대 한 골 관 절 종 양 학 회 지
J. of Korean Bone & Joint Tumor Soc.
Vol. 3, No. 1, March, 1997

Expression of the Multidrug Resistance Gene and its Product in Osteosarcomas of the Bone - Immunohistochemistry and In Situ Hybridization -

Hye-Rim Park, M.D. and Yong-Koo Park, M.D.*

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

-Abstract-

Resistance to combination chemotherapy remains challenge in the treatment of osteosarcoma. One of the mechanisms of multiple drug resistance is an increased expression of the multidrug resistance gene(mdr1). Expression of the P-glycoprotein(mdr-1 gene product) was studied immunohistochemically and the mdr-1 gene by in situ hybridization in 33 osteosarcomas relating to various prognostic factors. Thirty cases out of 33 osteosarcomas(90.9%) showed positive cytoplasmic reactions with P-glycoprotein and nineteen instances(57.6%) were strong positive(2+). The older(>20 years) and female patients revealed more intense immunohistochemical reactions rather than those of the younger and male patients. Osteoblastic and chondroblastic osteosarcomas revealed more strong immunohistochemical reactions compared to fibroblastic types. There were no significant staining differences between the type of bony involvement, Broder's grade and the presence of necrosis. On follow-up, the mean survival rate was decreased in the strong positive group, however, this was not statistically significant. In situ hybridization for mdr-1 gene revealed positive signals in 22 cases out of 29 osteosarcomas(75.9%). Chemotherapy was done in 15 cases out of 28 patients(53.6%). The results of immunohistochemistry and in situ hybridization were not correlated with the protocols for chemotherapy. However, this result should be confirmed by a larger scale study about mdr1 mRNA expression.

Key Words : P-glycoprotein, mdr gene, Immunohistochemistry, In situ hybridization, Osteosarcoma

※ correspondence author : Yong-Koo Park, M.D.,
Department of Pathology, Kyung Hee University Hospital, 1 Hoeki-dong, Dongdaemun-gu, Seoul, 130-702, KOREA

Introduction

Resistance to multiple chemotherapeutic agents is a common clinical problem in the treatment of cancer ; such resistance may occur in primary therapy or be acquired during treatment[2]. Drug resistance may be particularly important in the treatment of osteosarcomas. Despite an improvement in 5-year disease-free survival to 50-60% with chemotherapy, a substantial proportion of patients still have a systemic relapse, and most of these patients die. To improve treatment further, investigation of the mechanism of drug resistance is necessary[13].

The pattern of multiple drug resistance (MDR) involves cross-resistance to a wide range of structually and functionally unrelated lipophilic cytotoxic agents. Several of these drugs are used in the treatment of osteosarcoma, including Adriamycin. These multidrug-resistance cells contain an amplified gene, termed mdr1, that is transcribed into a 4.5 kilobase mRNA[2]. The protein product of this gene, a 170 kDa membrane protein(P-glycoprotein), functions as an energy-dependent drug efflux pump which helps to decrease the intracellular concentration of a wide variety of cytotoxic agents. Recently, intrinsic and acquired expression of the mdr1 gene has been detected in a variety of typically drug-resistant human tumors, which indicates that clinical MDR is likely to be important in human cancers[13].

We studied the expression of the P-glycoprotein immunohistochemically and mdr-1 gene by in situ hybridization in 33 osteosarcomas relating to various prognostic factors. The purpose of this study was to determine whether expression of P-glycoprotein and mdr-1 gene correlated with both chemotherapy and prognosis in osteosarcomas.

Materials and Methods

Osteosarcomas

Thirty three osteosarcomas of the bone 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, site, and information about chemotherapy and survival. All of the histologic slides were reviewed by two pathologists (HR Park and YK Park) and classified their histologic subtypes and Broder's histologic grading(grades Ⅰ-Ⅳ). According to the conventional histologic classification of osteosarcomas, 16 cases were osteoblastic types, 8 chondroblastic cases, and 9 fibroblastic cases. Presence of necrosis was also evaluated.

Immunohistochemistry

Antibody to P-glycoprotein(mdr-1 gene product, Immunotech, Marseille, France) was a mouse IgG monoclonal antibody. This monoclonal antibody reacts specifically with an extracellular epitope of the human 170 kDa P-glycoprotein(P-gp, P-170. mdr1 gene product).

Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method(DAKO LSAB kit, Carpinteria, CA). Briefly, 6-㎛ 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. The sections were exposed to the primary antibody(1:500 dilution) for 30 minutes at 37℃. After

washing with TRIS-buffered saline(DAKO, Carpinteria, CA), biotinylated link antibody was applied for 10 minutes followed by streptavidin peroxidase for an additional 10 minutes. Color development was performed with substrate-chromogen(3-amino-9-ethyl-carbazole) solution for 10 minutes.

Immunoreactions for P-glycoprotein were scored as follows : two positive(++), diffuse and strong positivity on tumor cells : one positive(+), focal and/or weak positivity on tumor cells : negative(-), tumor cells were negative.

In situ hybridization

Human mdr-1 oligonucleotide probe(Oncogene Science, Uniondale, NY, USA) is a 40 base single-stranded synthetic oligonucleotide. This sequence is of the antisense orientation and is derived from translated sequences near the 5′-end of the mdr-1 gene.

^{35}S-APT-labelled single-stranded antisense for cDNA was prepared with terminal deoxynucleotidyl transferase using a NEP-100 labelling kit(Du Pont, Boston, MA). ^{35}S-labelled probe was used for hybridization at a concentration of 5.6 kcpm/ml. Treatment of the slides and hybridization conditions were as previously described[11] with our modification. After hybridization, the section hybridized with mdr-1 were treated with 1M Tris, pH 8.0, 0.5M EDTA at 37℃ for 30 minutes. Sections were washed twice in 2X SSC and once in 1X SSC and once in 0.5X SSC. They were dehydrated in graduated ethanol and dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water. The dipped slides were placed in a well ventilated area for 4 hours to dry at room temperature, and were exposed at 4℃ in dessicated slide boxes. The exposed slides were developed in a D-19 developer for 5 minutes at room temperature, fixed in a fixative for 5 minutes and washed with water for 20 minutes. They were counter stained with Hemato-xylin-Eosin.

The positive signal was regared as more than 6 crowed grains on the nucleus compared to the background.

Statistics

Wilcoxon scores(rank sums) test and Kruskal-Wallis test were carried out to study the difference in the MDR gene product positivity between clinico-pathological factors. Kaplan-Meier(Product-limit) test was used for the survival estimates and Log-rank test and likelihood ratio test were used for the difference in the survival function estimates.

Results

The monoclonal antibody P-glycoprotein (mdr-1 gene product) showed diffuse granular positive immunoreactions in osteosarcoma cells. The staining reactions were cytoplasmic in all the instances with focal nuclear staining. The proportion and distribution of positive staining cells were variable from tumor to tumor but diffuse pattern in general. P-glycoprotein was focally noted in non-neoplastic osteoblasts and osteocytes of mature bony trabeculae. Thirty cases out of 33 osteosarcomas(90.9%) showed positive reactions with P-glycoprotein and three (9.1%) were negative. In nineteen tumors (57.6%), more than half of the tumor cells were strong positive(2+).

There was no significant different staining pattern between the histologic subtypes. The classical osteoblastic osteosarcomas revealed

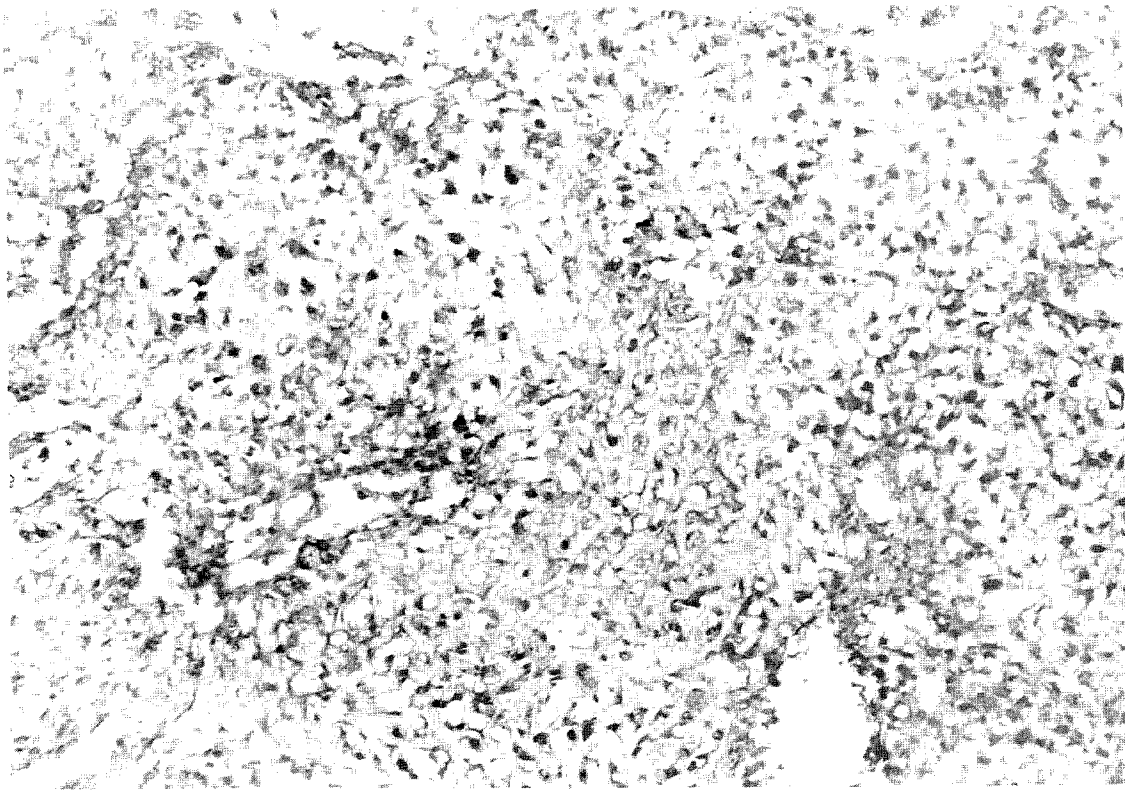

Fig. 1. Osteoblastic osteosarcoma showing intense cytoplasmic positive reaction with P-glycoprotein. Tumor osteoid is negative (× 100, ABC for P-glycoprotein).

Table 1. Expression of the mdr-1 gene product according to the patient age

Age	MDR gene expression			Total(%)
	0	1+	2+	
≤20 years	2(6.6)	8(26.6)	10(33.3)	20(66.6)
>20 years	1(3.3)	2(6.6)	7(23.3)	10(33.3)
Total	3(10.0)	10(33.3)	17(56.6)	30(100)

Missing = 3

diffuse positive staining in tumor cells. Adjoining osteoid and necrotic areas were negative(Fig. 1). In chondroblastic osteosarcomas, there was a tendency for intense stainability in the cellular, peripheral areas of chondroid lobules. The cases of chondroblastic type revealed patchy distribution of positive tumor cells and negative chondroid matrix(Fig. 2). In fibroblastic osteosarcomas, the spindle tumor cells as well as multinucleated giant cells were positive.

According to the patient age, the older patients(>20 years) revealed more 2+ cases(70%) than the younger patients(table 1). According to the patient sex, the female patients revealed more 2+ cases(71.4%) than the male patients(Table 2). As far as histologic type is concerned, osteoblastic and chondroblastic types revealed more 2+ cases(68.7% and 62.5%) than fibroblastic types(Table 3). However, these tendencies had no significant statistical differences. The staining patten revealed no difference according to the type of involved bone, Broder's grade and the presence of necrosis.

In situ hybridization for mdr-1 probe

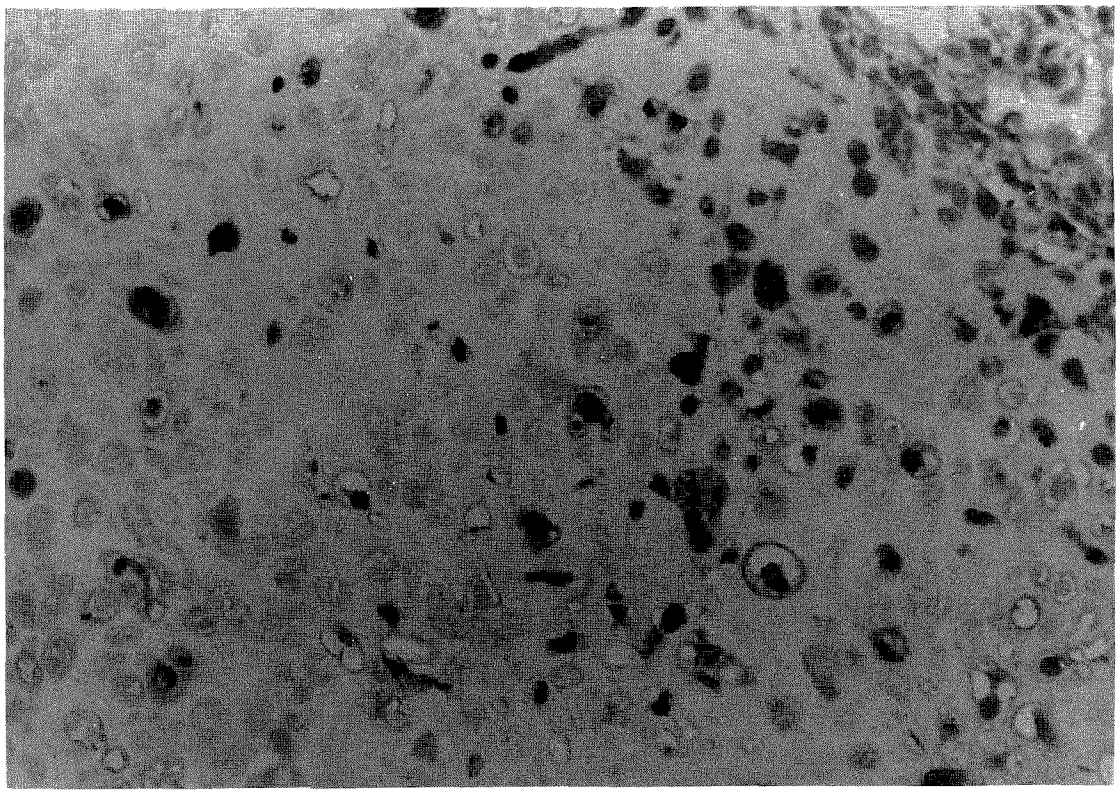

Fig. 2. Chondroblastic osteosarcoma showing patchy positivity. Some tumor cells with chondroid differentiation are negative (×200, ABC for P-glycoprotein).

Table 2. Expression of the mdr-1 gene product according to the patient sex

Sex	MDR gene expression			Total(%)
	0	1+	2+	
Female	1(3.3)	3(10.0)	10(33.3)	14(46.6
Male	2(6.6)	7(23.3)	7(23.3)	16(53.3)
Total	3(10.0)	10(33.3)	17(56.6)	30(100)

Missing = 3

Table 3. Expression of the mdr-1 gene product according to the histologic type

Type	MDR gene expression			Total(%)
	0	1+	2+	
Osteoblastic	0(0.0)	5(15.1)	11(33.3)	16(48.4)
Chondroblastic	2(6.0)	1(3.0)	5(15.1)	8(24.2)
Fibroblastic	1(3.0)	5(15.1)	3(9.0)	9(27.2)
Total	3(9.0)	11(33.3)	19(57.5)	33(100)

revealed positive signals in 22 cases out of 29 osteosarcomas(75.9%). The osteoblastic and fibroblastic osteosarcomas revealed diffuse positive signals on tumor cells(Fig. 3). In chondroblastic osteosarcomas, the peripheral cellular areas of spindle-shaped tumor cells revealed patchy positive signals. The tumor cells in central chondroid lobules were negative. These patterns on in situ hybridization in chondroblastic types were correlated with the staining patterns on immunohistochemistry. Seven cases(24.1%) were negative for mdr-1 signal, which were composed of necrotic area, thick osteoid and bony trabeculae. All three negative cases on immunohistochemistry revealed negativity on in situ hybridization.

Remaining four cases with negativity on

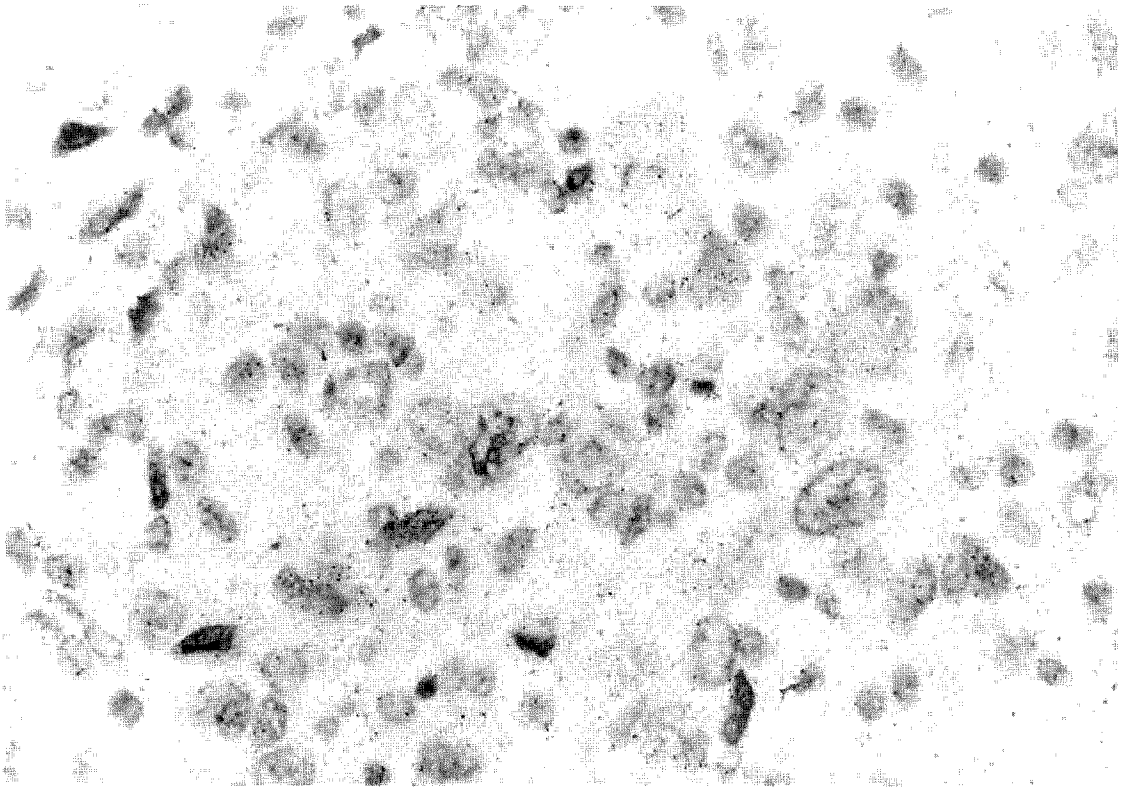

Fig. 3. In situ hybridization for mdr-1 gene reveals positive signals on most tumor nuclei (×400).

in situ hybridization were weak positive on immunohistochemistry.

Chemotherapy was done in 15 cases out of 28 patients(53.6%). Protocols for chemotherapy were MTX-A-CDDP(methotrexate, adriamycin, cis-platinum) in 6 cases, MTX-A-CDDP-BCD(MTX-A-CDDP plus bleomycin, cyclophosphamide, actinomycin-D) in 2 cases, MTX in 2 cases, and other protocols in 5 cases. The results of immunohistochemistry and in situ hybridization were not related with the protocols for chemotherapy. Among the patients receiving chemotherapy, 6 patients had had surgical procedure prior to the chemotherapy. Among these 6 patients, four were negative signal on in situ hybridization and three of them were alive on follow-up. Other two patient were positive with both immunohistochemistry and in situ hybridization. All the three patients receiving chemotherapy previously were strong positive on both in situ hybridization and immunohistochemistry.

Follow-up was available for 25 patients and the mean follow-up period was 60 months. Twelve patients were alive and thirteen were dead. According to the product-limit survival estimates, the patients with negative staining revealed 72 months of mean survival. The 1+ group of P-glycoprotein revealed 40.3±4.7 months of mean survival and the 2+ group 36.0±8.3 months. However, this result was not statistically significant. Ten patients were dead in 13 patients without chemotherapy(76.9%). Eight patients were alive in 15 patients with chemotherapy(53.3%). However, the mean survival according to the chemotherapy were

not significantly different.

Discussion

The resistance of tumor cells to chemotherapeutic drugs is a major obstacle to successful cancer chemotherapy. Multiple drug resistance (MDR) is one important mechanism through which tumor cells can become resistance to combination chemotherapy. High levels of expression of the mdr1 gene have been shown to be necessary and sufficient to confer the MDR phenotype in cell lines and transfection studies[13]. At present, there is no data to indicate the minimum level of mdr1 expression that might be expected to be clinically relevant[13].

In human cells, expression of the mdr1 gene, encoding a transmembrane efflux pump (P-glycoprotein), leads to decreased intracellular accumulation and resistance to a variety of lipophilic drugs (multidrug resistance : MDR)[5,9]. P-glycoprotein is expressed in many different types of tumors in humans[2,4,10] and in certain normal organs[2,12]. mdr1 expression has been frequently observed in human tumors after chemotherapy and in some but not all types of clinically refractory tumors untreated with chemotherapeutic drugs[9].

Drug resistance may be particularly important in the treatment of osteosarcomas. We studied expression of the P-glycoprotein (mdr-1 gene product) immunohistochemically and mdr-1 gene by in situ hybridization. Thirty cases out of 33 osteosarcomas (90.9%) showed positive cytoplasmic reactions with P-glycoprotein and in situ hybridization for mdr-1 gene revealed positive signals in 22 out of 29 cases (75.9%). Four cases revealed discrepant results, weak positivity on immunohistochemistry and negativity on in situ hybridization. They were composed of necrotic area, thick osteoid and bony trabeculae. Gerlach et al.[3] had described multidrug resistance cell lines overexpress P-glycoprotein often although not always, in conjuction with gene amplification.

In chondroblastic types, the tumor cells in the peripheral cellular areas were strong positive and the central chondroid lobules were negative or weak positive. This finding suggests that the peripheral spindle-shaped tumor cells may be main neoplastic components.

To study the problem of multidrug resistance, several laboratories have isolated cell lines resistant to the vinca alkaloids, doxorubicin (adriamycin), actinomycin D, and related agents[2]. In vitro selection for cells that are resistant to a single cytotoxic agent often yields cells that are simultaneously resistant to multiple toxic drugs. Several of these drugs are used in osteosarcoma protocols, including Adriamycin, which is generally regarded as the most effective agent in osteosarcoma treatment. Our protocols included adriamycin in 12 out of 15 chemotherapy patients and our results of mdr-1 gene product were not related with the protocols for chemotherapy.

All the three patients receiving chemotherapy previously were strong positive for mdr-1 gene and P-glycoprotein. In contrast, two out of 6 patients receiving surgery prior to chemotherapy were also positive for both. So, mdr-1 gene and P-glycoprotein was found in patients who had not received prior chemotherapy; this suggests that at least some osteosarcomas have intrinsic drug resistance[13]. Gerlach et al.[3] reported that tumors from six of 25 variable sarcoma patients displayed elevated levels of P-glyco-

protein by immunoblotting. Three of them exhibiting P-glycoprotein had not previously been exposed to chemotherapy, implying that overexpression of this marker and possible concomitant multidrug resistance may not depend only on selection during prior drug treatments. Resistance may be present at the initiation of chemotherapy or may develop during the course of a chemotherapy program, and can extend to structurally and functionally unrelated drugs. Lonn et al.[6,7] found that 4 out of 20 breast cancer patients with clinical stage-Ⅳ disease receiving endocrine treatment developed multiple gene copies of the mdr-1 gene in fine needle biopsied specimen by polymerase chain reaction. It also suggests that the event occurs without cytotoxic selection of cells with chemotherapy.

Follow-up data revealed the decreased mean survival rate in the strong positive group. These results suggest that the drug resistance in osteosarcoma may be related with the expression of mdr-1 gene and its strong expression may be a poor prognostic variable. Wunder et al.[13] detected various levels of mdr1 expression in 18 osteosarcoma specimens using the polymerase chain reaction and suggested that a high level of mdr1 expression may be a negative prognostic indicator in osteosarcoma, being associated with a poor response to treatment and an increased risk of relapse.

Chan et al.[1] revealed detectable P-glycoprotein appears to be an important adverse prognostic factor in children with soft tissue sarcoma and consistent absence of the protein is associated with a favorable prognosis using a semiquantitative immunohistochemical procedure. Weinstein et al.[12] detected P-glycoprotein immunohistochemically in 65 of 95 primary colon adenocarcinomas and reported an association of P-glycoprotein in colon carcinomas with enhancement of local tumor aggressiveness. however, Mayer et al.[8] reported that P-glycoprotein in colon carcinomas with enhancement of local tumor aggressiveness. However, Mayer et al.[8] reported that P-glycoprotein expression had no influence on survival in colorectal cancers. Our study was difficult to determine the precise prognositic implication due to a small number of patients and variable protocols for chemotherapy. We could confirm some possible role of mdr-1 gene and P-glycoprotein in osteosarcomas, however, this finding must be supported by a larger scale study with standardized chemotherapeutic protocols about mdr1 mRNA expression in osteosarcoma.

REFERENCES

1. Chan HSL, Thorner PS, Haddad G, Ling V : Immunohistochemical detection of P-glycoprotein: Prognositic correlation in soft tissue sarcoma of childhood. J Clin Oncol, 8:689-704, 1990.
2. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I : Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA, 84:265-9, 1987.
3. Gerlach JH, Bell DR, Karakousis C, Slocum HK, Kartner N, Rustum TM, Ling V, Baker RM : P-glycoprotein in human sarcoma: Evidence for multidrug resistance. J Clin Oncol, 5:1452-60, 1987.
4. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I : Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst, 81:116-24, 1989.
5. Juranka PF, Zastawny RL, Ling V : P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB J, 3: 2583-92, 1989.
6. Lonn U, Lonn S, Nylen U, Stenkvist B : Appearance and detection of multiple copies of the mdr-1

gene in clinical samples of mammary carcinoma. Int J Cancer, 51:682-6, 1992.
7. Lonn U, Lonn S, Stenkvist B : Reduced occurrence of mdr-1 amplification in stage-Ⅳ breast-cancer patients treated with tamoxifen compared with other endocine treatments. Int J Cancer, 53:574-8, 1993.
8. Mayer A, Takimoto M, Fritz E, Schellander G, Kofler K, Ludwig H : The prognostic significance of Proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer, 71:2454-60, 1993.
9. Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Audrulis IL, Gazdar AFm Willmann CL, Griffith B, von Hoff DD, Roninson IB : Quantitative analysis of mdr1(multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc Natl Acad Sci USA, 87:7160-4, 1990.
10. Nooter K, Herweijer H : Multidrug resistance(mdr) genes in human cancer. Br J Cancer, 63:663-9, 1991.
11. Simmons DM, Arriza JL, Swanson LW : A complete protocol for in situ hybridization of messenger RNAs in brain and other tissue with radiolabeled single-stranded RNA probes. J Histotechnol, 12:169-81, 1989.
12. Weinstein RS, Jakate SM, Dominguez JM, Lebovitz MD, Koukoulis GK, Kuszak JR, Klusens LF, Grogan TM, Saclarides TJ, Roninson IB, Coon JS : Relationship of the expression of the multidrug resistance gene product(P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. Cancer Res, 51:2720-6, 1991.
13. Wunder JS, Bell RS, Wold L, Andrulis IL : Expression of the multidrug resistance gene in osteosarcoma: A pilot study. J Orthop Res, 11:396-403, 1993.