5

Overexpression of the HER-2 oncogene does not play a role in high-grade Osteosarcomas

Jakob K. Anninga, Marc J. van de Vijver, Anne-Marie Cleton-Jansen, Petra M.P. Kristel, Antoni H.M. Taminiau, Marianne Nooij, R. Maarten Egeler and Pancras C.W. Hogendoorn

Eur J Cancer 2004; 40: 963-70

ABSTRACT

Purpose

The aim of our study was to determine whether or not expression of the tyrosine kinase receptor HER2 (also known as ErbB2/Her2/*neu*) is overexpressed in human osteosarcomas. We studied 15 biopsy and 18 resection specimens at the mRNA and protein levels.

Patients and methods

The HER2 status in the osteosarcoma specimens was assessed by immunohistochemistry (IHC) and quantitative Real-Time Polymerase Chain Reaction (PCR). In moderately immunopositive cases, fluorescent *in situ* hybridization (FISH) analysis was used in order to identify any possible gene amplification.

Results

Twenty-seven samples were evaluable for IHC, and only one case showed a moderately positive membrane staining. The remaining samples showed no staining or focal cytoplasmatic staining (2 samples). In the moderately positive case, FISH analysis showed no *HER-2* gene amplification. There was also no overexpression of HER2 mRNA, suggesting this sample was a false positive immunostain. HER2 mRNA expression was present in all samples at a similar level to that in the breast cancer cell line MCF7, which does not overexpress HER2 and was used a negative control.

Conclusion

This study shows that HER2 mRNA or membranous HER2 protein overexpression is absent in human osteosarcoma. We noted various inconsistencies in previous published studies, with regard to methodology and the interpretation of the results based on poor methodology. We therefore conclude that the positive data with regard to HER2 overexpression reported in these previous studies are not reliable. Our results suggest that the monoclonal antibody trastuzumab (Herceptin), directed against the HER2-receptor, is not likely to be an effective therapeutic agent in osteosarcoma.

INTRODUCTION

Osteosarcoma (OS) is the most common primary bone tumour, with an incidence of (on average) 6.5 patients per 10^6 children and 2.1 patients per 10^6 per year in adults. The peak incidence is between 10 and 19 years, and when it occurs after 40 years, it is usually associated with a pre-existing condition (1). Metastatic disease, large tumor volume, older age at presentation, axial site of the tumour, histological subtype of OS and histological response on preoperative treatment all have been associated with poor outcome (2-4). However, apart from metastatic disease and axial site, that occur in 10-20% of the cases, none of the other factors have been reliable enough to distinguish between high and low risk groups at diagnosis (2, 3). Chemotherapy induced tumour cell necrosis can be assessed only after surgery. Consequently, there is clearly a need to identify new predictive factors at time of diagnosis (2).

With the recent progress in the understanding of molecular biology of cancer, the cell surface receptor HER2 (also called $p185^{HER2}$) has suggested to be predictive for survival (5-7).

The *HER-2* gene (also known as ERBB2 or *neu* gene), located on chromosome 17q21 (8) encodes for a 185kD transmembrane receptor (9) and belongs to the epidermal growth factor (EGF) tyrosine kinase receptor superfamily (10). *HER-2* is an oncogene, and HER2 overexpression in vitro (11) and in human cancers, particularly in 25-30% of breast cancer patients, has been associated with disease behavior (12).

Overexpression of the normal HER2 receptor at the cellular membrane, above a critical level, results in cellular transformation and malignant cell proliferation in athymic mice (13). This oncogenic effect can be reverted by the use of monoclonal antibodies, directed against the HER2 protein (14). Furthermore, both in vitro and clinical studies have reported increased response rates to chemotherapeutic drugs when these are combined with anti-HER2 antibodies (15). Based on these results, and reports that *HER-2* is overexpressed in osteosarcoma, phase II trials have begun to study the efficacy of Herceptin® (trastuzumab), the commercial designation of humanized HER2 monoclonal antibody, in patients with relapsed or refractory osteosarcoma (6, 16) (www.cancer.gov/clinical_trials: MSKCC-99097/NCI-T98-0083 and COG-AOST0121).

Four studies have suggested that *HER-2* is overexpressed in osteosarcoma, however they report different correlations between *HER-2* overexpression and prognosis (5-7, 17). Furthermore, other investigators have not been able to confirm their conclusions (16, 18, 19). In order to clarify these conflicting results and to investigate whether trastuzumab is a suitable therapy in osteosarcoma, we studied the expression of the *HER-2* gene by assessing gene amplification, mRNA- and protein expressions of HER2 in 30 patients.

PATIENTS AND METHODS

Patients

All patients presented to the Department of Orthopedic Surgery of the Leiden University Medical Center with newly diagnosed high-grade osteosarcoma of the limbs (n=32) and the os ileum (n=1) from 1991 to 1999 (Table 1). Diagnosis was made on routine haematoxilin-eosin (HE) staining in 15 pre-treatment Yamshidi core needle biopsy specimens (group A) and in 18 resection specimens (group B) of the primary (n=12) or relapsed (n=6) tumour. If eligible, patients were offered participation in running European Osteosarcoma Intergroup (EOI) studies, such as Europeran Organization for Research and Treatment of Cancer 80861 (20)and 80871 studies (21), the EOI phase II study of intensive chemotherapy with granulocyte-colony stimulating factor (G-CSF) (22) or the recently closed EORTC 80931 trial (23). Patients who did not enter a trial (either refused or were not eligible) were offered short intensive courses of chemotherapy. One patient did not receive chemotherapy because of advanced age. Another patient was treated with doxorubicin only as palliative therapy. Histological response after pre-operative chemotherapy was determined in the resection specimens by a reference pathologist using a modified Huvos grading system. A good response was defined if less than 10% viable tumour cells were seen in the post chemotherapy specimens, whereas a poor response was present in cases where there were 10% or more viable tumour cells. Only patients with a poor response were selected in group B because HER2 status can only be assessed on viable cells, i.e. chemotherapy-resistant cells, and not on necrotic samples. HER2-status was assessed in the biopsy (group A), or resection (group B) specimens.

RNA extraction

RNA was isolated from 30 sections of 20 μm snap frozen fresh osteosarcoma tissue sections, using Trizol reagent (Invitrogen®) according to the manufacturer's instruction. For isolation of mRNA, only tissue containing more than 50% of tumour cells was selected.

Quantification of HER2 transcripts with TaqMan Real-Time PCR

HER2 expression was determined by quantitative real-time PCR (qPCR) using cDNA, synthesized from 2.5 μg reverse-transcribed total RNA in a 100 μl reaction containing 20 μl first-strand RT-PCR buffer (GIBCO), 10 μl 0.1 M dithiothreitol (DTT), 10 μl 10 mM deoxynucleotide triphosphate (dNTP), 25 μl 50 μM random hexamers (PE/Applied Biosystems), 100 U RNAsin (PE/Applied Biosystems), 500 U Superscript II reverse transcriptase (GIBCO). Incubation was for 10 min at room temperature, 60 min at 42°C and 5 min at 95°C. Porphobilinogen deaminase (PBGD), a housekeeping gene, was used as reference in a parallel reaction to quantify the relative results from real-time PCR for HER2. The primers and probe for PBGD were described previously (24). Primers for *HER-2* amplification, derived from Genbank accession number X03363, were 5'-GGC CTG CGG GAG CTG-3' (forward) and 5'-TCC GCT GGA TCA AGA CCC-3' (reverse) resulting in a product of 67 base pairs, detected by the probe (5'-TCC TTT CAA GAT CTC TGT GAG GCT TCG AAG-3' labelled with FAM and the quencher TAMRA. A PCR reaction consisted of 25 μl and contained 2.5 μl cDNA, 7.5 pMol of forward and reverse primer, 7.5 pMol of

TaqMan probe (PE/Applied Biosystems) and 12.5 µl TaqMan Universal PCR Mastermix (PE/Applied Biosystems). PCR was performed up to 50 cycles of 15 s 95°C and 1 min 60°C on a ABI PRISM® 7700 Sequence Detection System. SKBR3, a breast carcinoma cell line with 4-10 fold *HER-2* gene amplification and 128 fold over-expression of HER2-mRNA (25) was used as reference for HER2 expression. Serial dilutions of cDNA generated from SKBR3 mRNA resulted in a calibration curve for HER2 real time PCR values. Real-time PCR results from PBGD were used to quantify the amount of cDNA in each sample. The cell line MCF7 expresses normal levels of HER2-mRNA (25) and was used as a negative control for HER2 overexpression.

Immunohistochemical analysis

Paraffin-embedded, formalin-fixed tissue samples were used for immunohistochemical (IHC) analysis. These were retrieved from the department of Pathology. Bony specimens, that were resected, were decalcified according to routine laboratory methods, using formic acid. All IHC assays were performed on 5 µm tissue sections, mounted on APES coated slides. Plasma membrane associated staining for HER2 was performed using DAKO HERCEPTEST® (Glostrup, Danmark) according to the manufacturer's instructions. HER2 staining was scored as 0, 1+, 2+ or 3+, according to the scoring system provided with DAKO HERCEPTEST®.

FISH for *HER-2* gene amplification

One of the tumours showed a 2+ staining result for HER2. FISH was performed with a section from this specimen using the Vysis® FISH test kit for the detection of *HER-2* gene amplification, according to the manufacturer's instructions. Using a fluorescence microscope, the HER2 copy numbers and the centromere chromosome 17 copy numbers were counted in the tumour cells.

RESULTS

Patients

Patient clinical characteristics and outcome are listed in Table 1. Biopsy samples of 15 patients (group A) were studied. All 15 samples (ID no 1-15) were from primary tumours, three of which later relapsed (nos. 5, 12 and 15). Samples of group B consisted of 12 post chemotherapy resection of osteosarcomas or specimens of pulmonary (nos. 21 and 29), distant bone (no. 24) or locally (no. 17, 31 and 32) relapsed patients. From the latter 3 patients, biopsy samples at primary diagnosis are in the upper panel (nos. 5,12 and 15, respectively). The mean age of the patients in group A was lower (mean 22 years, range 7-48 year) than those in group B (mean 37 years, range 14-82 year). The localisation of the osteosarcomas was similar in both groups, mainly in the femur (in 80% and 72% in group A and B respectively). Other sites were the tibia in 2 cases in each group and in the humerus, clavicle and pelvis. Histological subtyping was high-grade conventional in all the cases in group A, except one sample that was high-grade osteoblastic. In group B, four samples were of the teleangiectatic, and one had a malignant fibrous histiocytoma (MFH)-like subtype (see table 1).

TABLE 1.
Patients clinical data and results of our study.

Id number	Age at Dx	Sample	Localization Tumour	Histological Subtype	Chemother Treatment	Response	Outcome	HER2 mRNA	IHC	FISH
Group A										
1	16	B	Ti	Co	Yes	PR	surv	0.025	0	
2	20	B	Hu	Ob	Yes	PR	surv	0.003	0	
3	9	B	Fe	Co	Yes	GR	surv	0.013	0	
4	7	B	Ti	Co	Yes	GR	surv	0.033	2+	no ampification
5	48	B	Fe	Co	Yes	PR	surv	0.031	0	
6	28	B	Fe	Co	Yes	PR	DOD	0.004	0	
7	20	B	Fe	Co	Yes	GR	DOD	0.004	0	
8	29	B	Fe	Co	Yes	PR	DOD	0.004	0	
9	20	B	Fe	Co	Yes	PR	DOD	0.034	0	
10	17	B	Fe	Co	Yes	NA	DOD	0.016	0	
11	20	B	Fe	Co	Yes	PR	DOD	0.028	0	
12	20	B	Fe	Co	Yes	PR	DOD	0.034	0	
13	33	B	Fe	Co	Yes	GR	DOD	0.008	NA	
14	19	B	Fe	Co	Yes	PR	DOD	0.002	0	
15	21	B	Fe	Co	Yes	GR	DOD	0.019	0	

Group B									
16	18	R	Fe	Co	Yes	PR	surv	0.001	no tumor
17	48	R	Fe	Co	Yes	PR	surv	0.020	0
18	20	R	Hu	Co	Yes	PR	surv	0.005	0
19	20	R	Fe	Co	Yes	PR	surv	0.029	0
20	14	R	Ti	Co	Yes	PR	surv	0.047	0
21	22	R	Cla	Co	Yes	PR	surv	0.030	no tumor
22	64	R	Fe	Te	Yes	PR	surv	0.011	0
23	82	R	Fe	Te	no	NA	surv	0.016	0
24	12	R	Fe	Co	Yes	NA	DOD	0.013	0
25	42	R	Ti	Co	Yes	PR	DOD	0.022	no tumor
26	26	R	Fe	Te	Yes	PR	DOD	0.013	NA
27	57	R	Fe	MFH	Yes	PR	DOD	0.038	0
28	30	R	Fe	Co	Yes	PR	DOD	0.049	0
29	64	R	Pel	Co	Yes	NA	DOD	0.001	0
30	36	R	Fe	Co	Yes	PR	DOD	0.027	0
31	21	R	Fe	Co	Yes	PR	DOD	0.012	0
32	20	R	Fe	Co	Yes	PR	DOD	0.105	0
33	71	R	Fe	Te	Yes	PR	DOD	0.018	NA
MCF7								0,014	

Abbreviations: B = biopsy, R = resection, Fe = femur, Ti = tibia, Hu = humerus, Cla = clavicle, Pel = pelvis, Co = high grade conventional, Ob = osteoblastic, Te = teleangiectatic, MFH = MFH-like, PR = poor response, GR = good response, Surv = survived disease, DOD = died of disease, NA = not available.

HER2 mRNA expression

HER2 mRNA expression was assessed by real-time PCR, using RNA from the osteosarcoma specimens. The mean absolute value for HER2 mRNA expression in group A is 0.017 (range 0.003 – 0.034) and 0.025 (ranging 0.001 – 0.105) in group B. The values of HER2 expression in both groups were similar to the HER2 expression in the breast cancer cell line MCF7 that has a HER2 expression value of 0.014. None of the tumour samples had values in the same range as the HER2-overexpressing cell line SKBR3, which was set at 1.0 in this study. All values fell within the range of normal HER2 expression, similar to the expression observed in normal breast tissues. Even the highest value of HER2 expression in group B (0.105) can be regarded as not being overexpressed particularly as no protein expression was seen in this sample.

Immunohistochemistry

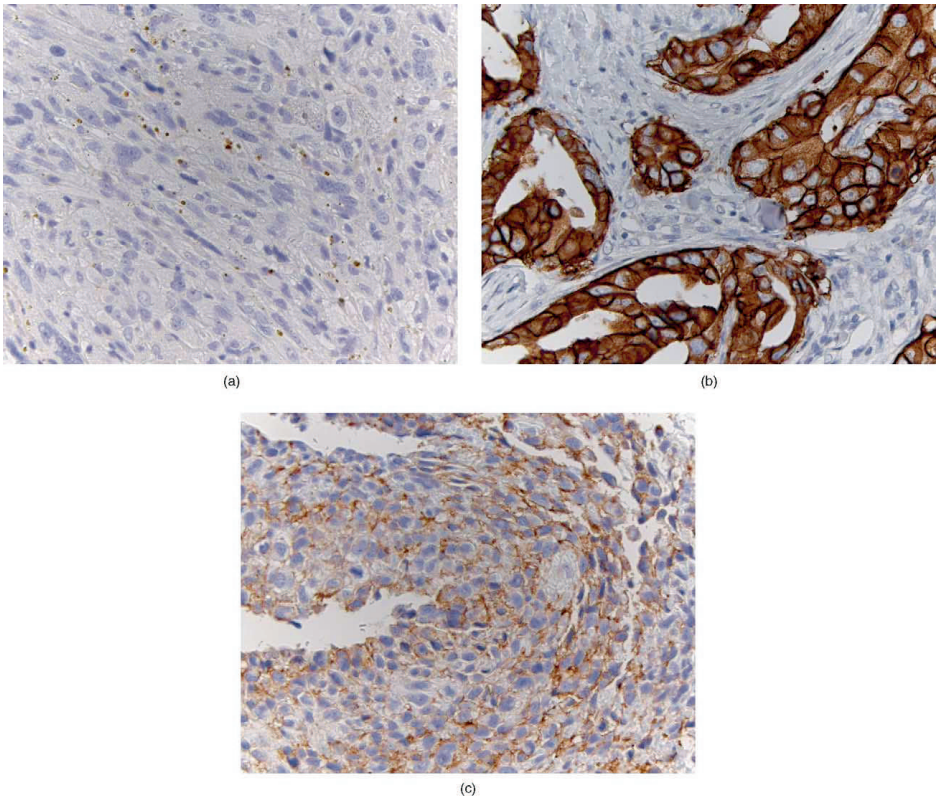
Three of the 33 samples were lost after immunostaining during retrieval procedures. In an additional three samples, no vital tumor was left on the histological section. IHC was not repeated in these samples. Nearly all of the samples showed no HER2 plasma membrane-associated staining. An example of negative immunostaining is shown in Fig. 1(a). Fig. 1(b) shows positive membrane staining in a control breast cancer sample with proven *HER-2* gene amplification. This represents a 3+ score. Only one osteosarcoma sample (patient no. 4) shows moderate positive immunostaining of the membrane, which was scored as 2+ (Fig. 1(c)). Focal cytoplasmatic IHC positivity was seen in two other samples, but as discussed previously, this was not considered as positive for HER2 overexpression.

FISH

Fluorescent *in situ* hybridisation (FISH) was performed in the osteosarcoma sample with 2+ positive membrane staining, and did not show any *HER-2* gene amplification.

FIGURE 1.

Immunohistochemical staining for HER-2 protein expression: (a) osteosarcoma negative for HER-2; (b) breast cancer sample positive for plasma membrane-associated HER-2 expression (score 3+); (c) osteosarcoma from patient no. 4 with moderate positive expression of HER-2 (score 2+).



DISCUSSION.

In this study, a single case of moderately (2+) positive membrane staining was recorded (fig. 1c). However, quantitative RT-PCR or FISH analysis could not confirm HER2-mRNA overexpression or *HER-2* gene amplification, respectively, suggesting this was a false- positive immunostain.

Usually, the HER2 protein is overexpressed as result of *HER-2* gene amplification and concomitant elevated mRNA expression (26). Nevertheless, protein overexpression has been

reported in the absence of gene amplification (12, 27-29). Interestingly, clinical studies suggest that cases with *HER-2* gene amplification have a poor outcome, whereas patients who show protein expression without gene abnormalities do not have an increased risk for a more aggressive disease course and death (30, 31).

HER2 status in osteosarcoma has been investigated in 8 other clinical studies (5-7, 16-19, 32) (Table 2).In five of these studies, HER2 overexpression was reported to occur in 42%-63% of the patients with primary non-metastatic osteosarcoma, and in 10%-58% of the cases that had pulmonary metastases at diagnosis (6, 7) or had relapsed (5, 17, 32).Three studies correlated HER2 overexpression with a poor response to pre-operative chemotherapy and a poor outcome (5, 6, 32). Remarkably, two other studies conclude that HER2 overexpression predicts better survival and is less frequent in metastatic disease (7, 17).

In four remaining osteosarcoma studies, including ours, no HER2 overexpression could be demonstrated (16, 18, 19).These inconsistent findings regarding the HER2 status and its significance in osteosarcoma raise questions about the reliability of some studies and may be explained by methodological differences.

The HER2 status in the published osteosarcoma studies has been assessed mainly by IHC. These studies differ considerably in use of antibody and quality controls, scoring systems, interpretation of positivity of the sample and validation of IHC result. In breast cancer, HER2 testing and standardization of its method has been an important issue, because only patients with HER2 overexpression are eligible for trastuzumab treatment (33).The quality of the antibody used is important, since a high rate (up to 40%) of false positive test results has been reported, due to a highly variable sensitivities (34). False positive cases are particularly noted when moderate (e.g. IHC2+) positivity occurs, and in these cases confirmation of the positive result with other tests is required (35).

Six different scoring systems to assess HER2 positivity have been used in the osteosarcoma studies (5-7, 17, 32). Interpretation of stained samples may have a high inter-observer variability and low rate of reproducibility (36, 37).This particularly occurs when the staining is heterogeneous, weakly positive, in non-malignant cells, cytoplasmatic staining or when retraction artifacts occur (38). Cytoplasmatic immunostaining is considered to be an IHC artefact (19, 39), and only complete membrane staining should be included when interpreting results (38). Only one of the five studies in osteosarcoma that scored membrane staining specifically, reported to have positive results (6). However, a poorly characterized antibody, 5B5, was used in this particular study and this antibody has not been used in other studies. Most of the ostesarcoma studies included the mandatory positive and negative controls for IHC, usually a patient sample with and without known HER2 overexpression. However, our series is the only one to use positive and negative cell lines as a control.

Validation of the IHC HER2 results by the use of other assays was done in four out of eight studies (5, 18, 19, 32).Validation assays included were immunoblotting (IB), single-stranded conformation polymorphism (SSCP) and Southern blotting (SB) (5), RT-PCR (19), and Fluorescent In Situ Hybridization (FISH) (18, 32). Except for one study that used FISH (32), no evidence for HER2 overexpression was found in the other validation analyses.

This confirms the results of our study, that showed no HER2 mRNA overexpression, assessed with a quantitative Real-Time-PCR technique, which is the only method, mentioned above that quantitatively assess HER2 mRNA expression (40).
FISH has proven to be an accurate and reproducible assay to detect *HER-2* gene amplification (41). Zhou and colleagues found *HER-2* gene rearrangement in seven of 12 tested samples, but unusual criteria were used to define the *HER-2* gene amplification (32). Accurate determination of low level *HER-2* gene amplification using FISH, requires assessment of *HER-2* copy number relative to chromosome 17 centromere number to distinguish between HER2 gene amplification and aneusomy of chromosome 17 (41), which frequently occurs in osteosarcoma (42). Furthermore, *HER-2* gene amplification in HER2-overexpressing breast cancers is usually observed in the most of the tumour cells (41).
Thus to conclude, our results show that HER2 does not play a role in the tumour biology of osteosarcoma and that pilot studies, using trastuzumab, as a drug with potential tumour-inhibiting properties, are not likely to benefit to patients with this bone tumuor.

TABLE 2.
Overview of clinical HER2 studies in osteosarcoma

	Number of		Immunohistochemistry				% HER2+ samples		HER2+ IHC samples related to		
Author (ref)	patients	samples	Antibody	Scoring system	Control	Other assays	IHC+	Other assay	PR (%)	EFS	OAS
Onda (5)	26	26	CB11 CBE1	Qualitative (M)	–	IB, SB, SSCP	42	0	67	–	HER+ 14% HER– 84%
Gorlick (6)	53	53	5B5 / Herceptest	Semi-quantitative (M)	+	–	45	ND	57	HER+ 47% HER– 79%	–
Akatsuka (7)	81	81	CB11	Semi-quantitative (M and/or Cy)	+	–	63	ND	58	HER+ 72% HER– 46%	HER+ 82% HER– 57%
Akatsuka (17)	19	38	CB11	Semi-quantitative (M and/or Cy)	–	–	PT 50 PuMet 10	ND	46	–	–
Zhou (32)	25	37	Ab3	Semi-quantitative (M and/or Cy)	+	FISH	PT 44 PuMet 58	67	NS	NS	NA
Kilpatrick (16)	41	41	CB11 Oncor	Semi-quantitative (M and/or Cy)	+	–	0	ND	NA	NA	NA
Maitra (18)	21	21	AO485	Semi-quantitative (M)	+	FISH	0	0	NA	NA	NA
Thomas (19)	33	66	AO485	Semi-quantitative (M)	+	RT-PR	0	0	NA	NA	NA
Present study	33	27	Herceptest	Herceptest guidelines; (M)	+	RT-PCR / FISH	1*	0	NA	NA	NA

M, Membrane staining; Cy, cytoplasmatic staining; IB, immunoblot; SB, Southern blot; SSCP, single stranded confirmation polymorphism; PT, primary tumour; PuMet, pulmonary metastases; PR, poor response to chemotherapy; EFS, event free survival; OAS, overall survival; NA, not applicable; NS, not significant. * Can be regarded as false-positive (see text).

REFERENCES

1. Raymond AK, Ayala AG, Knuutila S: Conventional Osteosarcoma., in Fletcher CDM, Unni KK, Mertens F (eds): World Health Organization Classification of Tumours. Pathology and Genetics of tumours of Soft Tissue and Bone. Lyon, IARC Press, 2002, pp 264-270
2. Davis AM, Bell RS, Goodwin PJ: Prognostic factors in osteosarcoma: a critical review. J Clin Oncol 12:423-431, 1994
3. Bielack SS, Kempf-Bielack B, Delling G, et al.: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776-790, 2002
4. Hauben EI, Weeden S, Pringle J, et al.: Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of the European Osteosarcoma Intergroup. Eur J Cancer 38:1218-1225, 2002
5. Onda M, Matsuda S, Higaki S, et al.: ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. Cancer 77:71-78, 1996
6. Gorlick R, Huvos AG, Heller G, et al.: Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 17:2781-2788, 1999
7. Akatsuka T, Wada T, Kokai Y, et al.: ErbB2 expression is correlated with increased survival of patients with osteosarcoma. Cancer 94:1397-1404, 2002
8. Coussens L, Yang-Feng TL, Liao YC, et al.: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230:1132-1139, 1985
9. Akiyama T, Sudo C, Ogawara H, et al.: The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232:1644-1646, 1986
10. Hynes NE, Stern DF: The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165-184, 1994
11. di Fiore PP, Pierce JH, Kraus MH, et al.: erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178-182, 1987
12. Slamon DJ, Clark GM, Wong SG, et al.: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177-182, 1987
13. Pierce JH, Arnstein P, DiMarco E, et al.: Oncogenic potential of erbB-2 in human mammary epithelial cells. Oncogene 6:1189-1194, 1991
14. Drebin JA, Link VC, Stern DF, et al.: Development of monoclonal antibodies reactive with the product of the neu oncogene. Symp Fundam Cancer Res 38:277-289, 1986
15. Pegram M, Hsu S, Lewis G, et al.: Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene 18:2241-2251, 1999
16. Kilpatrick SE, Geisinger KR, King TS, et al.: Clinicopathologic analysis of HER-2/neu immunoexpression among various histologic subtypes and grades of osteosarcoma. Mod Pathol 14:1277-1283, 2001
17. Akatsuka T, Wada T, Kokai Y, et al.: Loss of ErbB2 expression in pulmonary metastatic lesions in osteosarcoma. Oncology 60:361-366, 2001

18. Maitra A, Wanzer D, Weinberg AG, et al.: Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. Cancer 92:677-683, 2001

19. Thomas DG, Giordano TJ, Sanders D, et al.: Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. Clin Cancer Res 8:788-793, 2002

20. Souhami RL, Craft AW, Van der Eijken JW, et al.: Randomised trial of two regimens of chemotherapy in operable osteosarcoma: a study of the European Osteosarcoma Intergroup. Lancet 350:911-917, 1997

21. Bramwell VH, Steward WP, Nooij M, et al.: Neoadjuvant chemotherapy with doxorubicin and cisplatin in malignant fibrous histiocytoma of bone: A European Osteosarcoma Intergroup study. J Clin Oncol 17:3260-3269, 1999

22. Ornadel D, Souhami RL, Whelan J, et al.: Doxorubicin and cisplatin with granulocyte colony-stimulating factor as adjuvant chemotherapy for osteosarcoma: phase II trial of the European Osteosarcoma Intergroup. J Clin Oncol 12:1842-1848, 1994

23. Lewis IJ, Nooij MA, Whelan J, et al.: Improvement in histologic response but not survival in osteosarcoma patients treated with intensified chemotherapy: a randomized phase III trial of the European Osteosarcoma Intergroup. J Natl Cancer Inst 99:112-128, 2007

24. Faneyte IF, Kristel PM, van de Vijver MJ: Determining MDR1/P-glycoprotein expression in breast cancer. Int J Cancer 93:114-122, 2001

25. Kraus MH, Popescu NC, Amsbaugh SC, et al.: Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6:605-610, 1987

26. van de Vijver MJ, van de Bersselaar R, Devilee P, et al.: Amplification of the neu (c-erbB-2) oncogene in human mammmary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene. Mol Cell Biol 7:2019-2023, 1987

27. King CR, Swain SM, Porter L, et al.: Heterogeneous expression of erbB-2 messenger RNA in human breast cancer. Cancer Res 49:4185-4191, 1989

28. Parkes HC, Lillycrop K, Howell A, et al.: C-erbB2 mRNA expression in human breast tumours: comparison with c-erbB2 DNA amplification and correlation with prognosis. Br J Cancer 61:39-45, 1990

29. Pauletti G, Godolphin W, Press MF, et al.: Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. Oncogene 13:63-72, 1996

30. Farabegoli F, Ceccarelli C, Santini D, et al.: c-erbB-2 over-expression in amplified and non-amplified breast carcinoma samples. Int J Cancer 84:273-277, 1999

31. Riou G, Mathieu MC, Barrois M, et al.: c-erbB-2 (HER-2/neu) gene amplification is a better indicator of poor prognosis than protein over-expression in operable breast-cancer patients. Int J Cancer 95:266-270, 2001

32. Zhou H, Randall RL, Brothman AR, et al.: Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. J Pediatr Hematol Oncol 25:27-32, 2003

33. van de Vijver MJ: Assessment of the need and appropriate method for testing for the human epidermal growth factor receptor-2 (HER2). Eur J Cancer 37 Suppl 1:S11-S17, 2001

34. Press MF, Hung G, Godolphin W, et al.: Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res 54:2771-2777, 1994

35. Pauletti G, Dandekar S, Rong H, et al.: Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J Clin Oncol 18:3651-3664, 2000

36. Kay EW, Walsh CJ, Cassidy M, et al.: C-erbB-2 immunostaining: problems with interpretation. J Clin Pathol 47:816-822, 1994

37. Thomson TA, Hayes MM, Spinelli JJ, et al.: HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent in situ hybridization. Mod Pathol 14:1079-1086, 2001

38. Bilous M, Dowsett M, Hanna W, et al.: Current perspectives on HER2 testing: a review of national testing guidelines. Mod Pathol 16:173-182, 2003

39. Taylor SL, Rudland PS, Barraclough R: C-erbB-2 mRNA in breast cancer specimens that exhibit membrane or cytoplasmic immunoreactivity for c-erbB-2. Oncol Res 11:311-317, 1999

40. Bieche I, Onody P, Laurendeau I, et al.: Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clin Chem 45:1148-1156, 1999

41. Kallioniemi OP, Kallioniemi A, Kurisu W, et al.: ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. Proc Natl Acad Sci U S A 89:5321-5325, 1992

42. Bayani J, Zielenska M, Pandita A, et al.: Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. Genes Chromosomes Cancer 36:7-16, 2003