | - | - |
| 2 | 22/F | Ilium | C | 3 | + | + | +/- | - | + | - |
| 3 | 15/F | Tibia | F | 4 | - | +++ | ++ | ++ | - | - |
| 4 | 23/M | NA | C | 3 | - | + | +/- | - | - | + |
| 5 | 29/F | Tibia | O | 3 | - | + | +/- | - | - | + |
| 6 | 18/M | Femur | F | 3 | - | ++ | - | - | - | NA |
| 7 | 7/M | Femur | O | 3 | - | +++ | +/- | + | - | - |
| 8 | 42/F | Femur | C | 2 | - | ++ | ++ | - | + | + |
| 9 | 42/F | Femur | C | 2 | - | + | - | - | - | + |
| 10 | 8/M | Femur | O | 3 | - | +++ | +/- | - | - | + |
| 11 | 12/M | NA | O | 4 | - | + | - | - | - | + |
| 12 | 32/F | Femur | O | 3 | + | + | - | ++ | - | + |
| 13 | 43/F | Femur | F | 3 | + | +++ | +/- | ++ | - | + |
| 14 | NA | NA | C | 3 | - | +++ | ++ | - | + | + |
| 15 | 18/M | Tibia | C | 3 | - | + | ++ | - | - | + |
| 16 | 34/F | Maxilla | F | 2 | - | +++ | - | + | + | NA |
| 17 | 11/M | Femur | O | 3 | - | ++ | - | - | - | - |
| 18 | 20/M | Shoulder | C | 3 | + | + | ++ | + | - | - |
| 19 | NA | Femur | O | 3 | + | +++ | +/- | ++ | - | + |
| 20 | 20/F | Maxilla | F | 2 | - | +++ | - | + | - | + |
| 21 | 66/M | Femur | O | 3 | + | +++ | - | + | - | - |
| 22 | 13/F | Femur | C | 3 | - | ++ | ++ | - | - | - |
| 23 | 53/M | Pubis | F | 3 | - | +++ | ++ | ++ | - | + |
| 24 | 17/F | Humerus | O | 4 | - | +++ | - | ++ | + | - |
| 25 | 16/M | Ilium | O | 3 | - | + | - | - | - | + |
| 26 | 3/F | Femur | O | 3 | - | ++ | ++ | + | - | NA |
| 27 | 12/F | Femur | O | 3 | - | +++ | +/- | - | + | - |
| 28 | 17/M | Femur | O | 4 | + | + | - | - | - | + |
| 29 | 19/M | Tibia | F | 3 | + | +++ | - | + | + | NA |
| 30 | 13/M | Tibia | O | 3 | - | + | - | + | + | - |
| 31 | 13/M | Tibia | O | 3 | - | + | - | + | + | - |
| 32 | 17/M | Fibula | F | 3 | + | +++ | +/- | - | - | - |
| 33 | NA | NA | O | 4 | - | +++ | - | - | - | NA |

H: Histologic, F: Fibroblastic osteosarcoma, C: Chondroblastic osteosarcoma,
O: Osteoblastic osteosarcoma. NA: not available

tivity. The rates of apoptosis and chemotherapy treatment were not correlated. The results of immunostaining and clinical parameters were summarized as Table 1.

Positive rates of apoptosis were compared with necrosis. Among the 18 cases of negative apoptosis, only three cases revealed necrosis. Among the nine cases of one positive apoptosis, four cases showed necrosis and five cases revealed no necrosis. Among the 6 cases of two positive apoptosis, half of them showed necrosis. These results revealed that there was a strong correlation between the presence of necrosis and apoptotic positive rate (r= 0.31083).

Apoptosis was also compared with location of the bone involvement and histologic types. Four cases out of six involved flat bone showing grade one and two apoptotic positivity. However, 10 cases among the 23 instances involving long bone showing apoptotic positivity. Osteoblastic osteosarcomas were 16 cases among the 33 instances. Among them, seven

cases showed positive apoptosis with varying stainability. Chondroblastic osteosarcomas were 8 cases. Seven cases were negative for apoptosis and only one was one positive for apoptosis. Nine cases were fibroblastic osteosarcoma. Seven instances were positive for apoptosis with varying staining intensities.

Histologic grades were compared with apoptosis. Four cases were grade two osteosarcoma, half of them were negative for apoptosis. 23 cases were grade three osteosarcoma. Ten cases out of them showed grade one or two positive for apoptosis. Six cases showed grade four osteosarcoma. Half of the grade four osteosarcomas were negative for apoptosis.

All the thirty three osteosarcomas showed positive immunoreactions with PAb 1801 antibody which means either positive for wildor mutant type of p53 protein. PAb 1801 exhibited both intranuclear and intracytoplasmic granular positive reactions in osteosarcoma
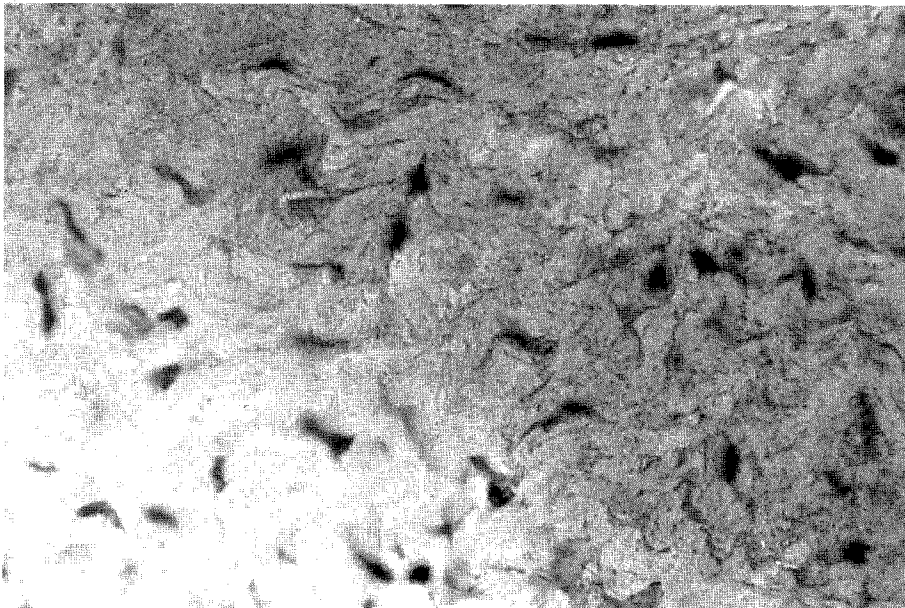


**Fig. 3.** Bcl-2 immunostaining of fibroblastic osteosarcoma counterstained with hematoxylin ( × 200). Some of the tumor cells showed grade two positive stainability.

cells. Non-neoplastic cells were negative for this antibody. Staining intensities of this antibody were quite prominent in most of the positive tumors. In fifteen instances (45.4 %, 15/33), more than half of the tumor cells were positive. 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 fibroblasltic osteosarcomas, the spindle tumor cells as well as multinucleated giant cells were positive. Expression of this antibody was not correlated with other clinical or pathological factors, such as age, location, histologic grade, and presence of necrosis (Table 1). There was no significant different staining pattern between the histologic subtypes. Among the thirteen cases of grade one positive p53, 11 cases were negative for apoptosis. Four cases were negative for apoptosis among the five cases of grade two p53 positive. Fifteen cases were grade three positive for p53 antibody. Seven cases among them showed grade one apoptosis positive, and five instances showed grade two apoptosis positivity. There was no significant differences between p53 staining intensity and the presence of chemotherapy. For Pab 240 antibody which means mutant type of p53, 8 cases out of 33 (244 %) were grade ++ positive staining. *Half of the PAb 240 positive cases showed variable degree of apoptosis.* Among the negative for Pab 240 cases, 8 cases showed variable degree of apoptosis (Table 1).

We perfomed anti bcl-2 immunostaining. 72.7% (24 cases out of 33) were negative for bcl-2 and 27.3% (9 cases out of 33) showed positive immunoreactions.

Among the 24 negative bcl-2 cases, 12 cases were negative for apoptosis, seven were one positive and the rest 5 were two positive for apoptosis. However, among the 9 bcl-2 positive cases, six cases were negative for apoptosis, two instances were one positive apoptosis and only one case showed two positive apoptosis.

The histologic grade of the osteosarcomas was also compared with bcl-2 immunoreactivity. Two cases of grade two osteosarcoma revealed bcl-2 positive and two cases of grade two were negative for bcl-2. Twenty-three cases were grade three. Among them 17 cases were negative for bcl-2 and six cases were positive for bcl-2. There were 6 cases of grade four osteosarcoma. Five instances were negative for bcl-2 and one case revealed positive for bcl-2.

Twenty-three cases revealed no necrotic area. 16 cases among them showed negative for bcl-2 and 7 instances showed positive for bcl-2. *10 cases showed necrosis.* Among them 8 cases showed negative for bcl-2 and only two instances showed positive for bcl-2.

Fifteen cases were treated with chemotherapy. Among them 13 cases showed negative for bcl-2 and two cases showed positive for bcl-2 immunostaining. Eight cases out of 13 cases which had not received any kind of chemotherapy showed negative for bcl-2 and the rest of five cases revealed positive for bcl-2 staining.

We also compared p53 immunoreactivity with bcl-2 immunostaining. Ten cases out of 24 negative bcl-2 showed one positive p53 immunostaining, four cases of bcl-2 negative showed two positive p53 staining, and the remaining 10 cases of bcl-2 negative showed grade three p53 immunoreactivity. Among the 9 bcl-2 positive cases, three instances showed grade one p53 positive immunoreaction, one case showed grade two, and the

remainig five cases revealed grade three p53 immunostaining.

## Discussion

The realization that apoptosis occurs in tumors is not new. More than 20 years ago it was suggested that apoptosis may account for much of the spontaneous cell loss known from kinetic studies to occur in many tumors[16, 18], and it has been clear for some time that its extent often is enhanced in tumors by well-established treatment modalities such as chemotherapy[4,8,9,32]

However, during the past few years, advances in understanding of the control of apoptosis at the molecular level have extended its potential oncologic significance far beyond the mere provision of a mechanistic explanation for tumor cell deletion.

Identification of apoptosis by light microscopy is difficult because cells undergoing the process may die individually and rapidly become phagocytized. Thus, some apoptotic cells may never be apparent in routine histologic sections, and early apoptotic nuclear changes are not distinguishable from lymphocytes and prophase mitotic cells by light microscopy alone. Despite these limitations, immunochemical methods can provide relevant estimations for the frequency and extent of the apoptotic process. Application of improved immunochemical techniques for identification of DNA double strand breaks in histologic sections has demonstrated that programmed cell death occurs spontaneously in a wide range of neoplasms[2, 25, 34].

The in situ identification of individual cells undergoing apoptosis is enabled by application of the recently described method of Gavrieli et al., identifying in situ DNA strand breaks by specific binding of terminal deoxynucleotidyl trnasferase(TdT) to exposed 3'-hydroxyl termini of DNA(Tunel method)[11]. Binding is followed by the TdT-catalyzed repetitive addition of several thousand normal or modified mononucleotides. This method permits the reliable quantification of DNA fragmentation in cells in suspension, as well as in tissue sections.

In this study we examined the occurrence of apoptosis in osteosarcoma patients who had or had not received chemotherapy. The rate of apoptosis and chemotherapy treatment were not correlated. Histologic grades, and the location of tumor in the long bones were also not correlated with the positive rates of apoptosis. Regarding the histologic subtype, there was a tendency that fibroblastic osteosarcomas exhibited more frequent apoptotic positive rates and chondroblastic osteosarcomas showed less frequent apoptosis. However, this was not statistically significant. There were trends of enhanced apoptosis in the case of positive necrosis. However, they were not statistically significant either. We also compared bcl-2 positivity and various other parameters. Histologic grade, presence of necrosis, and even positive rates of p53 were not correlated with positive rates of bcl-2.

A variety of anti-cancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cell populations, lymphoid tissues, and tumors[1,3,7,23]. The way in which anti-cancer drugs induce apoptosis is unknown.

Wild type p53 has two distinctive functions, inducing G1 arrest and apoptosis, particularly when cells are experiencing DNA damage[33]. Enhancement of apoptosis after induced expression of the p53 tumor suppressor gene is also evident[17]. Abnormali-

ties of the p53 tumor suppressor gene, ranging from complete deletion to point mutation, constitute some of the most frequently encountered genetic defects in human cancer[26]. Mutations of the p53 gene may have a close relationship with tumorigenesis in terms of apoptosis suppression[22,35]. In this study, we compared apoptosis positive rates with p53 positive rates. In cases of grade 3 positive wide type p53, enhanced apoptotic rates were recorded. This is statistically significant. In mutant type p53, the positive rates of apoptosis was variable and this is not statistically significant.

Bcl-2 is a 26 kD, putative membrane associated protein containing a hydrophobic carboxyl terminus that may locate it to the intracellular membranes while leaving the remainder of the protein in the cytosol[5,20]. Bcl-2 originally was proposed as a candidate proto-oncogene because of its location at a breakpoint in a chromosome translocation that occurs in a proportion of human B-cell lymphomas[20].

The involvement of Bcl-2 in p53 induced apoptosis was first suggested by the observation that p53 induced cell death can be prevented by Bcl-2 expression[37]. Miyashita et al[24]. reported that in p53 deficient mice, Bcl-2 expression is elevated accompanied by down regulation of Bax expression, particularly in the prostatic epithelium. These results have led to the hypothesis that wild type p53-dependent apoptosis occurs at least partly throught its suppressive effect on Bcl-2 expression or its function. In cases of bcl-2 negative, apoptosis positive rates tended to increase. However, it was not statistically significant. In cases which had not received any chemotherapy, there were less bcl-2 positive rates. There is evidence that stimulation of some cell lines by trophic cytokines

or increase in their level of expression of the bcl-2-proto-oncogene (the bcl-2 gene product inhibits apoptosis occurring in a variety of circumstances) can greatly increase their resistance to the apoptosis-inducing effect of anticancer drugs[6,10,21].

In the present study we have found that a significant proportion of osteosarcoma, a tumor in which resistance to chemotherapy often occurs, express high levels of wild type p53 and low levels of bcl-2.

This is the first report to study the occurrence of apoptosis in cases of osteosarcoma patients. · Enhanced occurrence of apoptosis was found in cases of wild type p53 +++ positive cases, and in the vincinity of necrosis. Also noted, increased apoptosis positive rates were found in cases of negative bcl-2 expression.

## REFERENCES

1) **Allan DJ, Harmon BV, Kerr JFR.** Cell death in spermatogenesis. In: Potten CS, editor. Perspectives on mammalian cell death. *Oxford:Oxford university Press*, 229-258, 1987.

2) **Arends MJ, Wyllie AH.** Apoptosis mechanism and roles in pathology. *Int Rev Exp Pathol* 32:223-254, 1991.

3) **Bansal N, Houle A, Melnykovych G.** Apoptosis Mode of cell death induced in T cell leukemia lines by dexamethasone and other agents. *FASEB J* 5:211-216, 1991.

4) **Bary MA, Behnke CA, Eastman A.** Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* 40:2353-2362, 1990.

5) **Chen-Levy Z, Cleary ML.** Membrane topology of the Bcl-2 proto-oncgnic protein demonstrated in vitro. *J Biol Chem* 25:4929-4933,1990.

6) **Collins MKL, Marvel J, Malde P, Lopez-Rivas A.** Interleukin 3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. *J Exp Med* 176:1043-1051, 1992.

7) **Collins RJ, Harmon BV, Souvlis T, Pope JH,**

Kerr JFR. Effects of cycloheximide on B-chrnoic lymphocytic leukemic and normal lymphocytes in vitro: induction of apoptosis. *Br J Cancer* 64:518-522, 1991.

8) **Cotter TG, Glynn JM, Echeverri F, Green DR.** The induction of apoptosis by chemotherapeutic ɐgents occurs in all phases of the cell cycle. *Anticancer Res* 12:773-780, 1992.

9) **Dive C, Hickman JA.** Drug-target interactions : only the first step in the commitment to a programmed cell death? *Br J Cancer* 64:192-196, 1991.

10) **Fisher TC, Milner AE, Gregory CD, Jackman AL, Aherne GW, Hartley JA, et al.** Bcl-2 modulation of apoptosis induced by anticancer drugs: Resistance to thymidylate stress is independent of classical resistance pathways. *Cancer Res* 53:3321-3326, 1993.

11) **Gavrieli Y, Ben, Sasson SA** Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493-501, 1992.

12) **Gobe GC, Axelsen RA, Searle JW.** Cellular events in experimental unilateral ischemic renal atrophy and in regeneration after contralateral nephrectomy. *Lab Invest* 63:770-779, 1990.

13) **Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ.** Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334-336, 1990.

14) **Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ.** Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* 88:6961-6965, 1991.

15) **Kerr JFR.** Shrinkage necrosis: A distinct mode of cellular death. *J Pathol* 105:13-20, 1971.

16) **Kerr JFR, Searle J.** A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain numerous mitotic figures. *J Pathol* 107:41-44, 1972.

17) **Kerr JFR, Winterford CM, Harmon BV.** Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 73:2013-2026, 1994.

18) **Kerr JFR, Wyllie AH, Curie AR.** Apoptosis: a basic biological phenomenon with wide-rainging implication in tissue kinetics. *Br J Cancer* 26:239-257, 1972.

19) **Kihara S, Shiraishi T, Nakagawa S, Toda K,** Tabuchi K. Visualization of DNA double strand breaks in the gerbil hippocampal CA1 following transient ischemia. *Neurosci Lett* 175:133-136, 1994.

20) **Korsmeyer SJ.** Bcl-2 initiates a new category of oncogenes:regulators of cell death. *Blood* 80:879-886, 1992.

21) **Lotem J, Sachs L.** Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor b1 and cancer chemotherapy compounds in myeloid leukemic cells. *Blooc* 80:1750-1757, 1992.

22) **Lowe SW, Ruley HE, Jacks T, Housman DE.** p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74:957-967, 1993.

23) **Martin SJ, Lennon SV, Bonham AM, Cotter TG.** Inductin of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J Immunol* 145:1859-1867, 1990.

24) **Miyashita T, Krajewski S, Krajewska M, et al.** Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 9;1799-1805, 1994.

25) **Moore JV. Death of cells and necrosis of tumors** In: Pottem CS. Perspective in Mammalian cell death. *Oxford: Oxford University press,*295-325, 1987.

26) **Oren M.** p53: the ultimate tumor suppressor gene? *FASEB J* 6:169-176, 1992.

27) **Raymond AK, Ayala AG.** Specimen management after osteosarcoma chemotherapy. *Contemp Issues Surg Pathol* 11:157-181, 1988.

28) **Rosen G, Marcove RC, Caparros B et al.** *Primary osteogenic sarcoma*: the rationale for preoperative chemotherapy and delayed surgery. Cancer 443:2163-2177, 1979.

29) **Sarraf CE, Bowen ID.** Proportions of mitotic and apoptotic cells in a range of untreated experimental tumors. *Cell Tissue Kinet* 21:45-49, 1988.

30) **Schiffer D, Cavalla P, Chio A, Giordana MT, Marino S, Mauro A, Migheli A.** Tumor cell proliferation and apoptosis in medulloblastoma. *Acta Neuropathol* 87:362-370, 1994.

31) **Searle J, CollinsDJ, Harmon B, Kerr JFR.** The spontaneous occurrence of apoptosis in squamous carcinomas of the uterine cervix. *Pathology* 5:163-169, 1973.

32) **Searle J, Lawson TA, Abott PJ, Harmon B, Kerr JFR.** An electron microscope study of the mode of

cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. *J Pathol* 116:129-138, 1975.

33) **Sherr CJ** G1 phase progression; *Cycling on cue. Cell* 79: 551-555, 1994.

34) **Shiraishi T.** Cell kinetic analysis of brain tumors using the monoclonal antibody Ki-67: in vitro and in situ study. *Acta Med Okayama* 43:187-201,1990.

35) **Tabuchi K, Fukuyama K, Mineta T, Oh-Uchida M, Hori K.** Alterned structure and expression of the p53 gene in human neuroepithelial tumors. *Neurol Med Chir* 32:725-732, 1992.

36) **Vauss DL, Cory S, Adams JM.** Bcl-2 gene pro-motes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440-442, 1988.

37) **Wang Y, Szekely L, Okan I, et al** Wild-type p53-triggered apoptosis is inhibited by bcl-2 in a V-myc-induced T-cell lymphoma line. *Oncogene* 8:3427-3431, 1993.

38) **Wyllie AH.** The biology of cell death in tumors. *Anticancer Res* 5:131-136, 1985.

39) **Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M.** Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 352:345-347, 1991.