Expression of Molecular Markers in the Tumor and Survival Prognosis in Osteosarcoma

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Immunohistochemical study of p53, VEGF, Flt-1/VEGFR1 Ab-1, EGFR, HER-2/neu, Bax, and Cox-2 expression in osteosarcomas was carried out in 40 patients aged 16-70 years. Expression of p53 was detected in 27.5% tumors, VEGF in 15%, Flt-1/VEGFR1 Ab-1 in 97.5%, EGFR in 52.5%, HER-2/neu in 32.5%, Bax in 77.8%, and Cox-2 in 32.3% tumors. Multifactorial analysis showed that the expression of HER-2/neu (p=0.004), p53 (p=0.01), and Cox-2 (p=0.04) in osteosarcomas significantly correlated with unfavorable prognosis for overall survival, while HER-2/neu (p=0.02) and Cox-2 (p=0.003) with relapse-free survival. Analysis of HER-2/neu, p53, and Cox-2 expression in the primary tumor should be taken into consideration in the treatment of patients with osteosarcoma.

Key Words: *osteosarcoma; expression; p53; VEGF; Flt-1/VEGFR1 Ab-1; EGFR; HER-2/neu*

Tissue, cellular, or molecular markers characterize the basic properties of tumor cells: unlimited proliferation, active neoangiogenesis and apoptosis, invasion, liability to metastatic growth [1,3,7]. Chemical method is assumed to be the most suitable for studies of protein expression in tumor tissues, including osteosarcoma tissue [4,5], and for better understanding of some stages in tumor development. The expression of Bax [8,14], Bcl-2 [11], HER-2/neu [7,12], p53 [7,14], VEGF and VEGF-R [6,9,13], and Cox-2 [10,15] proteins attracts special interest of scientists in immunohistochemical studies of bone tumors. The expression of ErbB-2 [12], VEGF [6], and Cox-2 [15] in the primary tumor is associated with poor survival prognosis for osteosarcoma patients. Published data are contradictory and necessitate further studies. The attitude of some authors to practical use of these parameters in cancer patients is skeptical because of a trend to overall adjuvant chemotherapy in this disease. On the other hand, two practical results can be expected from these studies: possibility of detecting the patients at a high risk of early relapses and/or metastases in need of adjuvant therapy or more thorough observation and evaluation of tumor sensitivity to certain therapies and creation of individual adjuvant treatment protocols for patients with disseminated process. One of the recent important practical results of studies of the molecular biology of tumors is the development of new drugs with target effects on these molecules, blocking the processes regulated by these molecules [2].

We carried out an immunohistochemical analysis of the expression of molecular markers (p53, VEGF, Flt-1/VEGFR1 Ab-1, EGFR, HER-2/neu, Bax, Cox-2) in osteosarcoma and of their relationship with the main clinical morphological characteristics of the disease and prognosis.

MATERIALS AND METHODS

Immunohistochemical studies were carried out in osteosarcomas of 40 patients (21 women and 19 men) aged 16-70 years. The osteoblastic (25 cases) and chondroblastic osteosarcomas (7 cases) predominated; telangiectatic variant was detected in 1 case. Parosteal

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osteosarcoma was detected in 5 cases, osteosarcoma (secondary) against the background of Paget disease in 1, and well-differentiated central osteosarcoma in 1 case.

Primary tumors were located in the femoral (21 case), tibial (7 cases), fibular (5 cases), humeral (4 cases) bones and in the ulnar and ileac bones and the rib (1 case each).

Poorly differentiated (G3) osteosarcomas predominated (33 cases); well-differentiated (G1) osteosarcomas were found in 7 cases.

The patients were distributed with consideration for the disease stage by the TNM system: T1N0M0 (*n*=4), T2N0M0 (*n*=22), T2N0M1 (*n*=2), T2N0M1a (*n*=2), T2N0M1b (*n*=3), T3N0M0 (*n*=2), T3N0M1 (*n*=1), T3N0M1a (*n*=3), and T3N0M1b (*n*=1).

Distribution of patients with osteosarcoma by clinical classification was as follows: 4 patients with stage IB, 3 with stage IIA, 19 with stage IIB, 2 with stage III, 8 with stage IVA, and 4 with stage IVB.

Morphological studies of the tumors was carried out using standard equipment and reagent kits, systems for immunohistochemical preparations staining on an Autostainer Plus Link (Dako) universal automated system by the streptavidin biotin peroxidase method using monoclonal antibodies (Table 1).

The parameters of expression of molecular biological markers in the control group were analyzed in 5 tissue specimens with foci of new bone in ossifying myositis.

The expression of the studied antigens (p53, VEGF, Flt-1/VEGFR1 Ab-1, EGFR, HER-2/neu, Bax, Cox-2) was studied by immunohistochemical method in specimens of osteosarcoma tissue obtained by biopsy or surgical removal of the primary tumor. Tissue specimens were fixed in 10% buffered formalin for 48 h and decalcinated by the standard method in 20% formic acid and 10% formalin. The material was processed in an automated mode in an Axcelsior vacuum processor (Thermo Scientific). After histological processing the material was embedded in paraffin and 3-4-μ sections were prepared. Special highly adhesive Dako slides were used in studies of tumor specimens. A pre-treatment modulus (PT-Link, Dako) for simultaneous deparaffination and rehydration of the sections and antigen decamouflage was used for manipulations with the sections. Endogenous peroxidase blocking, incubation with the first antibodies, visualizing reagent, and chromogen solution were carried out in an Autostainer Plus Link (Dako). The sections were poststained by hematoxylin and embedded in balm or synthetic medium by the standard methods.

The reaction in the cells of primary malignant osteosarcomas was evaluated by the semiquantitative method (by staining intensity and number of antigen-positive cells). The specific staining type, depending on the reaction product location in the cell (cytoplasmic, membrane, nuclear, mixed), was evaluated for each antigen.

The intensity of cytoplasmic reaction and number of stained cells were evaluated for Cox-2, Bax, VEGF, Flt-1/VEGFR1 Ab-1, and EGFR. The reaction in tumor cells was evaluated by the semiquantitative method by staining intensity and number of antigen-positive cells. Immunohistochemical reaction was evaluated as negative ("-": no reaction) and positive (>10% cells with the reaction of medium ("++") and high ("+++") staining intensity). Slightly positive immunohistochemical reaction ("+": <10% stained cells) and stromal reaction were neglected. The results of reactions with the antigens were expressed in percent as the number of stained cells per 100 examined tumor cells in 5-10 representative visual fields.

The results of reaction with the antigen located in the nucleus (p53) were expressed in percent as the number of stained nuclei per 100 examined tumor cells in 5-10 representative visual fields. The expression of p53 was considered negative if less than 10% tumor cells were stained and positive if the number of antigen-positive cell nuclei in the primary tumor reached 10%.

TABLE 1. Characteristics of Antibodies Used in the Study

Antigen	Antibody, clone	Firm	Working dilution	Protein function
p53	Mouse mAb DO-7	Dako	RTU	Cell cycle and apoptosis regulator
VEGF	Mouse mAb VG1	Dako	1:25	Angiogenesis stimulator
Flt-1/VEGFR1 Ab-1	Rabbit pAb	Thermo Scientific	1:25	Angiogenesis stimulator receptor
EGFR	Mouse mAb H11	Dako	1:200	Receptor tyrosine kinase
HER-2/neu	Rabbit pAb	Dako	1:550	HER-2/neu receptor
Bax	Rabbit pAb	Dako	1:50	Apoptosis stimulator
Cox-2	Mouse mAb	Dako	RTU	Arachidonic acid metabolism enzyme

For HER-2/neu protein the immunohistochemical reaction was considered positive only if tumor cell cytoplasm and membranes were stained (slight "+" and moderate "++").

RESULTS

No relationship between the incidence and levels of expression of molecular markers p53, VEGF, Flt-1/VEGFR1 Ab-1, EGFR, HER-2/neu, Bax, and Cox-2 in osteosarcomas and the main clinical morphological characteristics of the disease, such as patient's gender, tumor differentiation degree and location in skeletal bones, presence of distant metastases (M) was detected. No relationship between the expression of p53, VEGF, Flt-1/VEGFR1 Ab-1, HER-2/neu and patient's age and the tumor T factor was detected. On the other hand, EGFR(+), Cox-2(+), and Bax(+) osteosarcomas were significantly more incident in young patients. It is noteworthy that 21 patients with EGFR(+) osteosarcomsas were significantly younger than 19 patients with EGFR(-) tumors (28.7±3.1 and 42.2±4.7 years, respectively; p=0.02). The 11 patients with Cox-2(-) tumors were significantly older than patients with Cox-2(-) ones (45.5±6.3 and 31.7±3.4 years, respectively; p=0.04). The age of patients with Bax(-) osteosarcoma was 48.0±8.8 years, of those with Bax expression<50% 34.8±4.1 years, and of those with the expression of Bax≥50% 30.0±4.7 years (p=0.03). In addition, significant differences in the incidence of Cox-2(-) osteosarcomas with consideration for the T factor were detected (p=0.009; Table 2).

The expression of Bax reaching ≥50% (56.5%; 13 cases of 23) was significantly more incident in osteoblastic osteosarcomas than in other histological variants. The incidence of Bax(-) tumors was significantly (p=0.04) higher in patients with T3 tumors (57.1% cases). It is noteworthy that the expression of these markers was not detected in any of the control samples.

The expression of molecular markers in osteosarcomas is presented in Figs. 1-3.

The expression of p53 was studied in 40 osteosarcomsa and detected in 27.5% tumors. Overall and relapse-free survival medians were almost 2-fold lower in patients with p53(+) osteosarcoma. Analysis of overall survival curves of osteosarcoma patients has shown no relationship between the analyzed parameter and this protein expression in the tumor (Table 2). On the other hand, significant differences in relapse-free survival of patients with p53(+) and p53(-) osteosarcomas were detected (p=0.03; Table 3).

Hence, the expression of p53 in osteosarcoma should be regarded as an unfavorable predictor of early relapse.

The expression of EGFR, VEGF, Flt-1/VEGFR1 Ab-1 was studied in 40 osteosarcomas and found in 52.5, 15, and 97.5% tumors, respectively. No significant differences in overall and relapse-free survival of osteosarcoma patients with tumors expressing EGFR, VEGF, and Flt-1/VEGFR1 Ab-1 were detected.

The expression of Bax was studied in 36 osteosarcomas and detected in 77.8% tumors. A trend to differences in overall survival (p=0.05) in patients with different levels of Bax expression in osteosarcomas was detected. Relapse-free survival of osteosarcoma patients did not depend on Bax expression in the tumor (p=0.35; Table 3). The most unfavorable overall survival values were detected in osteosarcoma patients with Bax expression ≥50%.

The expression of Cox-2 was studied in 34 osteosarcomas and found in 32.3% tumors. The impact of Cox-2 expression levels in osteosarcomas for disease prediction remained not quite clear in unifactorial

TABLE 2. Expression of Cox-2 in Osteosarcomas with Consideration for T and M Factors

Parameter	N	Expression of Cox-2			
		Cox-2(-)		Cox-2(+)	
		abs.	%	abs.	%
T1	3	—	—	3	100
T2	25	6	24.0	19	76.0
T3	6	5	83.3	1	16.7
M0	23	5	21.7	11	78.3
M1a	8	4	50.0	4	50.0
M1b	3	2	66.7	1	33.3

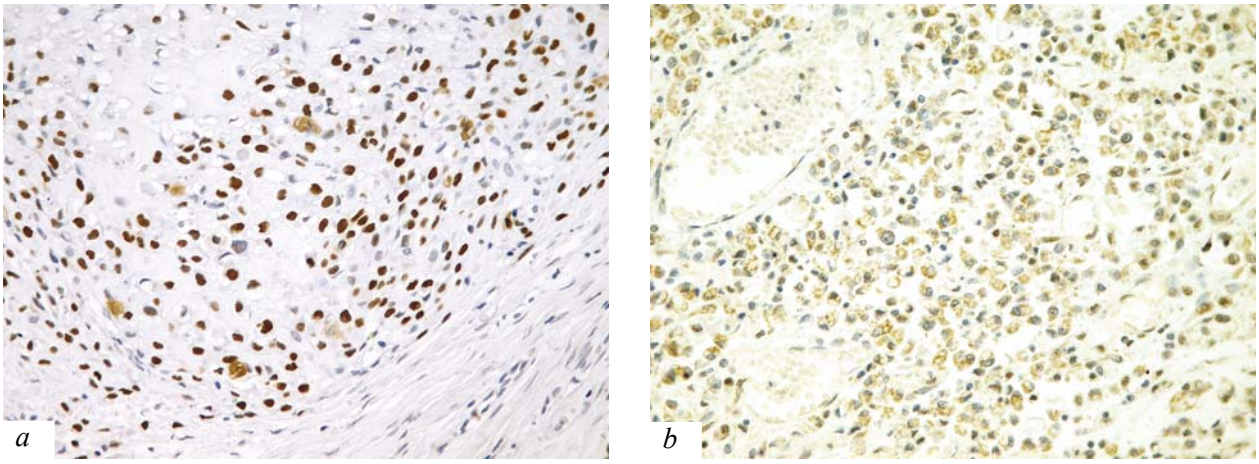

Fig. 1. Patient B., 19 years. Central classical osteosarcoma of the right humeral bone, grade 3 (chondroblastic variant). Fragment of the tumor with predominant chondrodifferentiation of atypical cells. *a*) positive nuclear expression of p53 in tumor cells. Immunohistochemical reaction, ×100; *b*) immunohistochemical reaction with HER-2/neu. Positive cytoplasmic and membrane staining of tumor cells, ×100.

analysis. No relationship between overall survival of osteosarcoma patients and the expression of Cox-2 in the tumor (p=0.9) was detected. However, relapse-free survival values were slightly better in the group of patients with Cox-2(+) tumors (p=0.3). The 3-year relapse-free survival was 37.3±11.9% in the group of 24 patients with Cox-2 (median 17.2 months) and 0% in the group of 11 patients without Cox-2 expression in

TABLE 3. Remote Results of Treatment of Osteosarcoma Patients and Tumor Expression of p53, Bax, and HER-2/neu

Expression	N	Survival, %			
		median, months	1 year	3 years	5 years
Overall survival (p=0.14)					
p53(-)	36	48.0	87.0±6.1	70.9±8.8	33.6±11.8
p53(+)	12	26.8	66.7±15.7	50.0±18.6	0
Relapse-free survival (p=0.03)					
p53(-)	35	28.2	77.8±7.6	47.3±10.3	47.3±10.3
p53(+)	12	12.3	54.3±17.6	0	0
Overall survival (p=0.05)					
Bax(-)	9	Not achieved	85.7±13.2	68.6±18.6	68.6±18.6
Bax <50%	17	47.9	94.1±5.7	78.4±11.2	24.5±14.4
Bax ≥50%	16	35.2	62.2±13.4	51.9±14.6	13.0±11.8
Relapse-free survival (p=0.35)					
Bax(-)	9	15.0	77.8±13.9	23.3±19.0	23.3±19.0
Bax <50%	17	17.5	80.2±10.4	48.1±13.9	24.5±14.4
Bax ≥50%	15	14.6	61.4±13.8	26.3±12.9	13.0±11.8
Overall survival (p=0.01)					
Her-2/neu(-)	27	49.3	92.6±5.0	78.7±8.5	45.8±11.6
Her-2/neu(+)	12	21.7	65.6±14.0	32.8±15.1	0
Relapse-free survival (p=0.03)					
Her-2/neu(-)	26	31.3	73.1±8.6	47.6±11.1	47.6±11.1
Her-2/neu(+)	11	8.7	54.5±15.0	0	

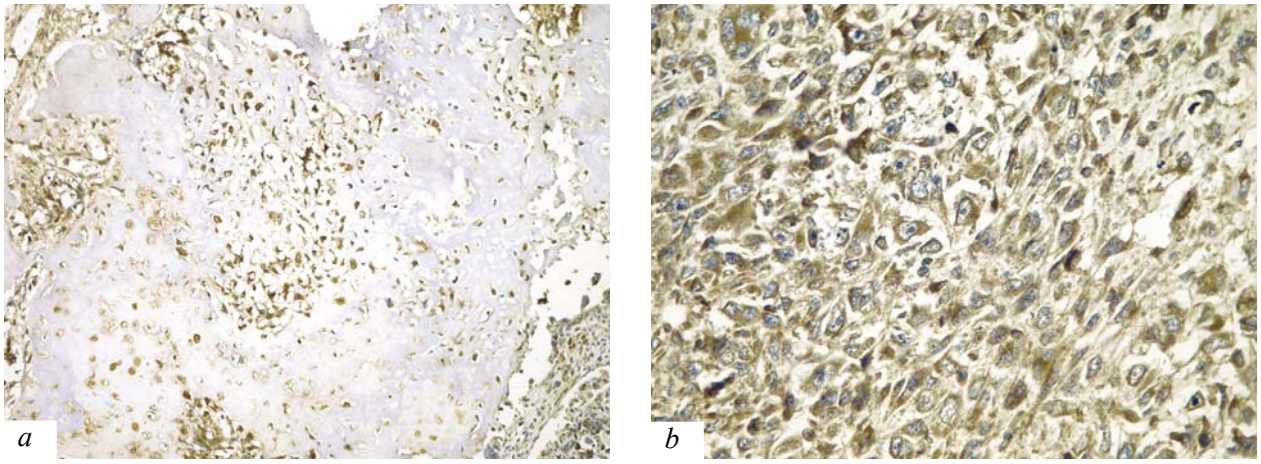

Fig. 2. Patient (female) N., 18 years. Central classical osteosarcoma of the right scapula, grade 3 (chondroblastic variant). *a*) positive cytoplasmic staining of tumor cells. Immunohistochemical reaction with VEGF, ×100; *b*) lobular structure of the tumor with islets of atypical cells with chondroid differentiation. Positive immunohistochemical staining for Flt-1/VEGFR1 Ab-1, ×100.

the tumor (median 10.4 months). However, these differences were seen only after 2 years of observation.

The expression of HER-2/neu was studied in 40 osteosarcomas and detected in 32.5% tumors. Overall survival of patients with HER-2/neu(-) and HER-2/neu(+) tumors differed significantly (p=0.01). The presence of this marker in osteosarcomas should be considered as a prognostically unfavorable factor. Similar results were found in the analysis of relapse-free survival (p=0.007; Table 3).

The results of multifactorial analysis indicate that the expression of HER-2/neu (p=0.004), p53 (p=0.011), and Cox-2 (p=0.04) in the tumor should be considered as independent factors of overall sur-

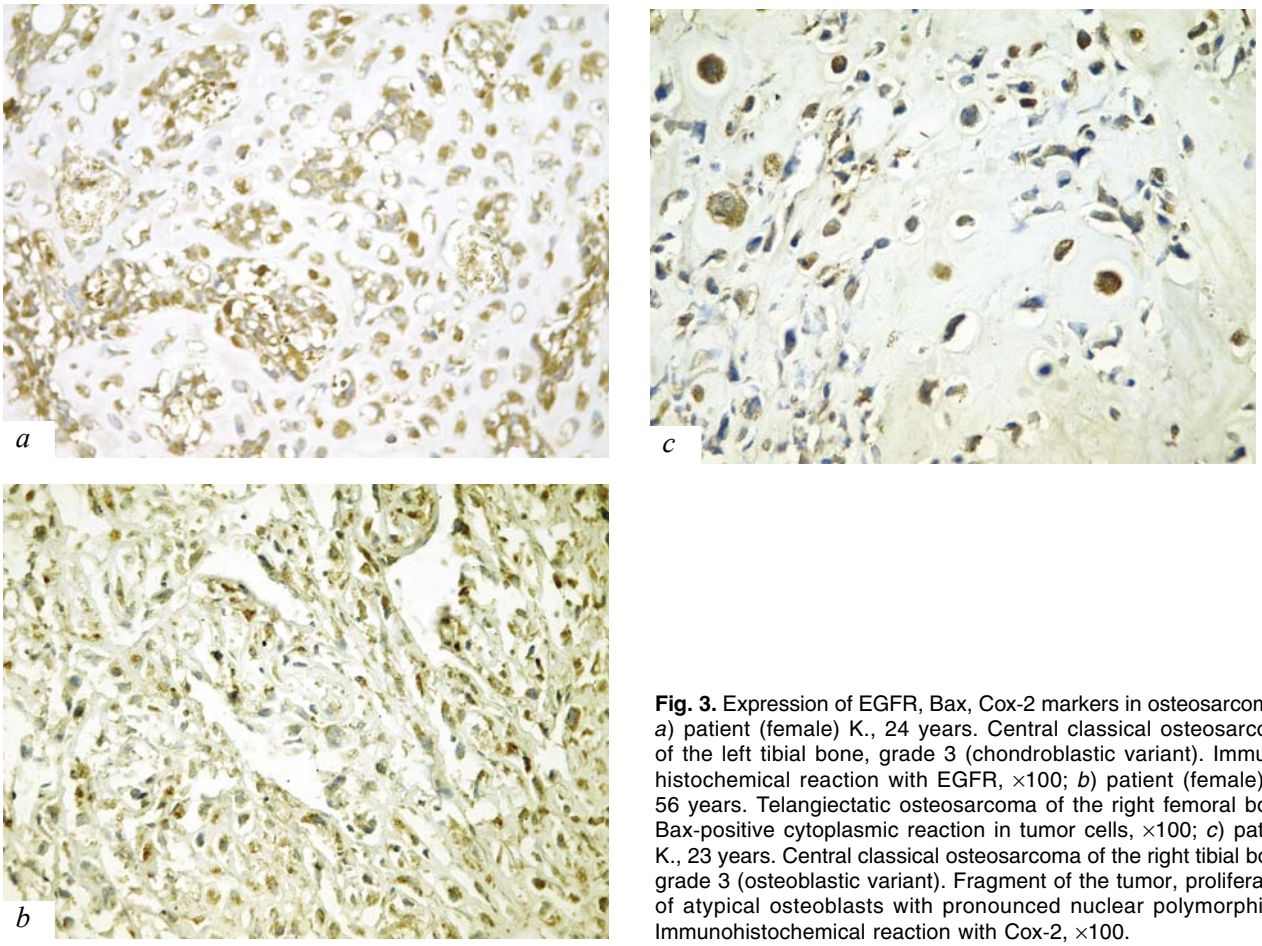

Fig. 3. Expression of EGFR, Bax, Cox-2 markers in osteosarcomas. *a*) patient (female) K., 24 years. Central classical osteosarcoma of the left tibial bone, grade 3 (chondroblastic variant). Immunohistochemical reaction with EGFR, ×100; *b*) patient (female) T., 56 years. Telangiectatic osteosarcoma of the right femoral bone. Bax-positive cytoplasmic reaction in tumor cells, ×100; *c*) patient K., 23 years. Central classical osteosarcoma of the right tibial bone, grade 3 (osteoblastic variant). Fragment of the tumor, proliferation of atypical osteoblasts with pronounced nuclear polymorphism. Immunohistochemical reaction with Cox-2, ×100.

vival prognosis for patients with osteosarcoma, while the expression of HER-2/neu (p=0.02) and Cox-2 (p=0.003) is significantly associated with relapse-free survival. By these data, a group of osteosarcoma patients with unfavorable prognosis can be distinguished, who need thorough observation and more aggressive treatment.

Hence, the expression of p53 was detected in 27.5% osteosarcomas, VEGF in 15%, Flt-1/VEGFR1 Ab-1 in 97.5%, EGFR in 52.5%, HER-2/neu in 32.5%, Bax in 77.8%, and Cox-2 in 32.3% cases. Analysis of the results of immunohistochemical study of the molecular markers expression in osteosarcomas revealed their relationships with the main clinical morphological characteristics of the disease (patient's gender, tumor differentiation degree and location in skeletal bones, presence of distant metastases, M factor). No relationships between the expression of p53, VEGF, Flt-1/VEGFR1 Ab-1, HER-2/neu with patient's age and T factor were detected. On the other hand, zero expression of Bax (-) and Cox-2 (-) was significantly more incident (p=0.04) in cases with greater dissemination of tumor process (higher T factor). The expression of EGFR (p=0.02), Cox-2 (p=0.04), and high expression of Bax (p=0.03) in osteosarcomas were significantly more incident in young patients. Unifactorial analysis has shown a relationship between worse relapse-free survival of patients with p53 (p=0.03) and HER-2/neu (p=0.07) expression and between overall survival and HER-2/neu expression (p=0.01). Multifactorial analysis has detected a relationship between the expression of HER-2/neu (p=0.004), p53 (p=0.01), and Cox-2 (p=0.04) in osteosarcomas and the overall survival prognosis and between HER-2/neu (p=0.02) and Cox-2 (p=0.003) expression and relapse-free survival. Hence, analysis of the expression of HER-2/neu, p53, and Cox-2 in the primary tumor should be taken into consideration in the treatment of patients with osteosarcoma.

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