

Evaluation of P-Glycoprotein, HER-2/ErbB-2, p53, and Bcl-2 in Primary Tumor and Metachronous Lung Metastases in Patients with High-Grade Osteosarcoma

Stefano Ferrari, M.D.¹
 Franco Bertoni, M.D.²
 Licciana Zanella, M.D.²
 Elisabetta Setola, M.D.¹
 Patrizia Bacchini, M.D.²
 Marco Alberghini, M.D.²
 Michela Versari, M.A.¹
 Gaetano Bacci, M.D.¹

¹ Chemotherapy Service, Department of Musculoskeletal Oncology, Istituti Ortopedici Rizzoli, Bologna, Italy.

² Pathology Service, Department of Musculoskeletal Oncology, Istituti Ortopedici Rizzoli, Bologna, Italy.

Address for reprints: Stefano Ferrari, M.D., Chemotherapy Service, Department of Musculoskeletal Oncology, Istituti Ortopedici Rizzoli, Via Pupilli 1, 40136 Bologna, Italy; Fax: (011) 39 051 6366 277; E-mail: stefano.ferrari@ior.it

Received October 20, 2003; revision received January 8, 2004; accepted January 22, 2004.

BACKGROUND. Investigation of the relation between primary tumor and metastatic disease is necessary for the identification of predictive factors for postrecurrence survival (PRS) in patients with recurrent osteosarcoma.

METHODS. Cellular levels of P-glycoprotein, ErbB-2, p53, and Bcl-2 expression were evaluated in primary tumor biopsy and metachronous pulmonary metastasis specimens from 19 patients with high-grade osteosarcoma. Results were analyzed for differences between primary tumor and pulmonary metastases and for correlations between expression patterns and survival.

RESULTS. Positive staining in lung metastases was noted in 68%, 53%, 32%, and 84% of patients for P-glycoprotein, ErbB-2, p53, and Bcl-2, respectively. These percentages were higher than those observed in primary tumor specimens for all genetic markers evaluated, with a significant difference in the percentage of patients with positive staining for P-glycoprotein (68% vs. 32%; $P = 0.05$) and a near-significant difference in the percentage of patients with positive staining for Bcl-2 (84% vs. 53%; $P = 0.08$). Patients with ErbB-2 expression in the primary tumor were more likely to have multiple metastases and shorter recurrence-free intervals compared with patients in whom ErbB-2 expression was not observed, whereas differences in P-glycoprotein, p53, and Bcl-2 expression were not related to differences in metastatic pattern. PRS was influenced by p53 expression levels in pulmonary metastases, with patients who had negative staining for p53 having a significantly better PRS rate relative to patients with positive staining for p53 (3-year PRS rate: p53-negative, 64%; p53-positive, 17%; $P = 0.008$).

CONCLUSIONS. In the current study of patients with high-grade osteosarcoma, most patients exhibited increased cellular expression of P-glycoprotein, ErbB-2, and Bcl-2 in recurrent pulmonary metastases compared with primary tumor. Further studies aimed at investigating the relation between altered p53 expression in lung metastases and postrecurrence survival are recommended. *Cancer* 2004;100:1936–42. © 2004 American Cancer Society.

KEYWORDS: osteosarcoma, metastasis, P-glycoprotein, HER-2/ErbB-2, p53, Bcl-2.

Chemotherapy has markedly improved the prognosis for patients with high-grade osteosarcoma, but despite receiving aggressive first-line treatment, approximately 30% of patients without overt metastatic disease at presentation have subsequent distant recurrences, usually in the form of lung metastases.¹

The metastatic pattern greatly influences postrecurrence survival; survival outcomes are more favorable for patients who experience recurrence following a long recurrence-free interval and for patients

with few pulmonary nodules.²⁻⁴ Osteosarcoma metastases originate from highly chemoresistant neoplastic clones, and because no effective second-line chemotherapy protocols are available at present, surgical removal of metastatic disease is crucial.²⁻⁶

To develop more effective strategies for the treatment of patients with recurrences from primary osteosarcoma, it is important to better understand the relation between primary tumor and metastatic disease. Evaluation of cellular levels of P-glycoprotein, ErbB-2, p53, and Bcl-2 in primary tumor and in metachronous pulmonary metastases could provide additional information regarding metastatic behavior in patients with high-grade osteosarcoma.

P-glycoprotein, a product of the multidrug resistance 1 gene (*MDR1*), is involved in processes that are responsible for the resistance of neoplastic cells to several agents,⁷ including doxorubicin, which currently is used in the treatment of osteosarcoma. Conflicting data regarding the significance of P-glycoprotein overexpression in the primary tumors of patients with osteosarcoma have been reported.⁸⁻¹⁰

The *c-erbB2* protooncogene encodes a protein that is structurally homologous to the epidermal growth factor receptor.¹¹ Overexpression of human *c-erbB2* has been shown to induce the malignant transformation of rodent fibroblasts.¹² Expression of the ErbB-2 protein is associated with a poorer prognosis in patients with nonmetastatic osteosarcoma,^{9,13,14} and the loss of ErbB-2 expression has been reported in metachronous pulmonary metastases from osteosarcoma.¹⁵

The *p53* tumor suppressor gene plays a key role in the suppression of abnormal cell proliferation by acting as a G1 cell cycle checkpoint control for DNA damage, and this gene also plays a critical role in apoptotic processes.¹⁶ The *p53* gene often is altered by mutations that inhibit its tumor suppressor activities, and through these mutations, *p53* can instead function as a tumor promoter.¹⁷ Alterations in *p53* are known to occur in approximately 20% of osteosarcomas.¹⁸⁻²⁰ Similar figures were reported for localized osteosarcoma and for osteosarcoma that was metastatic at the time of diagnosis.²⁰ In a previous study, no correlation was found between p53 expression and either histologic necrosis after preoperative chemotherapy or event-free survival in patients with nonmetastatic osteosarcoma.⁹

The Bcl-2 family of proteins, along with a number of complex mechanisms involving the receptorial and transcriptional triggering of these proteins, regulates various steps in apoptosis. Some members of this family, including Bcl-2, block cell death, whereas other members, such as Bax, promote apoptosis.²¹ An im-

munohistochemical study found moderate-to-strong expression of Bcl-2 in 81% of osteosarcoma cases analyzed.²² The significance of Bcl-2 expression differs in different tumor types: negativity with respect to Bcl-2 expression was associated with a poorer clinical outcome in patients with metastatic breast carcinoma²³; increased Bcl-2 expression in metastases relative to the primary tumor was observed in patients with clear cell renal carcinoma²⁴; and elevated expression of Bcl-2 was reported in unaffected tumor areas following treatment in patients with metastatic malignant melanoma.²⁵

We have performed a retrospective analysis of patients with recurrent disease in the form of lung metastases from high-grade osteosarcoma of the extremity. The aims of the current study were to investigate the expression of P-glycoprotein, ErbB-2, p53, and Bcl-2 in primary tumors and metachronous pulmonary metastases from these patients and to search for possible correlations of cellular expression with pulmonary metastatic pattern and postrecurrence survival.

MATERIALS AND METHODS

Patients with diagnoses of nonmetastatic osteosarcoma of the extremity who were treated between July 1995 and March 1999 were identified using the database of the Tumor Center and the Chemotherapy Service at the Istituti Ortopedici Rizzoli (IOR; Bologna, Italy).

Patients whose first recurrences involved metastases to the lung only and who underwent resection without receiving preoperative chemotherapy were included in the study. Biopsy specimens of primary tumors and lung metastases were obtained from our institution's pathology archives.

During the time period covered by the current study, two protocols for the treatment of nonmetastatic osteosarcoma of the extremity had been activated sequentially at our institution. The first was the Pilot-IOR protocol, which ran from July 1995 to February 1997, and the second was the Italian Sarcoma Group/Scandinavian Sarcoma Group 1 (ISG/SSG 1) protocol, which was activated in March 1997. The details of these two protocols are reported elsewhere.^{26,27} The eligibility criteria for these protocols were as follows: biopsy proven, classic, high-grade osteosarcoma of the extremity; patient age < 40 years; absence of metastases as confirmed by bone scintigraphy and computed tomography scanning of the lungs; an interval of < 1 month between biopsy and the start of chemotherapy; normal renal and hepatic function; and no previous chemotherapy or surgical treatment for the bone lesion. The treatment strategy

involved primary chemotherapy treatment, resection of the primary tumor, and postoperative chemotherapy. Both protocols called for chemotherapy with high-dose methotrexate, cisplatin, doxorubicin, and high-dose ifosfamide. The only difference between the two protocols was the cumulative doxorubicin dose (420 mg/m² in the Pilot-IOR protocol vs. 330 mg/m² in the ISG/SSG 1 protocol). Following chemotherapy, patients were monitored with computed tomography scans of the lung, which were performed every 3 months during the first 3 years after chemotherapy, every 4 months during the fourth and fifth years, and every 6 months thereafter.

Immunohistochemical Analysis

Sections were cut to 5 μ m thickness, deparaffinized, and rehydrated before being immunostained with the following antibodies: rabbit anti-human c-ErbB-2 oncoprotein (1:200 dilution; Dako, Glostrup, Denmark), monoclonal mouse anti-human p53 protein (clone D07, 1:100 dilution; Dako), monoclonal mouse anti-P-glycoprotein (clone C494, 1:200 dilution; Dako), and mouse anti-human Bcl-2 oncoprotein (clone 124, prediluted; Dako). Detection of primary antibodies was performed using a Dako autostainer in conjunction with a streptavidin-biotin alkaline phosphatase/red/rabbit/mouse detection system (for P-glycoprotein and Bcl-2) or a streptavidin-biotin peroxidase/diaminobenzidine/rabbit/mouse detection system (for p53 and ErbB-2) after pressure cooking of slides in citrate buffer. Slides were counterstained with hematoxylin and then dehydrated and coverslipped. Positive and negative controls were included in each experimental run.

Two pathologists independently reviewed the slides and were in agreement regarding the extent of immunohistochemical staining. Staining for P-glycoprotein and HER-2/ErbB-2 was observed in the cell membrane, whereas p53 staining was localized to the nucleus. For P-glycoprotein, HER-2/ErbB-2, and p53, cases were scored as 0 (no staining), 1+ (positive staining in 1–25% of cells), 2+ (positive staining in 26–50% of cells), 3+ (positive staining in 51–75% of cells), or 4+ (positive staining in 76–100% of cells); scores \geq 2+ were considered to be indicative of positive staining. For Bcl-2, any detectable staining was considered to be indicative of positivity.

For each patient, *accordance* with respect to a given marker was defined by the presence of the same expression pattern (positive or negative) in the primary tumor sample and the metastatic sample (including all nodules for patients with \geq 2 metastases). *Lack of accordance* was defined by the presence of different expression patterns in the primary tumor

sample and the metastatic sample or by the presence of both negative and positive staining (*mixed expression*) in lung metastasis samples.

Statistical Methods

The StatView statistical package (Version 4.5; Abacus Concepts, Berkeley, CA) was used in the statistical analysis of immunohistochemical data. Differences between groups were evaluated using the Fisher exact test or the chi-square test, as well as the Student *t* test when appropriate. Postrecurrence survival was calculated from the date of recurrence until death or most recent follow-up. Survival curves were calculated according to the method of Kaplan and Meier and compared using the log-rank test.

RESULTS

Of the 115 patients treated during the study period, 33 (28.7%) had their first recurrence in the lung, and 19 (57.5%) of these 33 fulfilled the inclusion criteria for the current analysis. Patient characteristics and data on pattern of first recurrence are summarized in Table 1. Ten patients had only one lung metastasis, three patients had two metastatic nodules, and three patients had three nodules. The remaining 3 patients had 4, 9, and 17 pulmonary nodules, respectively. After a median follow-up of 41 months (range, 24–53 months), 8 patients were alive, yielding an estimated 3-year postrecurrence survival rate of 49%.

Overall, 19 primary biopsy samples and 55 metastasis samples were evaluated immunohistochemically. Immunohistochemical staining results are summarized in Table 2. Positive immunostaining for P-glycoprotein was observed in 33 metastatic nodules (61%), whereas 14 (27%), 10 (19%), and 31 metastatic nodules (57%) exhibited positive staining for ErbB-2, p53, and Bcl-2, respectively.

Rates of accordance between primary and metastatic lesions are presented in Figure 1. With regard to P-glycoprotein, 5 of the 13 patients who had negative staining in the primary tumor also had negative staining in all metastasis samples; of the remaining 8 patients, 5 had positive staining in all metastasis samples, and 3 had mixed staining. Of the six patients with positive staining in the primary tumor, two also had positive staining in all metastasis samples; of the remaining four patients, one and three had negative and mixed staining, respectively, in their metastasis samples. Thus, overall, 7 (37%) of 19 patients exhibited accordance with respect to P-glycoprotein staining.

ErbB-2 accordance was noted in 8 patients (42%). Of the 13 patients who had negative staining in the primary tumor sample, 7 also had negative staining in all metastasis samples; of the remaining 6 patients, 4

TABLE 1
Patient Characteristics

Characteristic	
Age (yrs)	
Median	15
Range	6-34
Gender (%)	
Male	14 (74)
Female	5 (26)
Primary tumor	
Site (%)	
Femur	11 (58)
Tibia	6 (32)
Humerus	1 (5)
Other	1 (5)
Histology (%)	
Osteoblastic	12 (63)
Chondroblastic	4 (21)
Fibroblastic	2 (10.5)
Hemorrhagic	1 (5.5)
Surgery (%)	
Resection	18 (94.5)
Amputation	1 (5.5)
Lung metastases	
Recurrence-free interval (mos)	
Median	22
Range	13-67
Total no. of metastases	55
No. of metastases per patient	
Median	1
Range	1-17
Laterality (%)	
Unilateral	14 (74)
Bilateral	5 (26)

TABLE 2
Data on Immunohistochemical Staining in 19 Primary Tumor Samples and in Metachronous Lung Metastasis Samples

Marker	No. with positive staining (%)		P value ^a
	Primary tumor	Lung metastases	
P-glycoprotein	6 (32)	13 (68)	0.05
ErbB-2	6 (32)	10 (53)	0.32
p53	4 (21)	6 (32)	0.71
Bcl-2	10 (53)	16 (84)	0.08

^a Fisher exact test.

and 2 had positive and mixed staining, respectively, in their lung metastasis samples. Of the six patients with positive staining in the primary tumor sample, one had positive staining in all metastasis samples; two of the remaining five patients had negative staining in their lung metastasis samples, and three had mixed staining.

All four patients with p53-positive biopsy samples also had p53-positive metastatic nodules. Thirteen pa-

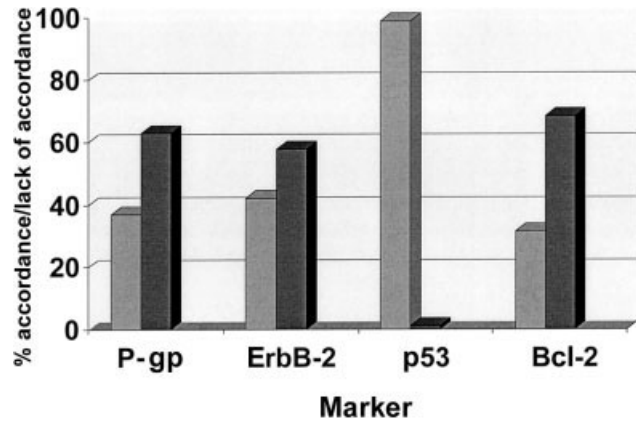


FIGURE 1. Immunohistochemical accordance between primary and metastatic osteosarcoma lesions. Light bars: accordance; dark bars: lack of accordance; P-gp: P-glycoprotein.

tients had negative staining in the primary tumor and all lung metastases. A p53-positive nodule was found in one patient who had negative staining in the primary tumor, and mixed expression was observed in the metastasis samples from another patient who had a p53-negative primary tumor. The accordance rate for p53 staining was 89%.

Six patients (31.5%) exhibited accordance with respect to Bcl-2 staining (negative staining in 1 patient and positive staining in 5 patients). Lack of accordance was observed in the remaining 13 patients (68.5%; negative primary tumor/positive metastases in 5 patients, positive primary tumor/negative metastases in 2 patients, and mixed metastatic expression in 6 patients).

Overall, 68% of patients (13 of 19) had positive staining for P-glycoprotein in at least 1 pulmonary nodule, 53% (10 of 19) had positive staining for ErbB-2 in at least 1 nodule, 32% (6 of 19) had positive staining for p53 in at least 1 nodule, and 84% (16 of 19) had positive staining for Bcl-2 in at least 1 nodule. These percentages were greater than those observed in primary tumor samples for all genetic markers evaluated, with a statistically significant difference with respect to P-glycoprotein staining (68% vs. 32%; $P = 0.05$) and a near-significant difference with respect to Bcl-2 staining (84% vs. 53%; $P = 0.08$) (Table 2).

Analysis of immunohistochemical staining of the primary tumor in relation to the number of lung metastases (Table 3) and the recurrence-free interval (Table 4) indicated that patients with ErbB-2-positive primary tumors more commonly had multiple metastases and shorter recurrence-free intervals compared with those who had ErbB-2-negative primary tumors. Differences in the expression of P-glycopro-

TABLE 3
Relation between Primary Tumor Immunohistochemical Data and Number of Lung Metastases

Immunohistochemical staining	No. of patients			P value
	Total	No. of lung metastases		
		1 (%)	≥ 2 (%)	
P-glycoprotein				0.87
Positive	6	3 (50)	3 (50)	
Negative	13	7 (54)	6 (46)	
ErbB-2				0.027
Positive	6	1 (17)	5 (83)	
Negative	13	9 (69)	4 (31)	
p53				0.3
Positive	4	3 (75)	1 (25)	
Negative	15	7 (47)	8 (53)	
Bcl-2				0.49
Positive	10	6 (60)	4 (40)	
Negative	9	4 (44)	5 (56)	

TABLE 4
Relation between Primary Tumor Immunohistochemical Data and Recurrence-Free Interval

	No. of patients	RFI (mos) ^a	P value
P-glycoprotein			0.98
Positive	6	27.3 ± 20	
Negative	13	27.1 ± 13	
ErbB-2			0.047
Positive	6	17.2 ± 2	
Negative	13	31.8 ± 16	
p53			0.57
Positive	4	23.2 ± 3	
Negative	15	28.2 ± 17	
Bcl-2			0.9
Positive	10	27.5 ± 15	
Negative	9	26.9 ± 16	

RFI: recurrence-free interval.
^a Mean ± standard deviation.

tein, p53, and Bcl-2 were not related to differences in metastatic pattern.

The probability of postrecurrence survival according to the expression of each genetic marker in lung nodules also was calculated (Table 5). Patients with negative p53 staining in lung metastases were found to have a significantly better postrecurrence survival rate compared with patients who had positive p53 staining in lung metastases (3-year postrecurrence survival rate: 64% [negative staining] vs. 17% [positive staining]; *P* = 0.008). For all other markers, there was no difference in postrecurrence survival between patients with positive staining and patients with negative staining in lung nodules.

TABLE 5
Relation between Lung Metastasis Immunohistochemical Data and Postrecurrence Survival

	No. of patients	3 yr PRS (%)	P value
P-glycoprotein			0.69
Positive	13	46	
Negative	6	62	
ErbB-2			0.9
Positive	10	48	
Negative	9	53	
p53			0.008
Positive	6	17	
Negative	13	64	
Bcl-2			0.51
Positive	16	51	
Negative	3	33	

PRS: postrecurrence survival.

DISCUSSION

In the current study, we examined the cellular expression of several genetic markers to explore the relation between primary tumor and lung metastases in patients with recurrent osteosarcoma. The study population included patients who achieved complete surgical remission and experienced recurrence in the lung only. To avoid possible confounding effects on immunohistochemical staining results, patients who received second-line chemotherapy before surgical treatment of lung nodules were excluded from the analysis. It is known that approximately 20% of patients with osteosarcoma experience recurrences at locations other than the lung and that > 30% of patients with metachronous metastases from osteosarcoma do not achieve complete surgical remission.²⁻⁵ Although the current study population consists of a selected subgroup of patients with a favorable prognosis and thus cannot be considered representative of the entire population of patients with recurrent osteosarcoma, some suggestions do arise from the data that were obtained.

Most patients had different expression profiles in lung metastases compared with the primary tumor (63% for P-glycoprotein, 58% for ErbB-2, and 67.5% for Bcl-2); however, primary tumor and lung metastasis specimens exhibited accordance with respect to p53 staining in 17 of 19 patients (89%). For the three markers for which a majority of patients had discordant expression profiles, and particularly for P-glycoprotein and Bcl-2, lack of accordance typically was attributable to gained expression in lung metastases. Thirty-two percent of patients had P-glycoprotein-positive primary tumors, whereas 68% had P-glycoprotein-positive metastases (*P* = 0.05). Similarly, 53% of pa-

tients had primary tumors that exhibited positive staining for Bcl-2, whereas 84% had metastases that were positive for expression of this marker ($P = 0.08$).

The significantly higher rate of P-glycoprotein expression in lung metastases recalls the well known phenomenon of chemoresistance in recurrent osteosarcoma^{3,4,6} and is consistent with data indicating that P-glycoprotein expression is a negative prognostic factor for patients with osteosarcoma.^{8,10} At the same time, this finding contradicts observations (made in osteosarcoma cell lines and animal models) indicating that osteosarcoma cells expressing P-glycoprotein have a less aggressive phenotype.²⁸ Regarding Bcl-2, increased expression in metastases relative to the primary tumor has been reported in patients with renal carcinoma,²⁴ and it has been found that expression of this marker in metastases may be related to resistance to systemic treatment and disease progression in patients with metastatic malignant melanoma.²⁵

All 4 patients with p53-positive primary tumors also had p53-positive metastatic lesions. In contrast, only 2 of the 15 patients with p53-negative primary tumors had p53-positive metastases; nonetheless, this result is not entirely consistent with the findings of Gokgoz et al.,²⁰ who concluded that p53 alterations occur before the development of metastases, rather than at a late stage of osteosarcoma progression. ErbB-2 expression was more common in pulmonary metastases than in primary tumors, with 6 of the 13 patients who had ErbB-2-negative primary tumors (46%) having at least 1 ErbB-2-positive lung nodule; nonetheless, the difference between primary tumors and lung metastases in terms of ErbB-2 expression was not statistically significant. These findings are in disagreement with those reported by Akatsuka et al.,¹⁵ who noted a loss of ErbB-2 expression in pulmonary osteosarcoma metastases, and they appear to support the idea that the *c-erbB2* protooncogene plays a role in the development of osteosarcoma metastases.^{9,13,14}

For P-glycoprotein, ErbB-2, and Bcl-2, mixed expression was observed in lung metastases in approximately two-thirds of all patients with multiple metastatic nodules. This observation reflects the biologic diversity of these metastases and their varied clonal origins²⁹; furthermore, from a clinical point of view, this finding may explain the observed variation in terms of response to chemotherapy in the study conducted by Nachman et al.³⁰

It is noteworthy that in the current study population, only cellular expression of ErbB-2 influenced the pattern of recurrence, as patients with ErbB-2-positive primary tumors had a shorter recurrence-free interval and an increased likelihood of multiple metastases. These findings confirm that HER-2/ErbB-2 plays a role

in metastatic processes in osteosarcoma. In other studies,^{9,13,14} it has been reported that patients exhibiting overexpression of ErbB-2 in primary tumor have a lower probability of event-free survival compared with patients whose primary tumors do not overexpress this marker. Based on the data obtained in the current study, it appears that when patients with positive ErbB-2 staining in the primary tumor experience recurrence, these patients tend to have a shorter recurrence-free interval and multiple lung metastases; this pattern of recurrence reflects the increased biologic aggressiveness of the malignancy.^{2,3,4}

The data obtained in the current study were analyzed for possible correlations with postrecurrence survival, and it was found that of the four markers investigated, only p53 exhibited a significant association with postrecurrence prognosis. Five of the 6 patients with positive staining for p53 in at least 1 pulmonary nodule died of disease, compared with 6 of the 13 patients with negative staining in all lung nodules (3-year postrecurrence survival rate: 17% vs. 64%; $P = 0.008$). These data appear to indicate that even if p53 alterations are not late events in osteosarcoma progression and instead occur before the development of metastases,²⁰ such alterations probably affect the biologic behavior of these metastases. It is noteworthy that in a previous study of patients with osteosarcoma, expression of p53 in the primary tumor was not found to be correlated with the event-free survival probability⁹; in contrast, the findings of the current study suggest that p53 expression, which appears not to have prognostic significance with respect to event-free survival for patients without overt metastases, does possess predictive value in terms of postrecurrence survival for patients with detectable metastases.

Although the current study involved a selected subgroup of patients, based on the data that were obtained, we can conclude that for most patients with pulmonary osteosarcoma metastases, the expression profiles of P-glycoprotein, ErbB-2, and Bcl-2 are different in these metastatic lesions compared with the primary tumor; these differences more often are attributable to gained, rather than lost, expression in the lung metastases. The current study also confirms that ErbB-2 plays a role in the metastatic process in osteosarcoma. Further studies are recommended to investigate the relation between p53 alterations in metastases and postrecurrence survival probability.

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